
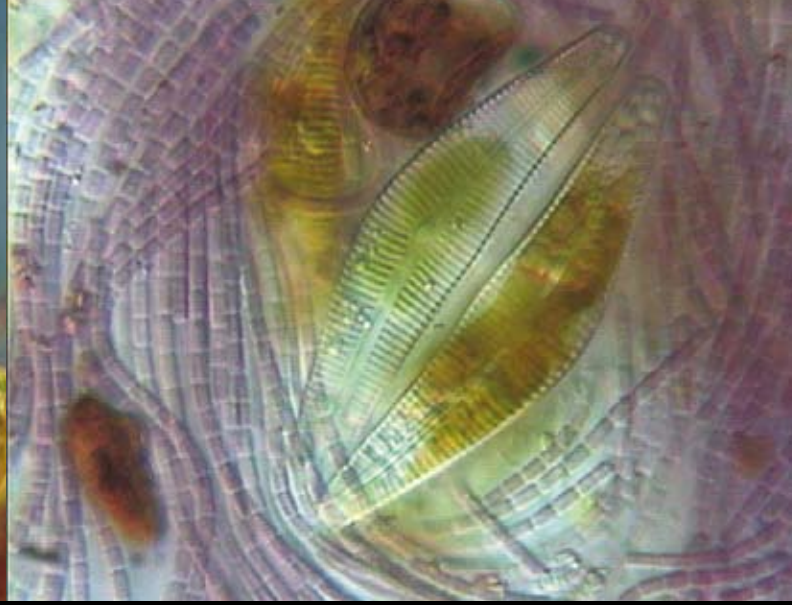
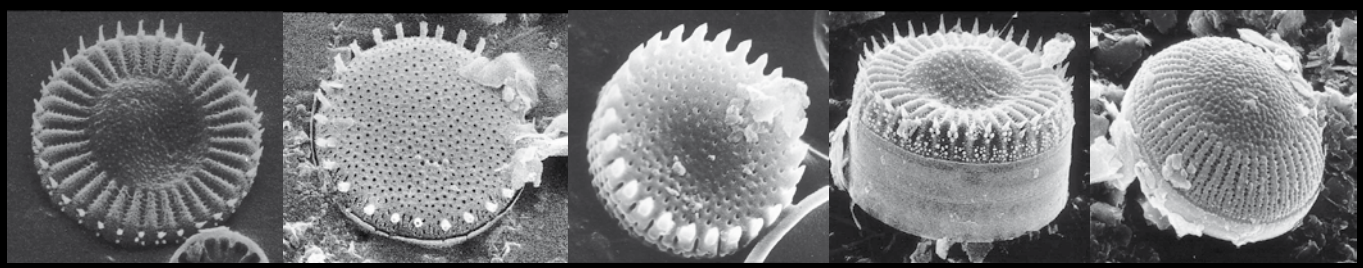

A Methods Manual for the Collection, Preparation and Analysis of Diatom Samples

Version 1.0

JC Taylor, WR Harding & CGM Archibald

TT 281/07

Water Research Commission

A Methods Manual for the Collection, Preparation and Analysis of Diatom Samples

Version 1.0

Report to the
Water Research Commission

by

JC Taylor*, WR Harding**
and CGM Archibald***

*** School of Environmental Sciences and Development**
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***** KZN Aquatic Ecosystems [KZNAE]**

WRC Report TT 281/07
January 2007

This report is part of a set on Diatoms. The other report is:

WRC report TT 282/07: An Illustrated Guide to Some Common Diatom Species from South Africa

Each report is provided with a DVD of
1. Training Videos for Diatom Field Sampling and Laboratory Practice
2. An electronic Diatom Taxonomic Key. Also, see plates in TT 287/07

These are obtainable from:

Water Research Commission
Private Bag X03
Gezina, Pretoria
0031 South Africa

The Methods Manual emanates from a Water Research Commission research project entitled: *Development of a Diatom Assessment Protocol (DAP) for River Health Assessment* (WRC project no K5/1588), for which DH Environmental Consulting was the Lead Consultant.

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TO THE USER:

This is a beta version manual for trial use in river biomonitoring programmes. As such the authors welcome any comments or suggestions regarding the content and usability.

Please note that subsequent versions of the manual will contain additional training and decision support aids, as well as image support for certain of the terms contained in the glossary.

Please forward comments or suggestions, or address queries to:

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FOREWORD

This method manual is intended for those who wish to become familiar with the methods of collecting diatom samples in a meaningful and repeatable manner, whether the outputs from these samples will be used for taxonomy or biodiversity studies or to infer water quality. The latter aim will be dealt with more extensively in this guide.

The application of diatom-based water quality monitoring has become a reality with the recent development of expertise in the fields of diatom taxonomy and ecology in South Africa. This, coupled with the support from government initiatives (e.g. the River Health Programme) as well as from the Water Research Commission, together with a growing interest amongst the scientific community of South Africa has sparked a new awareness of this particular field of study. Links with a number of international diatomists has also aided in the growth of knowledge and the verification of ecological and taxonomic data.

In addition, software packages are now available for index calculation, sample data archiving, capture of digital images, as well as simple and complex electronic diatom keys. The production of simple taxonomic guides, both in South Africa and around the world, has also lessened literature costs and allowed a more rapid and accurate approach for the assessment of water quality using diatoms. However, diatom-based monitoring is not a rapid field-based assessment technique and always includes a component which has to be completed in a laboratory. A sound taxonomic knowledge of the diatoms is required as well as the relevant set of microscopy skills.

BACKGROUND

There is a long and proud history of diatom research in South Africa, mainly as a result of the work of the late Dr Bela Cholnoky, a the pioneer diatom specialist. This is described in the WRC Report TT 242/04 – "*The South African Diatom Collection: An Appraisal and Overview of Needs and Opportunities*".

The WRC research project K5/1588, envisaged and planned by DH Environmental Consulting, and undertaken in collaboration with KZN Consulting and North West University, has resulted in a series of practical tools for the collection, processing and examination of diatom samples from South African Rivers.

Diatoms provide a valuable and well-understood means of biomonitoring – one which is focused at the base of the aquatic foodweb and highly representative of water quality. Although the need for careful microscopic examination and taxonomic identification of species is somewhat demanding, the technique is intended to provide a 'fourth leg' to the River Health Programme suite of monitoring tools (currently invertebrates, vegetation and fish).

Project K5/1588 has produced the following tools:

- A Methods Manual which details sampling procedures and the laboratory processing of samplings for slide mounting and microscopic examination. The content of the manual also provides general information pertaining to the occurrence of diatoms in aquatic systems. (WRC Report TT 281/07)
- An Illustrated Guide to some common diatom species from South Africa (WRC Report TT 282/07)
- Two DVD-quality videos that demonstrate the field and laboratory procedures described in the manual. These training videos will also be available on CD.
- A stand-alone software-based taxonomic key to the diatom species most commonly encountered in South African rivers and streams. This is an hierarchical, interactive tool that assists the user in learning more about diatoms and diatom taxonomy while seeking an identification for an observed species. The taxonomic key allows the user to undo incorrect entries, and includes photomicrographs in various formats that assist with confirming the final result.

The results of this project are dedicated to the memory of South African diatom specialist "Archie" Archibald.

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GLOSSARY

Aerophilic - Depending on free oxygen or air.

Areolae. - Perforation through valve with internal or external sieve membrane.

Autecology - The ecology of individual organisms or species.

Autotrophic - Capable of self-nourishment; said of all organisms in which photosynthetic activity takes place, in which inorganic constituents are transformed to cell material via photosynthetic activity, as opposed to parasitism or saprophytism.

Bio-film - A surface accumulation, which is not necessarily uniform in time or space, that comprises cells immobilised at a substratum and frequently embedded in an organic polymer matrix.

Centric diatom - Radially symmetric diatom; compare to pennate diatom.

Chlorophyll a - Chlorophyll *a* is quite often used as a surrogate measure of the amount of phytoplankton in a water sample. Comparing water bodies on the basis of chlorophyll *a* content implicitly assumes the algae are composed of equivalent amounts of chlorophyll though. The chlorophyll content of algae is usually about 0.5-1.5% of the dry wt. But increased amounts, up to 6% have been recorded in algae culture in weak light. Chlorophylls are "tetrapyrrolic molecules with a central magnesium atom and two ester groups", hence the need for micronutrients by plants and animals.

Chlorophyll *a* is the "master pigment" in bluegreen algae and higher plant photosynthesis (apparently some photosynthesizing bacteria can do it without chlorophyll *a*). It is chlorophyll *a* that ultimately captures energy from light (photons) and packages it as energy in chemical bonds for use by plants and eventually animals. There are other "accessory pigments" (such as chlorophylls *b*, *c*, and *d*, carotenoids, phycoerythrins, phycocyanins, and xanthophylls) which can trap light energy at shorter wave lengths and pass it along to chlorophyll *a* which absorbs at longer wavelengths. It is the unique combination of accessory pigments with chlorophyll *a* that help to distinguish certain groups of algae and higher plants from one another. For example, Euglenophyta are characterized by the presence of chlorophyll *a* and the accessory pigments b-carotene and the xanthophyll, lutein.

Chloroplasts - In eukaryotic organisms, the cellular organelle in which photosynthesis takes place.

Chrysolaminarin - (a glucose-mannitol polymer) carbohydrate food reserve.

Detritus - Living organisms constitute only a very small portion of the total organic matter of ecosystems. Most organic matter is nonliving and is collectively called *detritus*. Detritus consists of all dead particulate and dissolved organic matter. Dissolved organic matter is about 10 times more abundant than particulate organic matter. Much of the newly synthesized organic matter of photosynthesis is not consumed by animals, but instead enters the detrital pool and is decomposed.

Euphotic zone - The surface waters of rivers or lakes where enough light penetrates for photosynthesis to occur. The depth of the euphotic zone varies with the water's extinction coefficient, the angle of incidence of the sunlight, length of day and cloudiness.

Frustule - The valves and their associated girdle elements.

Girdle – The collective term for all structural elements between two valves.

Girdle bands - The elements of the girdle.

Girdle view - "side" view of a diatom.

Glides - Stream areas with low velocities and with a smooth surface. Water depth generally is less than half a meter.

Heterotrophic - Organisms that derive their nourishment from existing organic substances. Heterotrophs can be herbivores, carnivores, omnivores or detritivores.

Littoral zone - An interface zone between the land of the drainage basin and the open water of lakes. Most lakes of the world are relatively small in area and shallow. In such lakes, the littoral flora contributes significantly to the productivity, and may regulate metabolism of the entire lake ecosystem.

Wetland and littoral regions of freshwater ecosystems are commonly intensely metabolically active owing to the presence of aquatic macrophytes. Phytoplankton productivity is generally lower in the littoral zones, containing stands of aquatic macrophytes, largely because of competition for nutrients (including carbon) by submersed macrophytes, and by a reduction of light by macrophyte foliage. (Also see Macrophytes).

Macrophytes - The term *aquatic macrophyte* generally refers to the macroscopic forms of aquatic vegetation, and encompasses macroalgae (e.g. the alga *Cladophora*, the stoneworts such as *Chara*), the few species of mosses and ferns adapted to the aquatic habitat, as well as true angiosperms. Four groups of aquatic macrophytes can be distinguished as follows:

- ***Emergent*** macrophytes grow on water-saturated or submersed soils from where the water table is about 0.5m below the soil surface (supralittoral) to where the sediment is covered with approximately 1.5m of water (upper littoral).
- ***Floating-leaved*** macrophytes are rooted in submersed sediments in the middle littoral zone (water depths of approximately 0.5m to 3m), and possess either floating or slightly aerial leaves.
- ***Submersed*** macrophytes occur at all depths within the photic zone. Vascular angiosperms occur only to about 10m (1 atm hydrostatic pressure) within the lower littoral (infralittoral), and nonvascular macrophytes (e.g. macroalgae) occur to the lower limit of the photic zone (littoriprofundal).
- ***Freely floating*** macrophytes are not rooted to the substratum; they float freely on or in the water and are usually restricted to nonturbulent, protected areas.

Mucilage - A general term for complex substances composed of various types of polysaccharides, becoming viscous and slimy when wet.

Pennate diatom - Bilaterally symmetric diatom.

Periphyton -Refers to microfloral growth upon substrata in fresh waters. A much more explicit manner of expression is to refer to the organisms with appropriate modifiers descriptive of the substrata upon which they grow in natural habitats. These algal communities can be classified into,

- ***Epipelic algae*** as flora growing on sediments (fine, organic),
- ***Epilithic algae*** growing on rock or stone surfaces,
- ***Epiphytic algae*** growing on macrophytic surfaces,
- ***Epizooic algae*** growing on surfaces of animals, and
- ***Epipsammic algae*** as the specific organisms growing on or moving through sand.

(See also Phytoplankton).

Phytoplankton - The phytoplankton consists of the assemblage of small plants having no or very limited powers of locomotion; they are therefore more or less subject to distribution by water movements. Certain planktonic algae move by means of flagella, or possess various mechanisms that alter their buoyancy. However, most algae are slightly denser than water, and sink, or sediment from, the water. Phytoplankton are largely restricted to *lentic* ("standing") waters and large rivers with relatively low current velocities. (See also Periphyton).

Puncta - General term for pore/perforation through valve when substructure (i.e. sieve membrane) is unknown or lacking.

Raphe - Slit through valve along apical axis. Composed of (usually) two branches per valve.

Refractive index - The ratio of the speed of light in a vacuum to the speed of light in a medium under consideration.

Relative abundance - A measure of the ratio between different species in a population or community.

Riffle - Fast-flowing, shallow segment of a stream where the surface of the water is broken over rocks or debris.

Runs - Transitional segments of streams, between a riffle and a pool, with moderate current and depth.

Spines - Conical or forked solid external projection.

Striae - Rows of puncta/areolae, usually oriented along transapical axis, separated by unornamented ribs. Striae appear as dark lines under lower magnifications and as a series of dots (punctae) at higher magnification.

Valve - Siliceous part of the frustule containing most of the morphological features used to describe diatoms (taxonmically, morphologically, etc.). Each valve has two surfaces, the face and the mantle.

Valve face - Portion of the valve apparent in valve view (oriented to the valvar plane).

Valve mantle - Portion of the valve, differentiated by slope, that is apparent in girdle view (oriented to the apical plane).

SECTION 1: INTRODUCTION

1. History of diatom research in South Africa

Diatom research in South Africa can be divided into five distinct periods. The first period covers a span of some seventy years, beginning with Shadbolt's (1854) account of the diatoms from Port Natal, and continuing with brief reports and notes on odd specimens found in various samples sent to the leading diatomists of the day (e.g. Cleve 1894 and 1895; Grove, 1894).

The second period spanned the time between the two world wars and is characterised by accounts of diatoms found in the more general algological surveys made by a number of algologists, notably Felix Eugen Fritsch, Florence Rich and Edith Stevens (e.g. Fritsch and Rich, 1924, 1930).

The third period involved the most comprehensive study of diatoms in South Africa, and commenced after the arrival of Dr Bela Jeurno Cholnoky in South Africa in 1952. Cholnoky was a Hungarian refugee whose chief interest in life was the diatoms. Through his intensive and extensive taxonomic and ecological studies he built up the diatom collection of the then National Institute for Water Research (CSIR) in Pretoria, making it the centre for diatom research in this country. Cholnoky placed little faith in only the chemical analysis of water quality, arguing forcefully that *the chemical and physical characteristics of a water body could be determined more reliably and easily through a study of the diatom associations found living in it* (Cholnoky, 1968). His diatom investigations focussed, therefore, on two aspects – the taxonomy of the diatoms and their species specific autecology.

During this third period he also trained his successors, Dr R. E. M. 'Archie' Archibald who became his assistant in 1964; Dr Ferdi Schoeman who joined the institute in 1968; and Prof Malcolm Giffen of the University of Fort Hare. Dr Archibald and Dr Schoeman were trained in the ecology and taxonomy of freshwater diatoms while Prof Giffen was encouraged to study marine littoral and estuarine diatom taxa.

Following the death of Cholnoky in 1972, the fourth period saw a very fruitful partnership between Dr Archibald and Dr Schoeman in which new approaches to the taxonomy of diatoms were made, culminating in the production of "*The Diatom Flora of Southern Africa"*(Schoeman and Archibald, 1976- 1981). For each species included in this flora, samples of the type material were obtained and examined using traditional light microscopy techniques as well as electron microscopy. In this way the authors were able to check their identifications and fix the concepts of species according to their own observations of the type material. The resulting detailed descriptions and commentaries on each species, together with the first attempts to produce a diatom atlas correlating drawings, and both light and EM

photomicrographs earned high praise for the first six parts of this *Flora*. Unfortunately, the thorough treatment of each species was considered to be excessively costly and time consuming, resulting in the *Flora* being discontinued.

After the curtailment of the *Flora* there was a shift in the direction of diatom studies at the NIWR, and two lines of research were followed. The first of these adopted a purely taxonomic direction of study in which selected species were individually examined and thoroughly revised in the light of both type material and local material. Special attention was focussed on the genus *Amphora*, but other species in other genera were treated when and if material became available. The second line of research returned more to the style of investigation used by Cholnoky in his surveys from different regions but incorporated the new developments in electron microscopy and photomicroscopy.

At the end of 1986 Dr Schoeman left the NIWR, bringing to an end a fruitful partnership, in which he was the co-architect of so much that was achieved for over a decade. Continuing on the foundation laid by Cholnoky, the NIWR group had developed into the largest diatom research centre in the Southern Hemisphere at that time. Details of this collection have been discussed in Harding *et al.* (2004).

The third and fourth period of diatom research at the NIWR(CSIR) saw the strength of human resources engaged in diatom work rise from one to four full-time researchers as well as several for whom diatoms were a secondary interest, then decline again to one person, Archie Archibald, occupied full time with the help of an assistant. Following the untimely death of Dr Archibald in December, 1999 meaningful diatom research ceased at the CSIR. The Diatom collection was subsequently transferred to the CSIR in Durban under the care of Colin Archibald before his retirement in 2002.

The fifth or current period of diatom research in South Africa includes research undertaken under the leadership Prof Guy Bate (University of Port Elizabeth now Nelson Mandela Metropolitan University) which commenced with a study of diatoms in South Africa in the late 1990's. The research focussed on the ecological aspects of diatom assemblages for determining water quality and attempted to apply a descriptive European diatom index for South African conditions (Bate et al, 2002). This research continued with the publication of a Water Research Commission report relating freshwater, brackish and estuarine species to key water quality variables (Bate et al., 2004).

Also during this same period Dr Bill Harding commenced with an evaluation of the ex NIWR (now CSIR-Environmentek) diatom collection, as well as initiating further diatom studies by producing a set of protocols (of which this volume forms a part) by which diatom samples can be collected, prepared and the species in the samples identified – and by the use of which diatoms can form a valuable component of biomonitoring in South Africa. Diatom studies have also been undertaken in the last five years at the North-West University (Potchefstroom

Campus) where the application of numerical diatom indices to South African rivers was tested (Taylor, 2004). Other students are now engaged in both diatom ecological and taxonomic studies at M.Sc. and Ph.D. level. This revival of interest in the diatoms together with the production of standard protocols and the rigorous testing of numerical diatom-based indices, should culminate in the realisation of Cholnoky's prediction that diatom associations can be used to give a reliable and accurate indication of the chemical and physical characteristics of a water body.

2. How do you recognise diatoms in natural environments?

A common source of error in inferring ecological conditions using diatom communities arises from sampling from un-colonised substrata. Diatom communities may be detected on substrata by feel (slimy or mucilaginous) or may be seen as a thin golden-brown film covering substrata. In some conditions or at certain times of the year this film may become thicker and much more noticeable. The essential natural microhabitats are solid substrata, exposed damp sediments and the stems of rooted vegetation. Diatoms are also present in the seston or suspended component of the phytoplankton. Man-made and other objects (paper or plastic bags, pieces of wood) are also frequently colonised by diatoms.

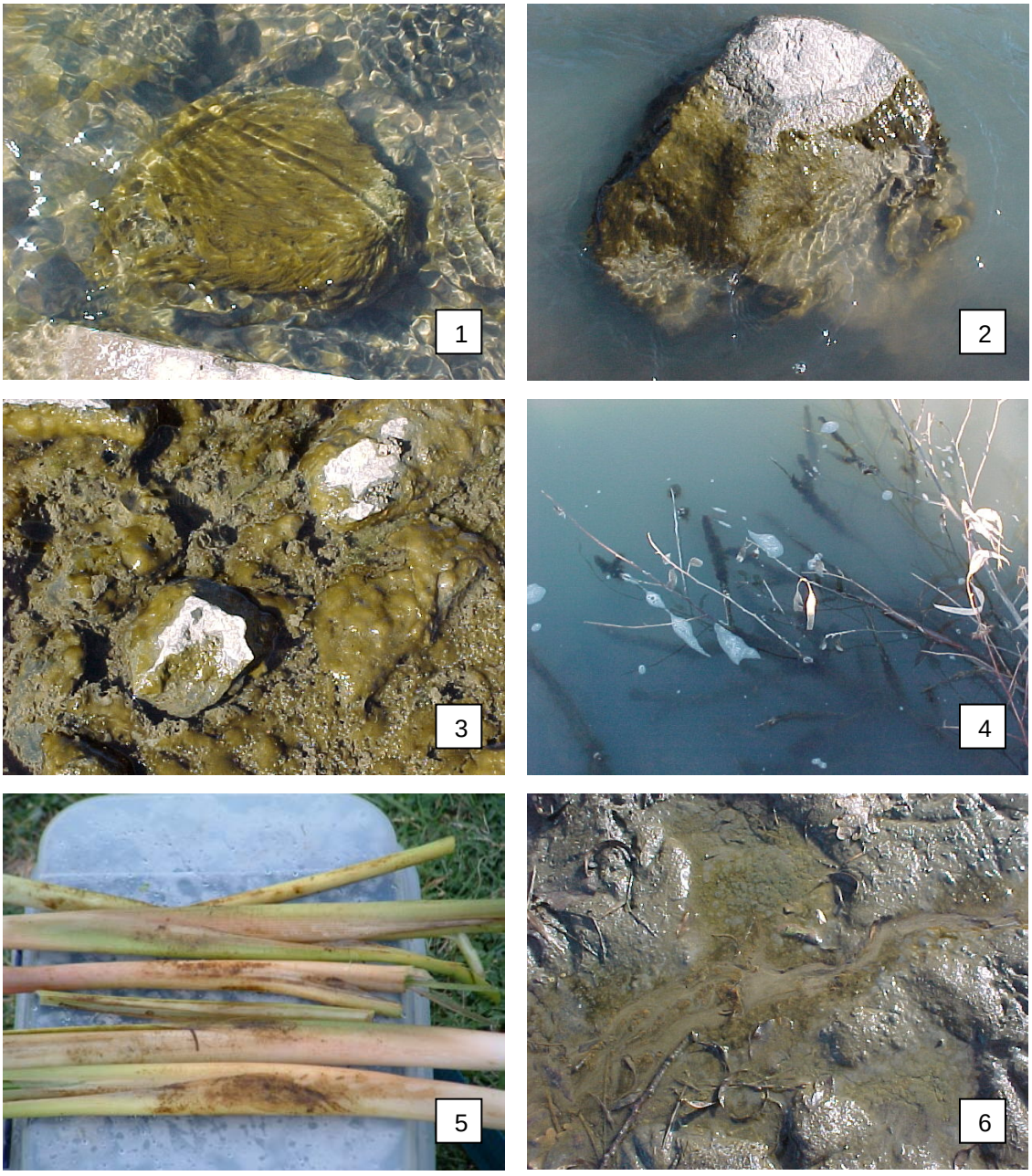


Fig. 1 and **Fig. 2** show a thick layer of diatom cells attached to boulders. **Fig. 3** shows a layer of diatom cells growing both on sediment and on pebbles. **Fig. 4** shows diatoms growing thickly around submerged tree branches. **Fig. 5** shows the film of diatoms to be found on the submerged stems of *Phragmites australis*. **Fig. 6** shows diatoms inhabiting sediments.

3. Diatoms – Living cells with a role in aquatic food webs

Diatoms are a key component of aquatic ecosystems and constitute a fundamental link between primary (autotrophic) and secondary (heterotrophic) production. Many micro-organisms feed on diatoms and in this way they are integrated into aquatic food webs. Diatoms are frequently used as bio-indicators, and if they are not investigated live they may be perceived simply as “glass boxes” used to give information about water quality. It is worth the time to study the living communities and to note the other algae and the interactions between the algae and other micro-organisms.

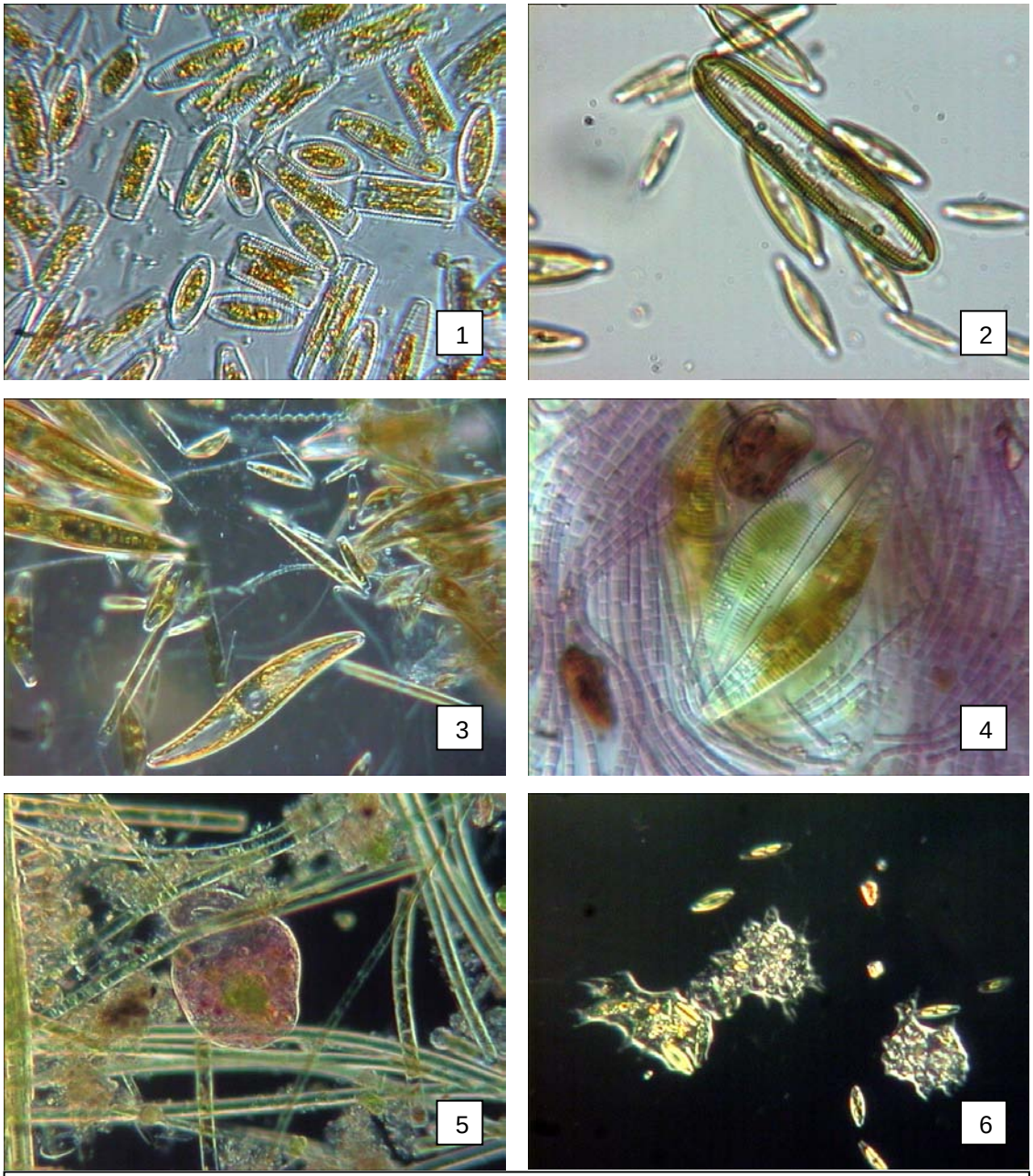


Fig. 1 a diatom community completely dominated by *Diatoma vulgaris* **Fig. 2** a sediment diatom community with *Navicula* spp. and *Pinnularia viridis*. **Fig. 3** mixed diatom community with large cells of *Gyrosigma* sp. **Fig. 4** shows cells of *Cymbella* sp. living in association with the blue-green algae *Oscillatoria*. **Fig. 5** shows the filamentous diatom *Aulacosiera granulata* being grazed by a protozoan. **Fig. 6** shows diatoms being grazed by *Amoeba* sp.

4. Diatoms – Colony formation and attachment

Diatoms release mucilage through various structures in the cell wall to facilitate locomotion or attachment of the cells to various substrata. Mucilage secretions can also be used to form colonies of various patterns. This material must be eliminated for microscopic detailed examination of the cell wall. After a diatom sample has undergone the necessary steps to prepare it for light microscopy at high magnifications all that can be seen is a silica structure. This skeleton or cell wall is typically referred to as the **frustule**. Chemical treatment eliminates all organic material from both inside as well as outside the cell walls.

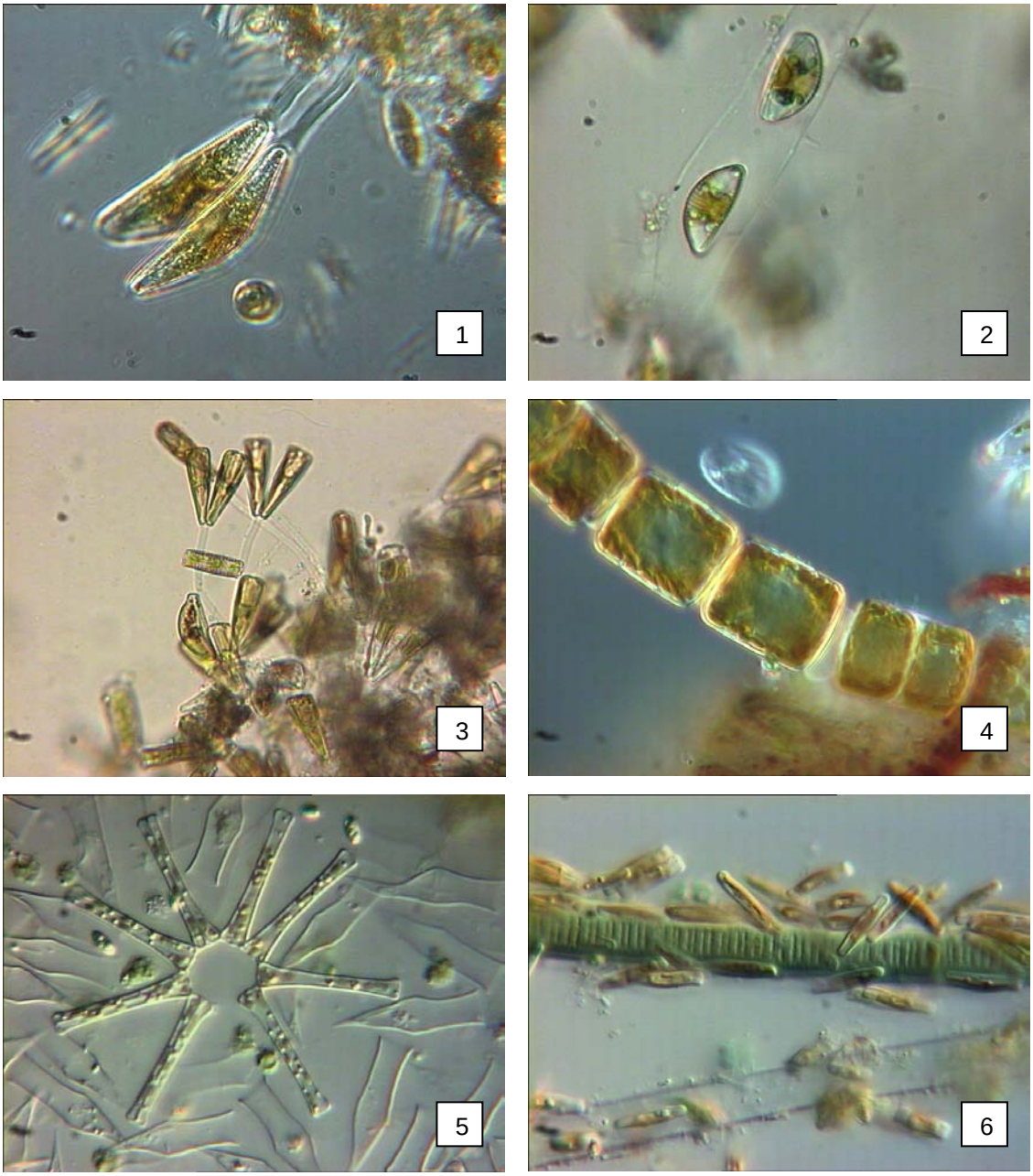
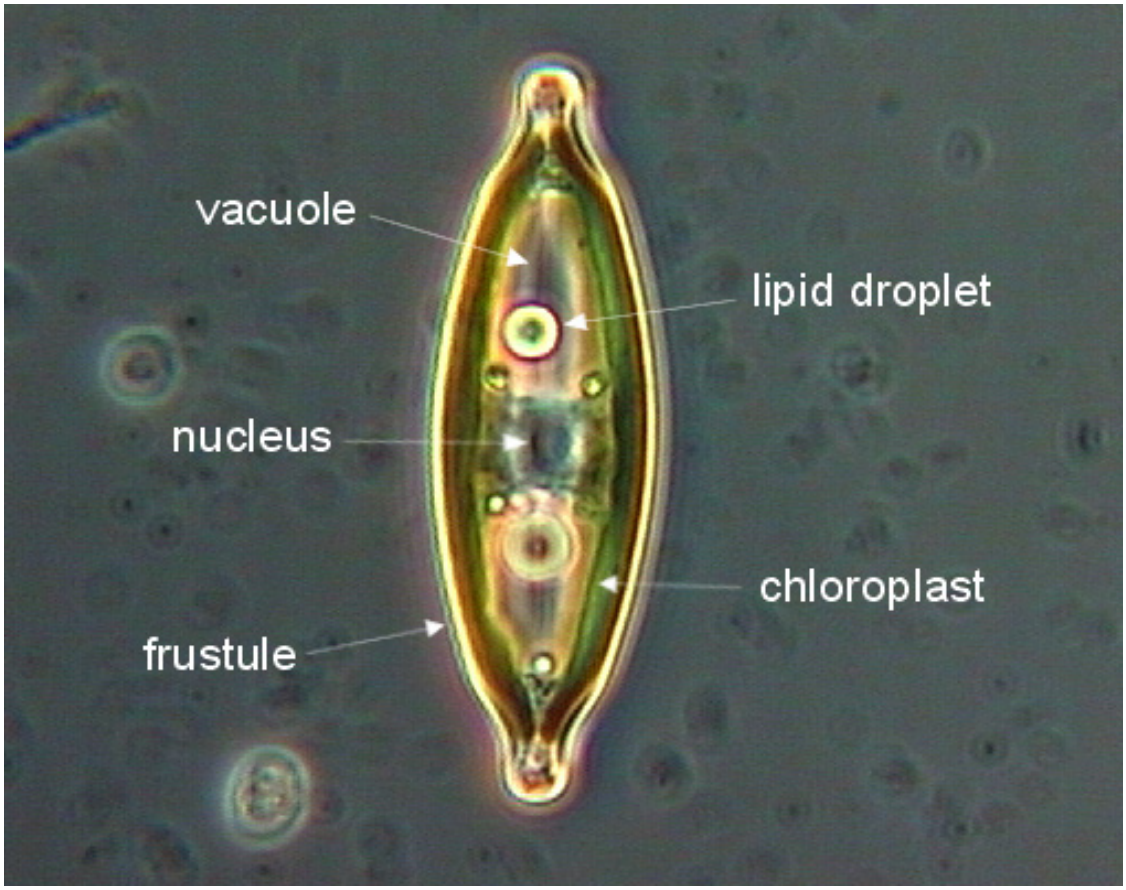


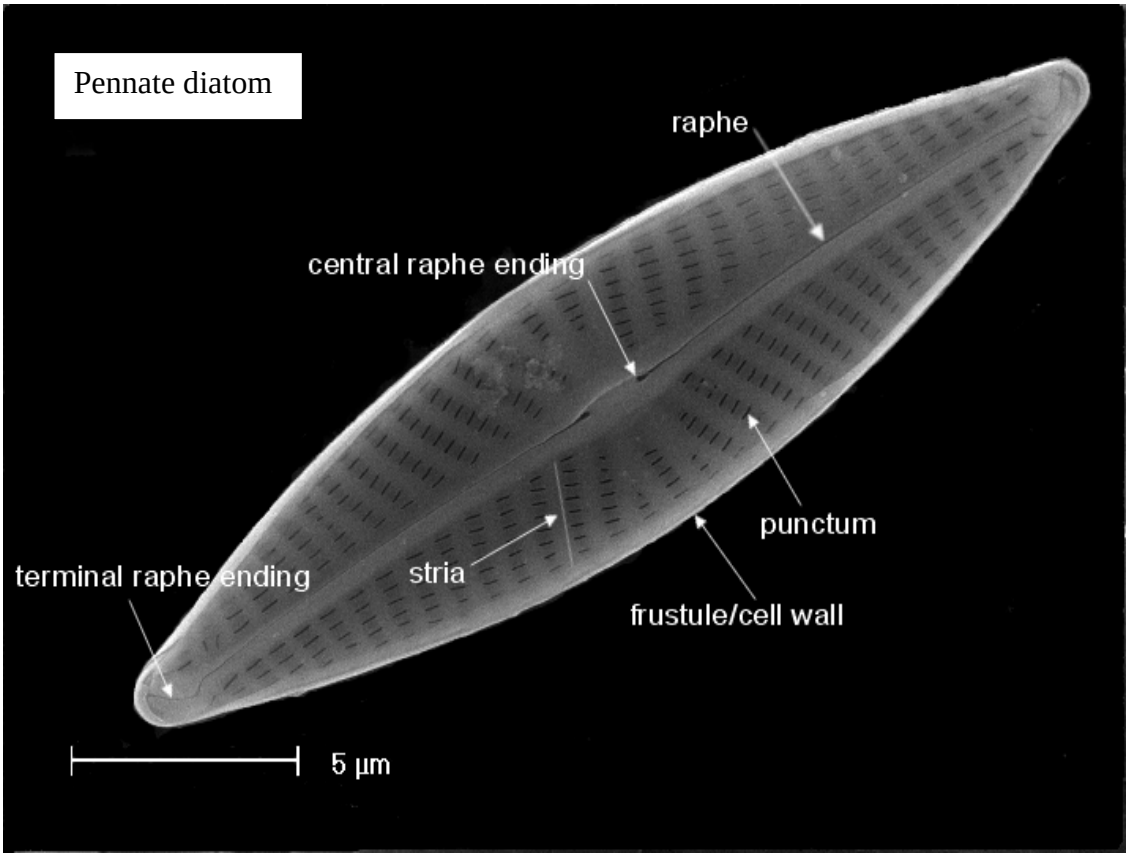
Fig. 1 shows the attachment of *Cymbella* sp. to a substratum with a mucilage stalks. **Fig. 2** shows *Encyonema caespitosum* inhabiting a mucilage tube. **Fig. 3** shows the dichotomously branching mucilage stalks to which cells of *Gomphonema* sp. are attached. **Fig. 4** *Melosira varians* with cells attached both to the substratum and each other by mucilage pads. **Fig. 5** stellar colonies of the diatom *Asterionella formosa*. **Fig. 6** *Achnanthidium minutissimum* attached by means of mucilage stalks to *Lyngbya* sp.

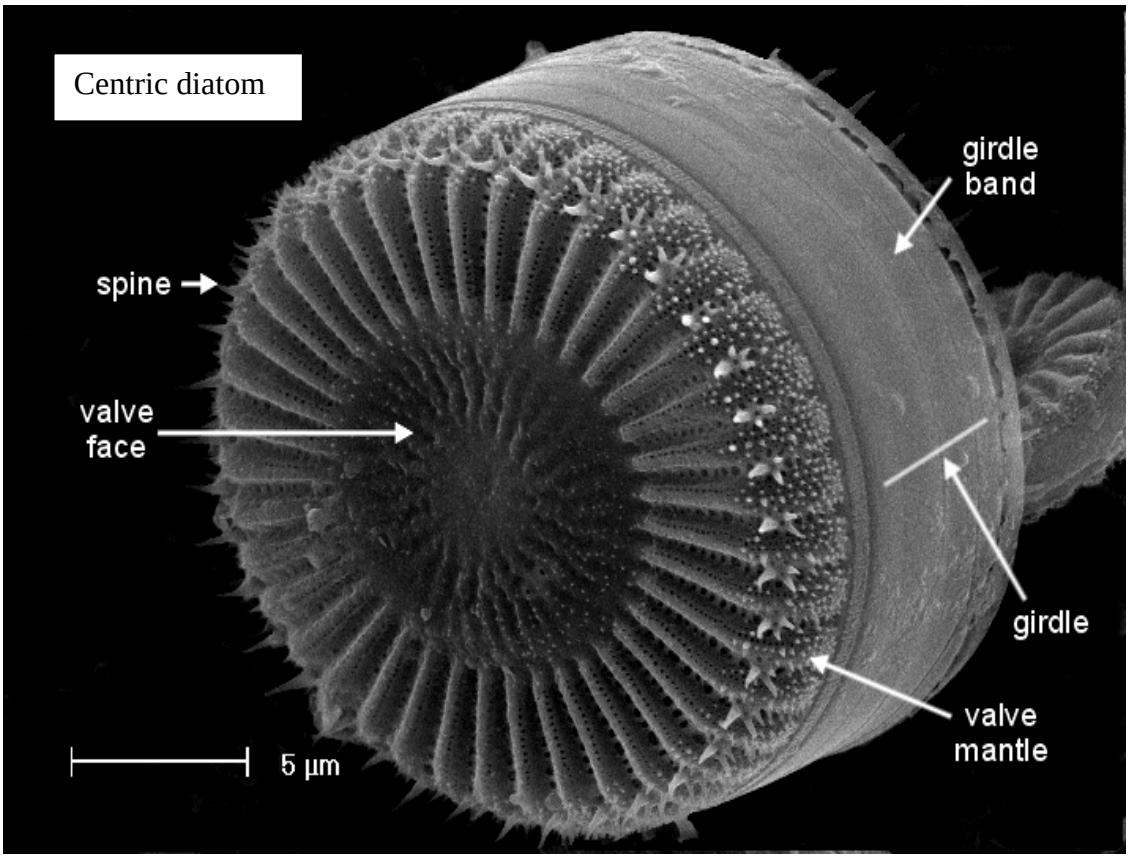
5. Diatom frustules – What do diatoms look like?

Diatoms are unicellular algae that occur mostly as single cells but some species form colonies. They have certain features which make them unique amongst the algae. The particular features include the siliceous cell wall (frustule) the possession of unique photosynthetic pigments and specific storage products (oil and chrysolaminarin). There are two groups of diatom common in freshwaters namely the **centric diatom** species which are in general circular in shape and adapted to live in the water column as part of the phytoplankton and the **pennate diatoms** that live in benthic habitats but are often temporarily re-suspended in the water column.



5.1 Pennate and Centric diatoms





6. What can you expect to see when viewing a prepared diatom slide?

A series of neatly aligned pictures that have been cropped and graphically enhanced are normally displayed to illustrate diatom taxa in books, manuals and guides. Whole cells are usually illustrated in valve view in such guides and most of the morphological characteristics are visible. Fragments or broken pieces are not normally shown. However, your slides will have diatom cells that are orientated at different angles, often lying obliquely or in girdle view and some may be damaged or fractured fragments. Different types of microscope illumination may also provide slightly different images to those found in routine identification guides.

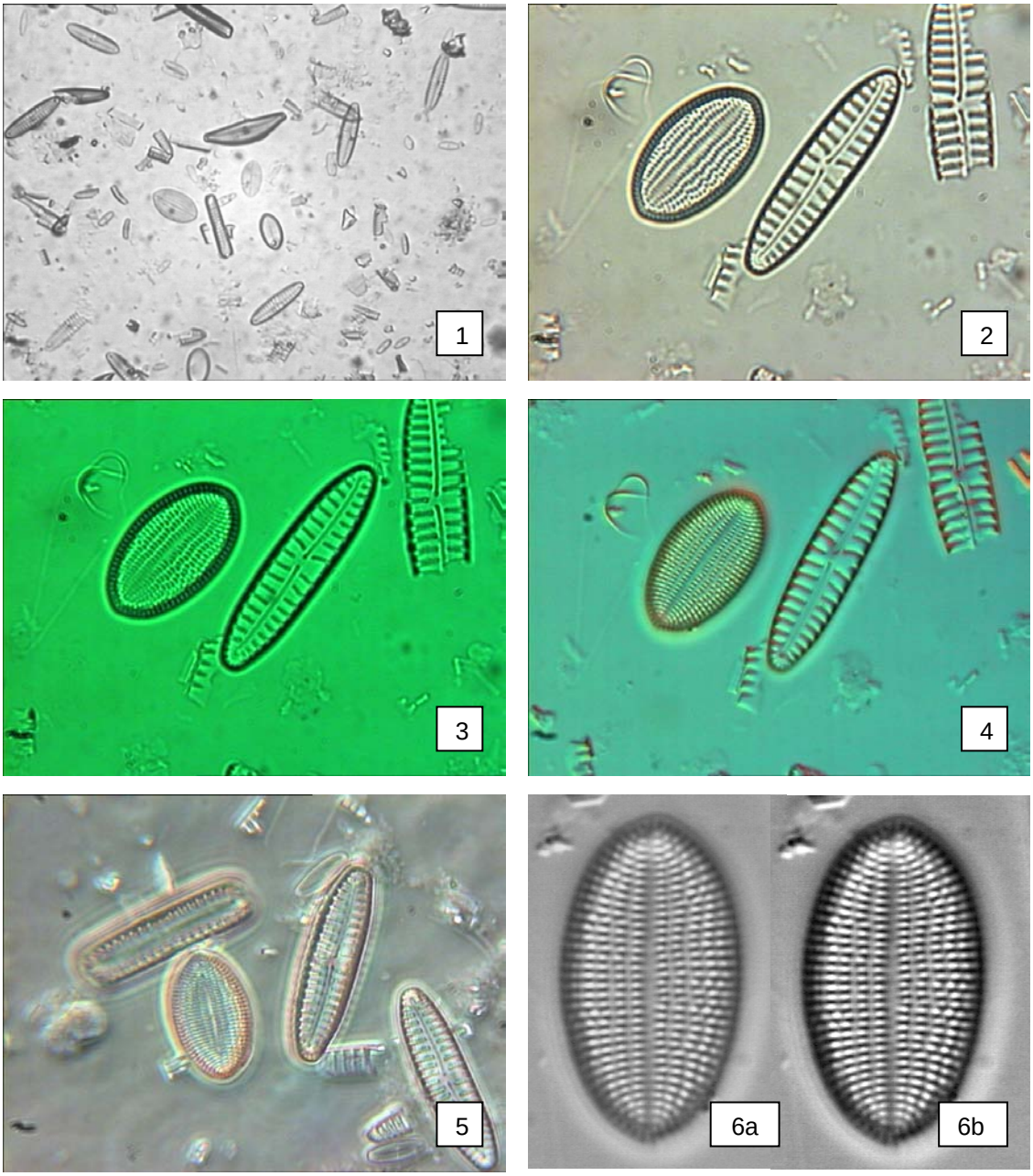


Fig. 1 shows a scattered slide mount of diatoms under low magnification. **Fig. 2** shows the same mount under high magnification (x1000) using incident light. **Fig. 3** shows the same as **Fig 2** but a green filter is used to increase contrast. **Fig. 4** shows the use of differential interference (DIC) optics. **Fig. 5** shows the use of Phase contrast optics and **Fig 6a** shows Fig. 4 correctly orientated, cropped and converted to greyscale, while **6b** shows digital enhancement and contrast correction.

SECTION 2: FIELD PROCEDURES

To facilitate the reading of this document references have been kept to a minimum. However it should be mentioned that the methods described below have been based on several key documents including: Kelly *et al.* (1998), CEN (2003), DARES (2004) and Taylor *et al*. (2005).

Health and safety of field operators (practical advice)

Diatom sampling should be both enjoyable and informative, however, there are attendant risks involved with these activities. The following points should be noted:

1. Always wear thigh waders or some other form of protection for your feet.
2. Always wear a life jacket while sampling.
3. Never sample in parts of the river which are out of your depth.
4. When sampling rivers which may be heavily polluted or polluted with faecal matter, be sure to always wear gloves.
5. When sampling in regions such as KwaZulu Natal, Limpopo and Mpumalanga care should be taken to avoid crocodiles and hippopotami. Great care should be exercised when sampling. These animals pose a very real threat to people and are attracted to activities at the sides of rivers and lakes.
6. In some rivers leeches may also be abundant.

1. Habitats for diatom-based water quality monitoring

The four distinct diatom assemblages that occur closely associated with particular microhabitats are generally recognised as the:

- Epipelon that frequents the surface of the sediments
- Epipsammon that occurs on and between the sand particles
- Epilithon that inhabit gravel, stone and bedrock
- Epiphyton that is attached to macrophytic plants

Diatom community structure is governed to some extent by substrata associations but there are other important influences on community composition, namely:

- Chemical constituents in the water
- Water turbulence and disturbance (mainly from floods)
- Resource supply (mainly from inorganic nutrients)
- Grazing by micro-organisms
- Light regime within microhabitats

1.1 Preferred substratum

Cobbles and small boulders (rocks) are the preferred substratum for monitoring diatoms in the riverine environment, and almost all diatom indices throughout the world can be applied to the community (i.e. the epilithon) that is found on this substratum.

The most important reasons for this choice of substratum can be summarised as follows:

- Cobbles and small boulders are generally widely available (riffles, cobble beds, benches and shelves), throughout the length of a river from headwaters to lowland stretches, and throughout the year.
- The type of stone sampled can usually be discounted when assessing the flora at a particular site.
- The performance of major diatom-based indices on this substratum is well understood.
- The ecology of the epilithon is better known than any other group.

1.2 Alternative substrata (in order of preference)

- Man made objects (bricks, pieces of concrete, bridge supports, cannel walls etc.).
- Emergent macrophytes, such as *Typha* spp. or *Phragmites* spp.
- Submerged macrophytes, such as *Potamogeton* spp, *Ceratophyllum* spp. etc. may be used as an alternative substratum.

1.3 Introduced substrata

If pebbles, cobbles, boulders or macrophytes are absent from the sample site, artificial substrata may be introduced into the stream. However sampling should **only** be attempted **if** they have been submerged for at least four weeks.

The advantages of using introduced substrata include:

- the ease of sampling from smooth surfaces,
- greater control over the exact area of sampling,
- standardisation of substrata,
- less contamination by macrophytic algal growth and
- the introduced substratum can be positioned exactly.

Some disadvantages to using artificial substrata include:

- The community will be somewhat unnatural and biased towards those diatoms which are fast growing and can attach to flat, smooth surfaces,

- depending on the period of exposure prior to sampling, the flora may not represent a 'climax' community,
- the smooth surfaces of some artificial substrata often lead to 'sloughing off' of the diatom film.
- substrata are often lost, removed or vandalised if the substratum is not fixed in position. An appropriate method and apparatus needs to be devised for each site.
- artificial substrata need to be immersed in the river for at least **four weeks** before sampling (although this period is dependent on the trophic status of the water). This causes a delay in the availability of data, as well as adding to the cost of the monitoring program as transport costs to and from the site in question are doubled.

  Further information about the use and application of artificial substrata can be found in Cattaneo and Amireault (1992), Gold *et al* (2002) and Lane *et al*. (2003).

2. Sampling for aquatic bio-diversity studies

All the methods mentioned in this manual can be used for sampling diatoms from different habitats for biodiversity studies. However, certain techniques are less suitable when sampling diatoms to infer water quality. Phytoplankton drifts downstream and thus is not as stable or reliable as the phytobenthos if an indication of a water quality impact at a specific point is required.

2.1 Cobbles and small boulders (rocks)

See section 1.1.

2.2 Phytobenthos ("Floatation method" for epipsammon and epipelon)

The epipsammon and epipelon are components of the phytobenthos and yield very diverse assemblages of usually motile diatoms. However, the "floatation method" discussed below does not allow for the inclusion in the analysis of non-motile diatoms. The method has the considerable advantage of extracting the **motile living fraction** of the diatom community for subsequent analysis of the assemblage. Samples taken from the epilithon may contain many attached and non-motile species which cannot be removed from the sample in the manner below.

2.3 Qualitative sampling of sediments

The common method, described by Round (1991) is to use 5 mm Ø glass tube about a meter long or more attached (splinted) to a rod (e.g. a broomstick) for deeper water at the margin of a river.

Sampling may be achieved as follows:

- Place a finger over the top end of the tubing and insert the bottom end under the water and rest it on the sediment.
- Release the finger pressure as the tube is drawn lightly over the sediment surface horizontally (for about one meter) - as if gently scraping a line on the surface of the sediment.
- The pressure of the water will push the sediment material (with diatoms) into the tube.
- Seal the top of the tube with your finger (to prevent loss of sample before you remove it from the water) and carefully swing the tube out and transfer the collected material into a sample bottle.

An alternative to this procedure in shallow water is to use a large syringe attached to the upper end of a flexible latex tube. The contact end of the latex tubing is cut at an angle to allow for oblique contact with the sediment containing the diatoms. Careful syringing action will ensure that diatom material is sucked up with some surface sediment and this can be discharged into an appropriate sample bottle until sufficient material is obtained.

Rapid qualitative sampling can also be achieved by scraping the surface of damp sediments to a depth of 1 cm in several smaller areas in the stream bed. The accumulated material can be stored in a damp environment within an ice-cream dish.

2.3.1 Quantitative sampling of sediments

To determine the biomass of a community (as reflected by chlorophyll measurements or cell counts) quantitative samples are required and may be collected as follows:

- Press the bevelled bottom end of a clear Perspex tube (~ 50 cm long and 20 mm in diameter) into the sediment or sand and carefully section out a 1 cm deep core.
- Remove the top 1 cm of the core containing surface diatoms using an extruder (i.e. push the sediment out from the lower opening upwards).
- The 1 cm surface core of the sediment sample usually retains its integrity as you remove the sample unless the grains are very large and loosely compacted or too dry.
- Typically, five 1 cm cores should be collected randomly for each sampling area.

Note: Cores collected in this manner can be used for chlorophyll ‘a’ analysis if placed into a bottle containing 90% acetone. If the habitat is available this is the most suitable technique for quantitative comparison of diatom populations between sites and over time.

2.3.2 Examination of fresh material and extraction of diatoms for acid treatment

The **living, motile component** of the sampled diatom population may be extracted and separated in the following manner:

- The fresh sediment/diatom mix is spread over the bottom of a petri dish or flat-bottomed plastic tray and the heavier sediment is allowed to settle for a few hours (e.g. overnight).
- The following day the excess supernatant is drained from the petri dish until the moist sediment is exposed.
- Several coverslips are allowed to gently 'float' and rest on the damp sediments for a 4 hour period of exposure to natural light.
- The coverslips are then carefully removed and gently rinsed to remove unwanted sand particles.
- The coverslips are placed on a clean slide for examination of diatom cells.

2.3.2.1 Alternative separation techniques

- *Tissue paper*

If the original sample contains large sand grains, it is advisable to place tissue paper between the coverslip and the sediment. This allows the passage of the motile diatoms on to the coverslip but prevents the transfer of unwanted sediment grains to the slide.

- *Submersed coverslips*

It is not necessary to remove all the supernatant from the fresh material if living diatoms are not required for initial examination. Coverslips are submerged and 'floated' on to the sediment/diatom mix after the material has settled in a tray / petri dish. Living diatoms actively adhere to the surface of the coverslip under the water. This technique ensures that sand grains are washed off the coverslips as they are carefully withdrawn and placed in a sample bottle containing ethanol for preservation or allowed to air dry for acid treatment.

2.4 Phytoplankton

Phytoplankton sampling can be achieved in one of two ways. The most simple method is to collect water in a two litre container, add preservative (Lugol's iodine), and allow the dead planktonic organisms to settle out. The sedimentation rate of most phytoplankton allows for complete settling within 16-24 hours from a two litre measuring cylinder.

Alternatively a plankton net may be used with a mesh size of not more than 25 µm. The plankton net should be dragged back and forth just below the surface of standing waters or held in the stream of moving waters for a few minutes. This should allow for the collection of

ample cells. The contents of the net should then be emptied into a wide-mouthed plastic storage bottle and preservative added if required (see Section 11 for further details).

In standing waters (such as dams, lakes, estuaries) one should be aware of vertical stratification of phytoplankton during certain times of the day. Where the system is deep enough (i.e. >10m) take a vertical haul with the plankton net over a 5 metre depth to cover the zone where light penetration is sufficient (euphotic zone) to encourage algal growth.

2.5 Terrestrial or soil diatoms

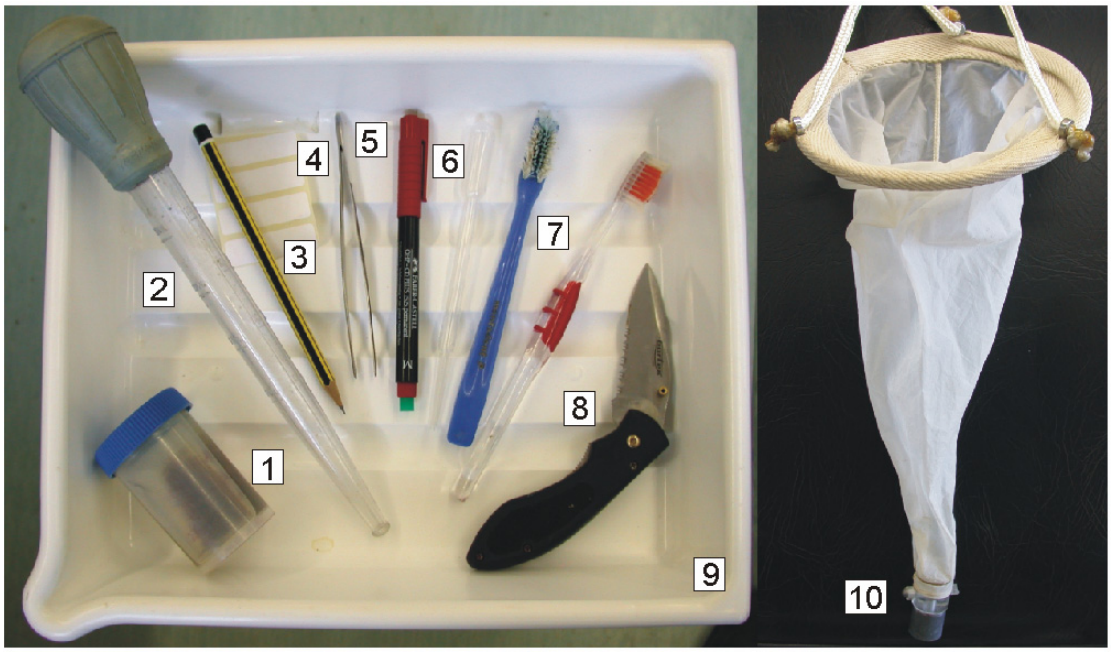
Soil diatoms and aerophilic diatoms have seldom been investigated in South Africa. These diatoms are an interesting group with many adaptations for the arid climate in which they live.

Soil diatoms can be collected from moist sub-aerial habitats, as well as from aerial and arid aerial habitats. Six sub-samples (± 5 cm^2) should be collected within a 10 m radius. Once the site has been selected detritus or other material covering the surface of the soil should be carefully moved aside. The soil should be collected to a depth of about 1 cm using a knife, spoon, perspex corer or other similar implement (a slightly concave butter knife is ideal!). The six sub-samples should, in total, amount to about 200 grams of soil. This soil should be stored in a paper envelope to prevent the build up of moisture which promotes the growth of undesirable fungi.

In order to separate the diatoms from the soil, a portion of the soil sample is placed in a sterile petri dish and wetted with distilled water until the soil is saturated. One or two wettings may be required before the soil becomes saturated depending on the amount of organic material present. Once the soil is saturated it should be left for several days exposed to light (but not in direct sunlight), where after pre-cleaned coverslips can be placed gently on the surface of the soil. After two weeks the coverslips can be removed and the living cells examined under the microscope. If cleaned material is required the coverslips may be treated using any of the methods detailed below under Laboratory Procedures. A simple method to check for the presence of diatoms is to invert the coverslips and heat them until the organic content is burned away (incineration). Permanent mounts can then be made in the standard fashion see Laboratory Procedures, section 2. Many soil diatoms are very small and are best examined under the scanning electron microscope (SEM) - see details of preparation techniques below.

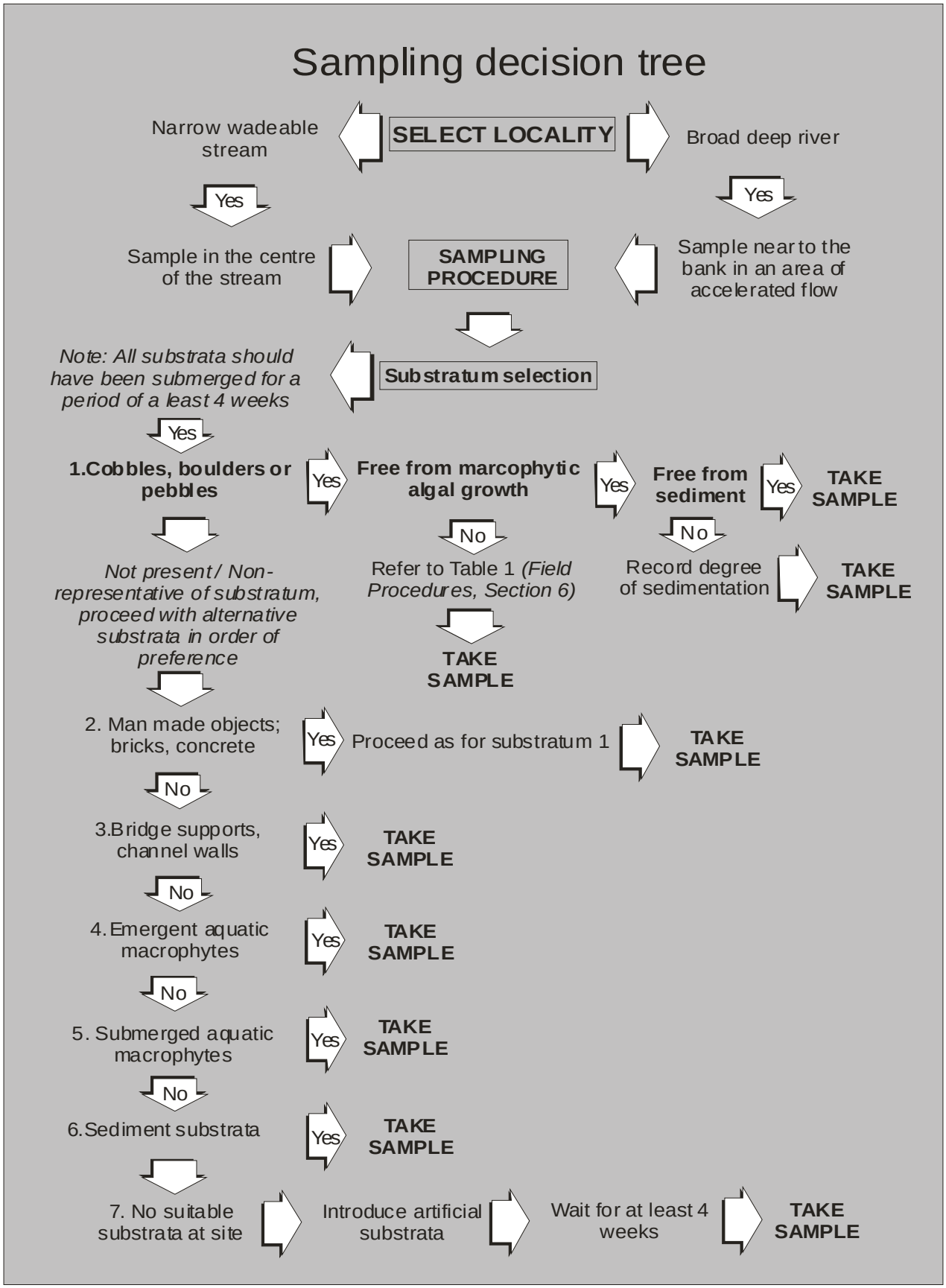
3. Toolkit for Fieldwork (field apparatus)

- Plastic tray / clean 2 litre ice cream dishes with lids
- Tooth brush or other similar brush
- Knife or spoon
- Envelopes (for soil samples)
- Wide mouth sample bottles (~100 ml)
- "Zip-lock" type plastic bags
- Plankton net
- Waterproof marking pen or labels
- Field note book/field record forms
- Pipettes
- Depth gauging 'broomstick' / rod
- 100 ml syringe fitted with latex tubing / Turkey baster
- Clear Perspex tubes (25 mm diameter)
- Camera



1: Wide-mouth sampling bottle ~ 100 ml. **2:** Turkey baster; useful for collecting sediment samples. **3:** Pencil and labels, ethanol does not dissolve pencil markings. **4:** Forceps for picking up filamentous algae and detritus. **5:** Water-proof marking pen. **6:** Plastic Pasteur-pipette useful for collecting small amounts of sediment. **7:** Toothbrushes for scrubbing solid substrata. **8:** Knife for cutting the stems of aquatic vegetation. **9:** White plastic tray with lip. **10:** Fine-mesh plankton net.

4. Decision 'tree' for sampling for water quality monitoring



5. Site selection for water quality monitoring - Principles

The number and location of sampling sites should be designed according to the extent and aims of the survey. Sites should be selected to provide representative samples, preferably where marked changes in water quality are likely to occur or where there are distinctive river features or human activities - for example confluences of sub catchments, major effluent or dam discharges, flow regime changes through abstraction or flow augmentation from interbasin transfers. Sampling both upstream and downstream of discharge points should be carried out if sampling is intended to monitor the effects of such disturbances. Sampling should extend for an appropriate distance downstream to assess the effects on the river and its potential recovery.

Experience has shown that, in South African inland waters, diatom communities are at the peak of their development in mid-winter to early spring. In addition, when sampling during the winter low-flow regimes (in summer rainfall regions), water levels are receding rather than rising and therefore the submerged substrata should have well-developed diatom communities. Care, however, should be taken to avoid sampling after heavy or prolonged rain events because scouring by high flows can displace diatom communities. Sampling conditions in rivers may be less favourable at the height of the wet season due to the frequency of flood events.

Sites for stream biomonitoring should be, **if possible**, in a “riffle”, where the water is flowing over stones. However, “runs” and “glides” are also suitable if these have suitable substrata

Sampling in riffles or areas of moderate or high water velocity ensures continuous exchange of the water surrounding the algae and prevents the build-up of a local chemical environment. Furthermore, it prevents sedimentation of drifting organisms and particles, with the result that only organisms living at that particular spot will be collected. The above recommendations have, however, been made with wadeable rivers in mind and may not be applicable at all times to deep rivers.

In broad, deep, slow-flowing rivers, such as the Vaal and Orange Rivers which are not wadeable, cobbles or other substrata may be collected close to the riverbank from riffles with flowing water or where flow is rapid enough (>20 cm sec^{-1}). The flowing water at the edge of the main stream (littoral zone) is assumed to be of the same physical and chemical quality as that in the main steam. Cobbles and boulders (but not macrophytes) should be gently agitated in the river for a few seconds before removal. This should remove any surface contamination, including small particles of organic matter and sediment.

The following aspects should be considered before selecting the reach and specific substrata to be sampled:

- Slight differences may occur between substrata from shallow water and those from deeper water although there is a reasonably uniform distribution of the diatom flora at any given sampling point. For this reason, sampling from depths greater than one metre should be avoided, especially in turbid rivers where the euphotic zone (zone of effective light penetration) may not extend to the riverbed. The performance of diatom indices is not affected at depths of up to 0.5 m, provided that this is still within the euphotic zone.
- Boulders with filamentous green algal growth should be avoided **if possible**, because these growths of algae may support other unique diatom communities. However, if the majority of the substratum is covered with filamentous green algae, sampling from un-covered substrata would be non-representative. If this is the case, follow the recommendations given below in Table 1.

TABLE 1 (DARES, 2004)	
Percent cover of filamentous green algae	**Number of cobbles**
< 15%	0
≥ 15 < 29	1
≥ 30 < 44	2
≥ 45 < 59	3
≥ 60 < 75	4
<75	5

- Boulders covered with a layer of sediment should also be avoided, **if possible**, as fine sediments may modify substrate conditions. However, in lowland rivers it may be difficult to avoid such boulders. The river bed in some coastal flood plains may be choked with sand and sediment.
- Although colonisation rates of diatoms are slower in fast-flowing than in slow-flowing rivers, current speeds of 0.1 to 1.6 $m.s^{-1}$ have little or no limiting effect on the performance of diatom indices.
- Repeated sampling at the same site requires the marking of sites with natural landmarks in relation to immovable structures such as bridges.
- To ensure the comparability of samples from other sites, the conditions of light, current velocity, substratum etc. should be as similar as possible.

6. Sampling locality details and field notes/forms

Sample Field Record Form (modified from DARES, 2004)

River: _____ Site: _____ Date: _____

DWAF #: _____ Sample collected by: _____

Co-ordinates: _____ Elevation:_____

Physical records

Width _____ **Depth:**_____

Substrate (record estimated percentage)

bedrock ☐ boulders/cobbles ☐ pebbles/gravel ☐

sand ☐ silt/clay ☐ peat ☐

Estimate percentage of boulders and cobbles covered by:

Filamentous algae: ☐ other macrophytes ☐

Shading (record estimated percentage)

Left bank None ☐ Broken ☐ Dense ☐

Right bank None ☐ Broken ☐ Dense ☐

Habitat Pool ☐ Run ☐ Riffle ☐ Slack ☐

Water clarity Clear ☐ Cloudy ☐ Turbid ☐

Bed stability Firm ☐ Stable ☐ Unstable ☐ Soft ☐

Time since last spate

< 3 days ☐ 3 - 7 days ☐ 7 - 14 days ☐ > 14 days ☐

not known ☐

Photograph Facing upstream _____ Facing downstream _____

NB *It is important to include an immovable structure in a photograph as a referencefor future comparison e.g. a bridge*

Use the reverse of this sheet for sketch map and other comments

7. Useful water quality variables and information to collect concurrently with diatom samples for diatom index validation.

Note: *The choice of which of the following variables need to be sampled will depend on the design and outcomes of the particular study as well as monetary constraints.*

7.1 Hydrological characteristics of the stream

7.1.1 Stream velocity

7.1.2 Channel depth

7.1.3 Channel breadth

7.2 Physical variables

7.2.1 Water temperature

7.2.2 Turbidity

7.3 Physico-chemical variables

7.3.1 pH, Conductivity/Total dissolved solids (TDS)

7.3.2 Nutrients

7.3.2.1 Orthophosphate-phosphorus (PO_4-P), Total phosphate (TP)

7.3.2.2 Ammonium-nitrogen (NH_4-N), Nitrite-nitrogen (NO_2-N), Nitrate-nitrogen (NO_3-N), Total Kjeldahl nitrogen (TKN)

7.3.3 **Major Cation/Anions** ***(****Budget constraints may provide for Conductivity values only; Chlorides, sulphates and/or potassium may be essential to detect human intervention.*

7.3.3.1 Magnesium (Mg^{2+}), Calcium (Ca^{2+}), Sodium (Na^{+}), Chloride (Cl^{-})

7.3.3.2 Sulphates (SO_4^{-})

7.3.4 Measures of Oxygen and Organic matter

7.3.5 Oxygen saturation

7.3.6 Chemical Oxygen Demand (COD) *(preferred parameter for assessing performance of sewage/industrial effluents and is aligned with DWAF monitoring programmes).*

7.3.6.1 Biological Oxygen Demand - 5-day (BOD_5), Total Organic Carbon (TOC)

8. Choice of substrata (detail)

Sampling should be representative rather than random. Operators should first decide which areas in a river reach should be excluded and then search within the remaining areas for substrata with obvious diatom growths, either by appearance or by feel.

Diatom growths can be identified by a golden-brown coloured mucilaginous layer on the substratum or, if this is not visible, by the feel of the rocks, which will be slimy or slippery because of the mucilage exuded by the diatoms for locomotion or attachment (see Introduction).

8.1 Samples should be taken from five or more cobbles (diameter > 64, ≤ 265 mm) or small boulders (> 256 mm diameter) **where possible**.

8.2 It is also acceptable to sample vertical faces of man-made structures such as quays and bridge supports in the absence of appropriate stones at a particular site. Other hard man-made surfaces, such as bricks, can also be sampled.

8.3 Alternative substrata, such as submerged or aquatic macrophytes, can also be sampled, providing the stems are permanently submerged and not contaminated with sediment. The type of macrophyte from which the sample is taken should always be noted because it is important to sample the same species or, if this is not possible, the same morphological type of macrophyte.

A useful identification guide for the identification of aquatic macrophytes is:

Gerber A, Cilliers CJ, van Ginkel Cand Glen R (2004) ***Easy identification of aquatic plants. A guide for the identification of water plants in and around South African impoundments****. Department of Water Affairs, Pretoria.*

This publication is available from:

Director; Resource Quality Services (RQS)
Department of Water Affairs and Forestry
Private Bag X 313, Pretoria 0001

Tel: 012 808 9500
or:
Annelise Gerber
gerbera@dwaf.gov.za

8.4 In order to compare downstream community composition, it is important to sample from similar substrata along a river, as diatom communities vary according to substratum

8.5 Samples should be taken in such a way as to obtain the greatest possible degree of uniformity between sites.

9. Sampling

9.1 Solid substrata

9.1.1 Five to ten cobbles, boulders, pebbles or other substrata of similar proportions should be collected from a reach of at least 10 m in the river or stream.

9.1.2 Gently rinse the substrata in the stream and carefully place in a sampling tray on the river bank, together with about 50 ml of stream water.

NB If time limitations and safety factors are a concern; the cobbles/stones can be placed in a large dish (e.g. an ice-cream container) and removed from the site for attention in a safer environment.

9.1.3 Diatoms should be removed by vigorously scrubbing the **upper surface** of the substratum with a small brush (e.g. clean toothbrush) to dislodge the diatom community. Some diatomists prefer to scrape the substrata with a knife or a spoon as these implements are easier to clean and reduce the possibility of contamination between sites.

9.1.4 Only the upper side (the side most exposed to flowing water) of boulders should be scrubbed to avoid contamination with sediment that might be present on the undersides of the cobbles.

9.1.5 The resulting diatom suspension is then poured into a **labelled** wide-mouth plastic sample bottle of 100 ml capacity or greater.

9.1.6 Care should be taken to avoid equipment contamination between sites by rinsing both the toothbrush and the plastic tray in the river both **before and after** taking the diatom sample.

9.2 Sampling from emergent aquatic macrophytes

9.2.1 The emergent macrophyte stem is cut with a knife above the water line.

9.2.2 A plastic bottle is then inverted over the remainder of the stem and the stem is cut slightly above the point where it emerges from the sediment.

9.2.3 The bottle is inverted and brought to the bank.

9.2.4 This procedure needs to be repeated until five stems have been collected.

9.2.5 Scrubbing and removal of the diatom communities can then proceed in a similar fashion to that described above for solid substrata (see 10)

9.3 Sampling from submerged aquatic macrophytes

9.3.1 Select replicates from five different plants growing in the main flow of the river.

9.3.2 Each replicate, consisting of a single stem plus associated branches of the plant from the lowest healthy leaves to the tip, should be placed in a plastic bag together with 50 ml of stream water. Diatoms should be visible as a brown film associated with the macrophytes (see Introduction).

9.3.3 The plants should be shaken vigorously and squeezed in the plastic bag and the resulting brown suspension poured into a sample bottle.

10 Preservation of diatom material and labelling samples

Fresh diatom samples should be stored in the following manner:

- In a refrigerator if the period of storage is to be less 24 hours.
- If the samples are not going to be analysed immediately the samples may be fixed with **Lugol's iodine**, which may be used for short-term storage. *(Lugol's iodine is preferred if material is to be examined prior to cleaning and should be added to reach a final concentration of 1% by volume).*
- Lugol's Iodine can be prepared by dissolving 2 g potassium iodide and 1 g iodine crystals in 300 ml distilled water.
- An alternative to Lugol's iodine is **ethanol.** Ethanol should be added to reach a final concentration of 20% by volume. Adding ethanol to a sample will destroy the chloroplasts.
- Ethanol is recommended for the long term preservation of un-cleaned material.
- **Formalin** is **NOT RECOMMENDED** as a preservative although it is commonly used for other algal samples. It should be **avoided** for diatom samples as it is **carcinogenic** and in addition, even very weak formalin solutions might damage the fine structure of diatoms.

SECTION 3: LABORATORY PROCEDURES

To facilitate the reading of this document references have been kept to a minimum. However it should be mentioned that the methods described below have been based on several key documents including: Kelly *et al.* (1998), CEN (2004), DARES (2004) and Taylor *et al*. (2005).

Health and safety of laboratory staff (general comments)

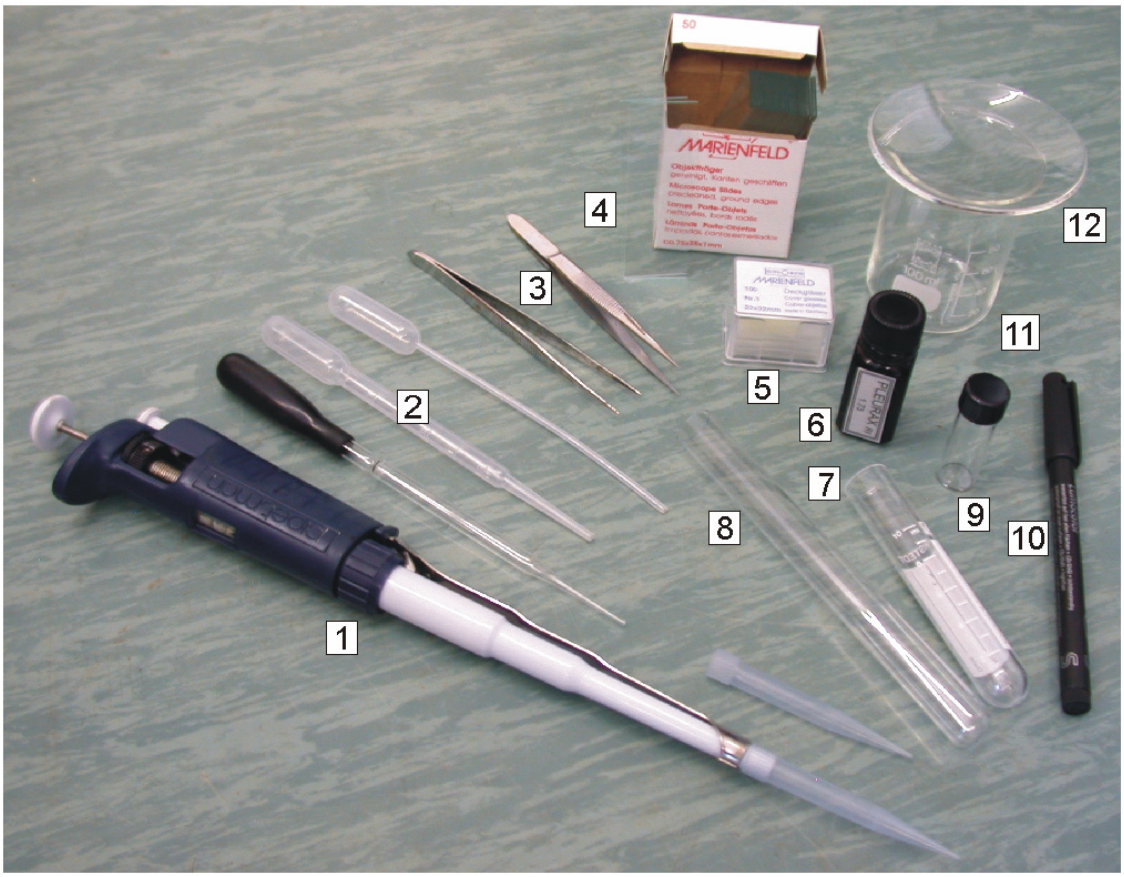
The specific health and safety risks involved in each of the diatom preparation methods will be dealt with below. However, several general rules apply for safe laboratory practice:

- Always work in a well ventilated room.
- If a reaction or procedure results in the production of vapors, fumes or smoke **ALWAYS** work in a fume cabinet.
- Proper care and attention should be paid to the storage and handling of dangerous chemicals.
- Do **NOT** dispose of hazardous chemicals or any other chemical compounds into the municipal sewage system.
- Avoid the use of dangerous chemicals if other less dangerous chemicals may be substituted.
- Work areas in the laboratory should be adequately delineated and carry the appropriate warning and advisory signs

1. Preparation

1.1 Toolkit

- Beakers (easy to clean) - (50, 100, 250 ml)
- Watch glasses to cover beakers (prevent cross-contamination between samples)
- Test tubes
- Pasteur pipettes (a cheap alternative is drinking straws)/Micro- Pipette with disposable tips
- Hot Plate for heating diatom material (inside a fume cabinet)
- Vortex mixer (optional)
- Separate hot-plate/slide dying bench for curing slides (inside a fume cabinet)
- Reagents (clearly marked and correctly stored)
- Waste bottles for disposal of hazardous compounds.



1: Micro-pipette 1 ml with disposable tips **2:** Glass and plastic Pasteur-pipettes (2-3 ml). **3:** Forceps. **4:** Microscope slides. **5:** Coverslips. **6:** Diatom mountant. **7:** 10 ml plastic gradated centrifuge tube. **8:** 15 ml glass test tube. **9:** 4 ml glass sample storage bottles with rubber seal inside cap. **10**: Water-proof fine marking pen. **11**: Heat-resistant glass beaker ~ 100 ml. **12:** Watch glass.

Section Summary

Preparation:

1. Pre-preparation examination for live cells.
2. Cleaning of cells:
    a. In laboratory equipped with a fume cabinet: $KMnO_4$ + hot HCl method, hot H_2SO_4 + HNO_3 (2:1) method, hot H_2O_2 method.
    b. In **WELL VENTILATED** laboratory without fume cabinet: Cold H_2O_2.
    c. Rinsing: Centrifuge available: centrifuge with distilled water until sample is circumneutral (4-5 runs for 10 min. at 2500 rpm)
    d. No centrifuge available: decant supernatant using an aspirator. Resuspend sample using distilled water and allow to settle for 8 hours (repeat 4-5 times)
3. Slide preparation:
    a. Concentrated diatom solution diluted with distilled water until only slightly cloudy,
    b. Add 1-2 drops of 10% NH_4Cl to dilute solution to prevent clumping of cells,
    c. 1.5 – 2 ml of dilute solution is placed on cover slip, depending on size of cover slip,
    d. Sample allowed to air-dry (Takes approximately 24 hrs),
    e. Cover slip heated to drive off excess moisture and sublimate NH_4Cl,
    f. Sample mounted with high-resolution mountant.

Archiving:

1. Cleaned samples should be stored in ethanol, at a concentration high enough to prevent the growth of bacteria and fungi and to prevent the dissolution of silica.
2. Slides should be stored flat until mountant is dry.
3. All relevant information on the location, date of collection, substratum and collector should be stored both with the sample and the slide, not simply a reference number.

1.2 Pre-preparation examination of freshly sampled material

A quick examination of unpreserved fresh diatom material should be performed on return to the laboratory to assess whether the diatom assemblage consists of predominantly of live cells (*It should be noted dead cells will also form part of the bio-film and are not necessarily washed away under natural conditions).*

If the majority of the diatoms in the freshly collected material are registered as dead cells (empty frustules with no chloroplasts) the sample should be discarded, as further analysis will not give a true reflection of recent water quality at the particular sampling site.

1.3 Cleaning techniques with rationale

Any method of preparation of diatoms for microscopy is acceptable, as long as the cleaned material meets the following criteria:

- ***Concentrations of cells in the cleaned sample should match as closely as possible the concentration of cells collected in the original sample.***
- The organic matter in the sample should be completely removed.
- Foreign matter should either be absent or insufficient to cause problems during the enumeration or identification of the specimens.

1.3.1 Decalcification

Decalcification is ONLY necessary if samples are to be later treated with nitric or sulphuric acid, as these acids combine with calcium causing the formation of an insoluble precipitate. This stage can be omitted if you are sure that the sample does not come from a site with any calcareous rock in the catchment or if using the Hot HCl and $KMnO_4$ method (*recommended technique*)

HEALTH AND SAFETY

Hydrochloric acid is **CORROSIVE** and **OXIDATIVE**. Do not perform any analysis using this chemical outside of a fume cabinet.
When handling HCl wear acid resistant gloves, goggles and a lab coat.

1.3.1.1 On return to the laboratory, allow the samples to settle for 24 hours;

1.3.1.2 Pour off the supernatant liquid taking care not to loose any diatom material;

1.3.1.3 Shake the remaining suspension well and pour 5-10 ml (depending on the concentration of the material) into a glass beaker;

1.3.1.4 In a fume cabinet, add a few drops of dilute HCl (e.g. 1 M) and agitate gently - the material should effervesce as the carbonates are reduced to CO_2. [*If the sample does not effervesce on addition of HCl there is not a significant amount of Ca in the sample and it is not necessary to continue with decalcification*];

1.3.1.5 Continue adding dilute HCl, and agitate the beaker gently until effervescence stops;

1.3.1.6 Pour the solution into a centrifuge tube (10 ml);

1.3.1.7 Add distilled water to 1 cm below the rim of the centrifuge tube and centrifuge to remove the acid;

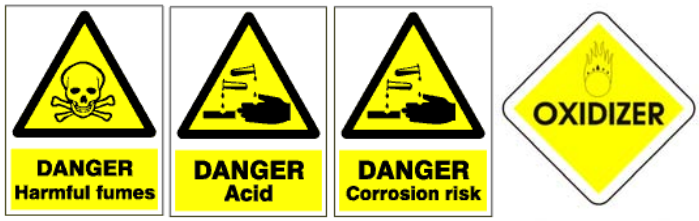
1.3.1.8 The samples are rinsed by centrifuging with distilled water at 2500 rpm for 10 minutes;

1.3.1.9 After centrifugation the supernatant is decanted and the washing is repeated a further 4 times until the sample is circumneutral.

1.3.2 Hot HCl and $KMnO_4$ method (*recommended technique*)

This method is recommended by the authors as it has yielded good results with samples taken from throughout South Africa, which usually have a high content of organic material. In addition, there is no need to remove calcium (1.3.1) before processing the samples as in the other techniques below (1.3.3 – 1.3.4).

HEALTH AND SAFETY



Hydrochloric acid is **CORROSIVE. CHLORINE GAS** is emitted when combined with potassium permanganate. Potassium permanganate is an **OXIDATIVE AGENT**. Do not perform any analysis using these chemicals outside of a fume cabinet. When handling HCl wear acid resistant gloves, goggles and a lab coat.

1.3.2.1 Allow the diatom sample to settle for 24 hours after return to the laboratory;

1.3.2.2 Decant the clear supernatant liquid from the sample bottle taking care not to loose any of the diatom material;

1.3.2.3 Shake the sample well and pour 5 to 10 ml (depending on the concentration of the material) of thick suspension into a heat-resistant beaker;

1.3.2.4 Mark the beaker clearly with the sample number in several places;

1.3.2.5 Add 10 ml saturated potassium permanganate ($KMnO_4$) solution, mix and leave for a period of 24 hours;

1.3.2.6 In a fume cabinet, add 5-10 ml concentrated HCl (32%), **taking care not to inhale the gasses released**. Cover the beaker with a watch glass and heat on a hot plate at 90°C for 1 to 2 hours until the solution becomes clear (usually the solution will have a yellowish colour when clear);

1.3.2.7 After oxidation of organic material, add 1 ml of hydrogen peroxide to check if the oxidation process is complete and no organic material remains, in which case the hydrogen peroxide will not cause lasting foaming;

1.3.2.8 When oxidation is complete, the samples are allowed to cool and are then transferred to 10 ml centrifuge tubes. Before pouring the diatom and acid samples from the beakers, the beakers are vigorously swirled, the aim of the rotary movement being to re-suspend the diatoms, whilst causing the stone and heavier sand particles to fall to the bottom of the beaker;

1.3.2.9 The samples are rinsed by centrifuging with distilled water at 2500 rpm for 10 minutes;

1.3.2.10 After centrifugation the supernatant is decanted and the washing is repeated a further 4 times until the sample is circumneutral;

1.3.2.11 The supernatant should be poured off in a single movement, and care should be taken not to lose any diatom material. After pouring off the supernatant fluid the diatoms and small particles of sand at the bottom of the tube are loosened by means of a jet of distilled water from a wash bottle. More water is then added until reaching the required volume in the centrifuge tube;

1.3.2.12 After the last wash, the diatoms are again loosened by means of a jet of distilled water and then poured into small glass storage vials bearing the necessary sample information. *It is important to store diatom samples in glass as opposed to plastic vials, as glass releases silica, which counters the dissolution of diatom valves.*

Alternatively, the excess acid and soluble chlorides can be washed out by a series of timed **decantations**.

1.3.2.13 The beaker is filled with distilled water to within 1 cm if the top and the solution is allowed to settle overnight;

1.3.2.14 The clear supernatant is decanted;

1.3.2.15 After each decantation, the remainder is swirled to get it into suspension and the beaker is again filled with distilled water;

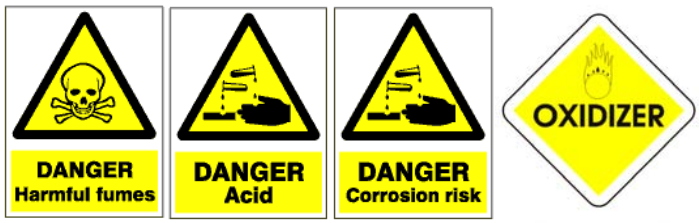
1.3.2.16 This is repeated until the suspension is clear and it no longer turns blue litmus paper red (i.e. the sample is circumneutral).

The supernatant may be decanted using an aspirator attached to a water suction pump or by siphoning.

Tip: an aspirator can conveniently be made by heating and bending a glass Pasteur pipette into a 'u'-shape.

1.3.3 Hot HNO_3/H_2SO_4 method

HEALTH AND SAFETY



Both Nitric acid and Sulphuric acid are highly **CORROSIVE OXIDISING** agents.
Do not perform any analysis using these chemicals outside of a fume cabinet.
When handling HNO_3 or H_2SO_4 wear acid resistant gloves, goggles and a lab coat.

1.3.3.1 Allow the diatom sample to settle for 24 hours after return to the laboratory;

1.3.3.2 Decant the supernatant from the sample bottle taking care not to loose any of the diatom material;

1.3.3.3 Check the sample for the presence of calcium and decalcify the sample if necessary (see section 1.1);

1.3.3.4 Mix the diatom suspension carefully and pour a sub-sample (~ 5 to 10 ml) into a beaker. The size of the sample is dependent on the sample density, which can be judged by the visible concentration of suspended material;

1.3.3.5 Mark the beaker clearly with the sample number in several places;

1.3.3.6 Add 5 ml of a strong acid mixture (HNO_3 + H_2SO_4, 2:1) and place the beakers on a hot plate. The beakers should be covered with a watch glass to prevent contamination between flasks if boiling becomes too vigorous and splashing occurs;

1.3.3.7 Heat the samples at 90°C for 2-3 hours, depending on the amount of organic matter in the sample;

1.3.3.8 Rinse the samples and test for organic material as in points 1.2.7 – 1.2.12 in the previous method.

1.3.4 H_2O_2 Methods

Hydrogen peroxide is much gentler than acid as it is not as corrosive. It is best used with samples that require little cleaning, and where corrosion should be limited, as in SEM studies. The choice of technique (either hot or cold) depends on the availability of a fume cabinet. If

one is available the peroxide can be boiled and, if not, a cold method should be used, **but only** in a well-ventilated room.

Hot H_2O_2 method

Health and Safety

Hydrogen peroxide is an **OXIDISING** agent. Do not perform any analysis using this chemical outside of a fume cabinet if heat is to be used to speed the reaction. When handling H_2O_2 wear acid resistant gloves, goggles and a lab coat.

1.3.4.1 Allow the diatom sample to settle for 24 hours after return to the laboratory;

1.3.4.2 Decant the supernatant from the sample bottle taking care not to loose any of the diatom material;

1.3.4.3 Check the sample for the presence of calcium and decalcify the sample if necessary (see section 1.1);

1.3.4.4 Mix the diatom suspension and place 5 to 10 ml of the suspension in a beaker;

1.3.4.5 Mark the beaker clearly with the sample number in several places;

1.3.4.6 Add 20 ml H_2O_2 and heat on a hot plate at 90°C for 1 to 3 hours;

1.3.4.7 The beakers should be covered with a watch glass to prevent contamination between flasks if boiling becomes too vigorous and splashing occurs;

1.3.4.8 Add a few drops of HCl and leave to cool;

1.3.4.9 Rinse the samples as in method 1.2.7-1.2.12.

Cold H_2O_2 method

PAY ATTENTION TO THE HEALTH AND SAFETY WARNING FOR THE PREVIOUS METHOD

1.3.4.10 Proceed as in method 1.3.4, above, with the exception of using a hotplate;

1.3.4.11 Cover beaker with watch glass and leave for a minimum of four days;

1.3.4.12 Rinse the samples as in method 1.2.7-1.2.12.

1.4 Other methods (Incineration)

The above methods (1.3.1 – 1.3.4) will completely destroy the organic material both inside and outside of the diatoms cells including the organic materials used for colony formation. Thus, by using these methods it will only be possible to observe single cells in the final preparations. To make permanent slides which retain colony formation requires the use of a different technique known as incineration. This technique may also be useful for particularly delicate and weakly silicified diatoms that would be destroyed or badly damaged using other techniques. The technique of incineration is described below:

- Place 2-3 drops of fresh concentrated diatom suspension onto a coverslip.
- Add distilled water until the surface of the coverslip is almost covered.
- Gently heat the coverslip on a hot plate until all the liquid has evaporated.
- Increase the heat to 350°C and continue to heat the sample.
- Watch carefully for the changes in the colour of the diatom material.
- The sample will first become brown as the chlorophyll and other organic material is incinerated.
- The sample will then turn black as all the organic material is converted into carbon.
- As the carbon is incinerated the remaining material will turn a whitish colour.
- When the sample is clean, i.e. has a white appearance, allow the coverslip to cool and remove it from the hotplate.
- Gently rinse the upper surface of the coverslip to remove any salts or other precipitates.
- Heat the coverslip again until all the moisture has been removed from the sample.
- The sample is now ready for mounting in a high resolution diatom mountant.

2 Preparation of diatom slides

Health and Safety

Many diatom mountants and the solvents used for these mountants contain harmful or carcinogenic chemical compounds. Always make slides in a fume cabinet or under a fume hood and never inhale the fumes and smoke released during this procedure.

As with of the cleaning of diatoms any method slide preparation is acceptable, as long as the slide meets the following criteria:

- The distribution of valves on the cover slip should not be significantly clumped, but be evenly dense, without significant edge effects, over the whole area of the coverslip.
- Ideally, there should be 5-15 valves, but not less than 1 and not more than 25 valves, per field of view when viewed at 1000 x magnification.
- The mountant should be properly cured, with no air bubbles, and should spread right to the edge of the coverslip.

Most of the ultra-structural details of diatoms lie at the limit of resolution of light. In addition, all generally used mounting media used in cytology have a refractive index similar to that of diatom valves, with the result that slides with diatoms mounted in these media are too low in contrast for satisfactory investigation. For this reason diatoms must be enclosed in a medium of higher refractive index than that of the diatom valves. Four types of mounting media are generally used: “Hyrax” r.i. (refractive index) 1.71; “Naphrax” r.i. 1.69, “ZRAX”, r.i. 1.7 and “Pleurax”, r.i. 1.73

Naphrax is available from:
Brunel Microscopes Ltd,
Chippenham, SN14 6QA.
England

ZRAX is available from:
Professor W. P. Dailey
dailey@sas.upenn.edu
or
Mr. Klaus Kemp
Microlife Services,
Blautannen,
Wickham Way
East Brent,
Somerset, TA9 4JB
England
Tel: +44 1278 760 411
klaus.kemp@onetel.net
www.diatoms.co.uk

Pleurax is available from:
Mr. Jonathan Taylor
School of Environmental Sciences
Division Botany
North-West University
(Potchefstroom Campus)
Private Bag X6001
Potchefstroom, 2520
Tel: 018 299 4305
plbjct@puk.ac.za

2.1 Preparation

Principle: Slides should be free of contamination by other diatomaceous material and *should display an assemblage of diatoms that is as close as possible, in terms of composition, to that of the original sample.* For this reason strewn slides are used almost exclusively.

Note: It is always necessary to keep the sample well mixed or shaken, as the larger diatom cells will tend to settle out of solution quicker than the smaller cells and thus the community counts will be skewed and unreliable (a mechanical vortex mixer is very useful for this purpose).

2.1.1 Slides and cover slips should be scrupulously cleaned with detergent soap and stored in ethanol until needed.

2.1.2 Using a pipette, a portion is drawn from a well-shaken numbered vial of cleaned material. The cleaned diatom suspension is diluted until it appears only slightly cloudy to the naked eye.

Note: *Obtaining the correct dilution is a matter of practice as the concentration of diatom cells in the solutions will always differ from sample to sample.*

2.1.3 A single drop of ammonium chloride (NH_4Cl; 10% solution) is added for every 10 ml of diluted diatom suspension to neutralise electrostatic charges on the suspended particles and reduce aggregation. This is a very important step; if the diatoms and other particles clump together this destroys the random distribution of the cells on the slide which will in turn have an effect on the results of the analysis.

2.1.4 Using a pipette ~1.5 ml of this suspension is placed on a clean, dry cover-slip (22 x 32 mm). The amount of solution used will vary according to the size of the coverslip.

2.1.5 After being placed on the cover slip the diatom suspension should be allowed to dry at room temperature in a dust free environment. It should not be disturbed until dry, because *vibration causes clumping of the diatom valves*.

2.1.6 The drying of cover slips on a hot plate is **not** recommended because the resultant convection currents form more or less concentric rings of diatoms, with consequent overlying.

2.1.7 After the water has evaporated, diatom-coated cover-slips are placed on a hot plate at ~350ºC for 3 minutes to drive off the excess moisture and to sublimate the residual ammonium chloride.

2.1.8 After the cover slips have cooled, they can be briefly examined under 400 x magnification to determine if the concentration of diatoms in the solution was correct. At least 10, but not more than 40, valves should be visible per field. When the sample is finally viewed at 1000 x magnification there should ideally be between 5 and 15, but not more than 20, valves visible in each field. If the concentration is too high or low, steps 1-7 need to be followed again, using a more or less dilute suspension, before proceeding further.

2.1.9 After the diatom-coated cover slips have been allowed to cool, one or two drops of mountant are placed onto each by means of a glass rod or pipette.

2.1.10 A previously cleaned glass slide is then lowered onto the cover slip, inverted, and then heated at 90-120 °C on a hot plate until the mountant 'boils' and all the solvent evaporates.

2.1.11 The solvent of the mountant should be evaporated quickly. If this is not done a ring of exuded medium will harden around the edge of the cover slip, while the mountant under the cover slip remains more or less viscous.

2.1.12 Under no circumstances should the mountant be heated for too long, or at too high a temperature, because then it will turn dark in colour.

2.1.13 Depending on temperature and the quality of the mountant, it will be necessary to heat the slide on the hot plate for two to five minutes.

2.1.14 After the mountant has boiled for this length of time, but while it is still viscous, the hot slide is quickly removed from the hot plate, and laid on the work bench.

2.1.15 The cover slip is then manoeuvred into position. If this operation is not successful the first time, the slide need only be re-heated for another few moments and the positioning repeated.

2.1.16 When the slide is thoroughly cooled, all the mountant should be hard and brittle and capable of being easily chipped off with the point of a scalpel.

2.1.17 Surplus medium, which has been exuded and has set round the edge of the cover slip, may be carefully removed with the point of a scalpel or cotton bud soaked in solvent, after which the slide is then wiped clean with a soft rag soaked in the particular mounting medium's solvent (iso-propyl alcohol for "Pleurax" and toluene (which is carcinogenic) for "Hyrax").

2.1.18 The cover glass may then be ringed with shellac cement or Bio-seal®.

2.1.19 The slide should be carefully labeled. Important details to be included on the slide label are: Date of collection, site location and co-ordinates, habitat, collector and type of mountant.

2.1.20 The slide is then ready for microscopic examination.

3. Preparation for Electron Microscopy (EM)

Diatoms provide an excellent subject for electron microscope studies. Both scanning electron microscopy (SEM) and transmission electron microscopy (TEM) are useful but **not essential** tools for diatom taxonomy. An impression of the three dimensional structure of diatoms is gained using SEM, while the higher resolving power of TEM makes it useful for examining fine structures and small, thin-walled diatoms (e.g. *Fistulifera saprophila* Lange-Bertalot & Bonik). The silica frustule of diatoms is very robust and no special techniques such as those used for soft-bodied algae are required to prepare these cells for electron microscopy.

2.2 Preparation for SEM

- Freshly collected diatom material can simply be air dried onto a small coverslip (Ø 12 mm) or filtered through a 2.5 µm Millipore® filter.
- Cleaned material can be prepared in a similar manner.
- When air dry the sample should be placed in a dessicator containing silica gel for 24 hours to make sure that it is completely dehydrated before continuing with further preparation.
- When dry the coverslip or filter should be mounted on an aluminium microscope stub with carbon tape and sputter coated with gold palladium.
- The samples are now ready for examination.
- 10 – 15 kV is usually adequate voltage for examining diatoms.

2.3 Preparation for TEM

- The sample is suspended (or diluted) into water **if possible** and adsorbed onto a formvar film which is attached to a copper specimen grid.
- Once the diatom material has been adsorbed onto the film surface, the excess sample is blotted off and the grid is covered with a small drop (5 µl) of stain solution (Aqueous Uranyl Acetate).
- This is left on the grid for a few seconds and then blotted off.
- The sample is dried and examined in the TEM.

4. Archiving

- It is important to retain a portion of the original sample throughout the preparation stage until the final slide has been made and examined under a microscope.
- After slide preparation, a portion of the cleaned suspension should be preserved and stored in a labeled vial with ethanol added to reach a final concentration of more than 20% by volume, to prevent the growth of micro-organisms.
- Alternatively, two or three drops of a 5% aqueous solution of phenol (c**aution: this chemical is carcinogenic**) may be added. The archiving of diatom material is necessary in case further slides need to be made or if other workers wish to verify the results of a diatom community analysis after mounting and examining the sample themselves, or if SEM studies on the material are to be undertaken.
- Finally, a slide should be stored in a herbarium or diatom collection to facilitate cross-referencing. Diatom slides provide a permanent historical record of water quality conditions at a site and should be stored in order to ensure that they can be accessed for future analyses. It is recommended that at least two slides are prepared from each sample. One of these should be lodged in the appropriate national herbarium, for future reference. This is:

  The North-West University,
  Potchefstroom Campus,
  School of Environmental Sciences and Development,
  Division Botany,
  Private Bag X6001,
  Potchefstroom, 2520

  Type material should be lodged at:

  The Natural History Museum
  Cromwell Road
  London, SW7 5BD
  UNITED KINGDOM

  Diatom slides sent to such institutions should be labeled with the following minimum information:

  - Location (place name not just a number)
  - Co-ordinates if available
  - Date on which the sample was collected
  - Substratum
  - Name of the sampler
  - Type of mountant
  - Date on which the slide was prepared.

5. Enumeration and simple biometrics

Diatom counts, when analysed may be used to infer the average water quality at a particular sampling site. Once the sample has been counted in the correct manner the data can be entered into a computer data-base ("Omnidia", Lecointe *et al*., 1993) from which several calculations can be made using a specific equation or using the sum of the water quality optima of all the species in the sample.

Simply, each diatom species used in the calculation/equation is assigned two values; the first value reflects the tolerance or affinity of the diatom to a certain water quality (good or bad) while the second value indicates how strong (or weak) the relationship is. These values are then, in addition, weighted by the abundance of the diatom in the sample i.e. how many of the particular diatom in the sample occur in relation to the total number counted. Below is the formula of Zelinka and Marvan (1961) on which most diatom indices are based:

$$index = \frac{\sum_{j}^{n}=1 \quad a_j s_j v_j}{\sum_{j}^{n}=1 \quad a_j v_j}$$

Where $\mathbf{a_j}$ = abundance (proportion) of species **j** in sample, $\mathbf{v_j}$ = indicator value and $\mathbf{s_j}$ = pollution sensitivity of species **j**. The performance of the indices depends on the values given to the constants **s** and **v** for each taxon and the values of the index ranges from 1 to an upper limit equal to the highest value of **s**.

The aim of counting diatom units is to produce semi-quantitative data from which ecological conclusions can be drawn. With this in mind it is important to know how many valves to count to get a reliable estimation of the relative species composition at a specific sampling site. The total number of valves to be counted for each sample varies according to the purpose of the analysis and according to the need to produce statistically good results. The statistical precision of percentage counting depends on the frequency of the taxon in the sample count in relation to the size of the sample count. In a South African study Schoeman (1973) made a series of experimental counts in which 200, 300, 400, 500 and 800 valves per sample were counted and their relative abundance calculated. When only 200 valves were counted, compared to when 800 valves were counted, the percentage differences of the relative abundances of individual species were often as high as 6-7%. However, the results obtained from counting 400 as opposed to 800 valves differed by only 1-2%. For this reason he concluded that counting 400 valves was satisfactory for the calculation of relative abundance of diatom species. Similarly, Battarbee (1986) demonstrated that there were marked differences in the percentages between counts of 100 and 200 valves, while there was little difference between counts of 400 and 500. For this reason he recommended that a count of 300 to 600 may be used for purposes of routine analysis. This range is supported by Prygiel *et al.* (2002) who, in an inter-comparison exercise, found that diatom index scores were not

affected at counts of 300 and above. Hence, it is recommended that, for diatom community analysis in South Africa, 400 diatom valves should be counted in each sample.

Different conventions have been evolved for the enumeration of diatoms, using either valves or frustules as the basic unit, or not distinguishing between valves and frustules. The effect that such conventions have on the final results has not been evaluated, but is likely to be small. However, it is important that the convention used be specified in advance and kept consistent. In the case of small diatoms, such as some *Achnanthidium* and Naviculoid species, it may not be possible to distinguish with certainty between intact frustules and isolated valves on all occasions.

Suggested rules for counting diatoms can be summarised as follows:

- Counts of diatom valves on slides should be made using a microscope equipped with incident light (bright field illumination), phase contrast optics or differential interference (Normarski) optics (DIC) at a magnification of 1000 x and higher (i.e. 100 x oil immersion objective in combination with a 10 x eyepiece).
- The eyepiece graticule or other measuring equipment must be calibrated against a stage micrometer prior to the analysis to allow for measurement of dimensions and taxonomic features.
- Either the field of view or the grid of a graticule is used as the area defining the limits of the count. All diatoms visible in the field of view (or within the grid of a graticule) are identified and counted before moving along either a horizontal or vertical traverse to the next field, or selecting a new field of view at random.
- The edge of the dried sample suspension is recommended as the position to begin counting, but if this rule is to be adopted, ensure that there are no significant “edge effects”.
- A rule is needed to cover situations where a diatom lies only partially inside a defined counting area. For example, such a rule might include taxa that are only partially visible at the upper but not the lower margin (in the case of vertical traverses) or the left but not the right margin (in the case of horizontal traverses). The precise form of the rule is less important than the consistency of its use when analysing samples.
- Whether a horizontal or vertical traverse is used, it is important that each subsequent traverse does not overlap with the previous one. No diatom valve should ever be counted twice. The distance that the stage is moved on each occasion must also account for any diatoms only partially visible in the field of view.
- If sample analysis is unlikely to be completed in a single session, then it is useful to record the position of each traverse. This ensures that subsequent traverses do not overlap with those already completed.
- Each individual specimen encountered is counted as a single unit, with no differentiation between a valve and a frustule.
- Girdle bands (copulae) should **not** be enumerated as being representative of diatom taxa.

- Occasional filaments should be recorded as the corresponding number of diatom units. If a large number of diatom units are found in filaments, a new preparation technique, using a more aggressive mix of oxidising agents, should be considered.
- In order to eliminate the risk of including separate fragments of broken valves or frustules, a consistent approach must be decided on before starting a project. Valves should be counted only if approximately three quarters is present, or alternatively broken valves may be excluded from the count altogether. Since the scale of physical damage during the sampling and preparation stages is unlikely to be significant, the presence of many small fragments of diatoms may indicate that dead diatoms are being washed in from upstream sites.
- A diatom may not be identifiable for a number of reasons, including the presentation of a girdle view, the presence of overlying material obscuring the view, or the taxon not being recognised by the analyst. If many valves are obscured, then new slides should be prepared using more dilute suspensions.
- Some taxa are identifiable from girdle (side) views, either because the girdle view is particularly characteristic (e.g. *Rhoicosphenia abbreviata*) or because the girdle view can be assigned with confidence to a particular taxon by "matching" it with corresponding valve views of taxa found in the sample. However, this is not always possible and, in cases of doubt, the analyst should record the girdle views at the lowest level to which they can be assigned with confidence (e.g. "unidentified *Gomphonema* sp.", "unidentified pennate girdle view").
- This convention should also be applied to other individuals found on the slide but not identifiable by the analyst. A large number of such individuals may indicate a problem either with the slide preparation or the identification skills of the analyst.
- As most diatom indices presume that all taxa in a sample are identified, it is recommended that *not more than five per cent of the total count should comprise unidentifiable individuals*. If a diatom unit cannot be identified for any reason, photographs, digital images or detailed drawings should be made. Notes should also be taken of the shape and dimensions of the diatom unit, striae density and arrangement (at the centre and poles), shape and size of the central area, number and position of punctae and arrangement of raphe endings.
- For some purposes, especially biodiversity studies, it is useful to continue to scan the slide after the required number of diatom units has been counted, and any taxa encountered that were not included in the count should be identified and recorded as "present".
- A further scan using a medium power magnification (e.g. 400 x) may also be appropriate in order to note any larger taxa (e.g. *Gyrosigma* spp.) which may be missed at higher magnifications.

6. Counting records (electronic and manual methods)

A *pro forma* count sheet with a list of taxon names and space beside each on which the counts can be made or a laboratory notebook organised in such a way that taxon identities and numbers can be clearly recorded. Such sheets should be clearly marked with the details of the sample, the date of the analysis and the analysis's name.

Alternatively a computer program with facilities for direct entry of data can be used. These programs (e.g. "Opticount") allow the names taxa to be assigned to the different keys of a computer keyboard. Each time a specific key is hit the program will record a single individual of that specific taxon. Such programs greatly facilitate and speed counting and the storage and archiving of counting records.

7. Microscopy

A compound light microscope, equipped with a mechanical stage and 100 x oil-immersion lens is required for examining diatom slides. Use of a phase contrast or differential interference (Nomarski) condenser is recommended, although brightfield light will give satisfactory results. The microscope must incorporate facilities for measurements (e.g. an eyepiece graticule) with a resolution of at least 1 µm. Apparatus for photomicroscopy or video capture are useful for taking pictures of difficult specimens. Image Analysis software is also recommended for capture, storage and presentation of diatom images as well as facilitating the measurement of cell dimensions. The eyepiece graticule, or other measuring equipment, must be calibrated against a stage micrometer prior to the analysis. The results of this calibration must be displayed in a position where users of the microscope can easily consult them. The second eyepiece may be equipped with a second graticule to aid enumeration.

8. Image capture, analysis and archiving

A problem affecting light microscopy of diatoms is diffraction. Many of the features of diatom frustules – stria, puncta, spines, etc – are very small, approaching the same dimensions as the wavelengths of visible light (c. 0.5 µm for blue-green light) and this means that diffraction is the major factor determining whether or not structures can be seen. This in turn means that microscopes must be of good quality and, perhaps even more importantly, they must be used well if some of the fine details necessary for identification of diatoms are to be observed.

A further peculiarity of 'diffraction limited systems' is that small structures, like puncta (pores) or spines or the raphe system, appear in focus more than once, in different horizontal planes. For example, in a normal light microscope as one focuses through a specimen

vertically, a feature may first appear in-focus dark against the background, then it will virtually disappear, and then it will appear in-focus again, but now light against the background. Neither of the in-focus images is 'correct'. Both are artifacts of diffraction at the interface between two transparent materials of different refractive index. In many ways, it seems more reasonable to choose the 'white-spot focus' for small pores and the raphe slit, because these are immaterial features – the absence of silica – whereas the 'dark-spot focus' seems more appropriate for solid structures like spines and warts. However, such choices are arbitrary and observers choose on the basis of aesthetics and convention. So, for example, it is more common to photograph most pennate diatoms with the striae (the lines of pores) and raphe in 'dark-spot focus'. However, *Epithemia* is usually photographed with the pores in 'white-spot focus' (Kelly *et al*., 2005).

Image analysis is the most advanced technology for a broad range of functions such as digital image acquisition, image processing, sample analysis, database archiving and results/report documentation. A recommended software package is "analySIS" which has several expansion versions and configurations (Website: www.soft-imaging.net). Version 3.1 provides live overlay, 3-D surfaces, automatic scale bar and many other functions. It is relatively user-friendly but expert advice is required for the most benefit.

9. Sources of variation in diatom community analysis

When implementing monitoring programs based on assessments of diatom community composition, we in South Africa have the advantage of looking to European and other studies to identify sources of error in advance. Several sources of error, in all stages of the analysis, have been highlighted by Prygiel *et al*. (2002) in an inter-laboratory comparison exercise. According to these authors sampling appears to be a very important step. *When the sampling protocol is not strictly adhered to, variability in the end result of the analysis can be very high.*

Sampling-induced variations include:

- Sampling from exposed substrata,
- from areas subjected to water level change,
- from areas of low-velocity flow as compared to other parts of the river and
- sampling from stones covered by abundant filamentous algae.

Laboratory- and counting-induced variations may include:

- The use of high temperatures when drying slides, leading to clumping of diatom valves,
- the settling out of large taxa during the preparation of consecutive slides from a single sample.

The main source of variability is, however, the identification of individuals, that is why biological quality controls focus mainly on counts and misidentification. Diatoms are suitable for such controls and proposals have been made by Kelly (1999).
Prygiel *et al*. (2002) made recommendations which should be seen as the way forward for South Africa in terms of quality control and the validation of diatom analysis data:

- Most of the variability due to sampling and slide preparation can be avoided by organising comparisons between different studies. Such comparisons are very useful because, with field and laboratory approaches, they make operators aware of the consequences of not following protocols. They are also useful because they highlight some taxonomic problems.
- Diatoms are good subjects for photomicrography and therefore most diatomists use the internet to check problematic identifications. This approach should be encouraged by formalising expert-practitioner exchanges, by creating iconographic databases, and by organising regular workshops to allow updating of knowledge.
- The archiving of permanent slides also facilitates the creation of reference collections which are particularly useful for the identification of difficult species.

10. Recommended software packages

Package	**Applications/comments**	**Available from:**
Opticount	A counting program especially designed for the enumeration of algae species and calculation of biomass based on species counts.	DoMix Software Dr. Dominik Hepperle Dorstrasse 20 D-18249 Klein Raden Germany info@do-mix.de http://science.do-mix.de/software.php
Omnidia	Diatom-based index calculation and archiving both diatom sample information and species counts. This is a very valuable tool containing about 11 000 diatoms species with synonyms and references.	CLCI Mrs. Catherine Lecointe : Fax: +33 5 53 61 26 73 Give your computer type (Mac or PC and Windows version) Payment can be made by cheques or international postal order PRICE: Euros 701,27 (07/2005) clci@club-internet.fr http://membres.lycos.fr/rimetfrederic/links.html
TaxIBD	A pictorial key containing the 209 species used for the calculation of the French *Indice Biologique Diatomées* (IBD). This is a simple key accompanied by photographs and a	l'Agence de l'Eau Artois Picardie 200 Rue Marceline BP 818 59508 Douai Cedex France

	short description of the taxa used in the index.	
Common Freshwater Diatoms of Britain and Ireland: A multi-access key	This electronic key is based on LUCID software and contains 379 river diatom species commonly encountered in Britain and Ireland. This key provides an introduction to the morphology of the diatoms, a detailed description of each taxon included accompanied by photographs and ecological information.	UK Environment Agency http://www.environment-agency.gov.uk
Common Freshwater Diatoms of South Africa: A multi-access key.	This electronic key is based on LUCID software and contains ~ 200 river diatom species commonly encountered in South African fresh waters. This key provides a detailed description of each taxon included, accompanied by photographs, drawings and ecological information	Water Research Commission Private Bag X03 Gezina 0031 Mrs Rina Winter Publications Telephone: 012 330 0340 Fax: 012 331 2565 orders@wrc.org.za http://www.wrc.org.za
AnalySIS	Allows for image capture, measurement, processing and archiving	Wirsam Scientific & Precision Equipment Mrs. Colleen Syrett 23 Menton Road Richmond 2092 Johannesburg Telephone: 011 482-1060 Fax: 011 726-6094 e-mail: wirsamjb@wirsam.com http://www.soft-imaging.net

11. Key reference works

There are several key reference works as well as simpler illustrated keys and introductory volumes on the diatoms that may be useful when first commencing the study of diatoms, these include the following:

Title and authors	Available from:
Suesswasserflora von Mitteleuropa: Band 2: Bacillariophyceae. Tiel 1-4. K Krammer and H Lange-Bertalot. (1986-1991)	Koeltz Scientific Books Po Box 1360 D-61453 Königstien Germany E-mail: koeltz@t-online.de www.koeltz.com
Suesswasserflora von Mitteleuropa: Band 2: Part 5: English and French translation of the keys. K Krammer and H Lange-Bertalot: Bacillariophyceae. (2000)	Koeltz Scientific Books Po Box 1360 D-61453 Königstien Germany E-mail: koeltz@t-online.de www.koeltz.com
Guide méthodologique pour la mise en œuvre de l'indice Biologique Diatomées NF T 90-354. Agences de l'Eau-Cemagref de Bordeaux, mai 2000, 134 pages + Clés de détermination (89 planches) + cédérom français-anglais (tax'IBD). J Prygiel and M Coste M. (2000)	l'Agence de l'Eau Artois Picardie 200 Rue Marceline BP 818 59508 Douai Cedex France
Identification of common benthic diatoms in rivers. MG Kelly (2002)	FSC Publications Field Studies Council Preston Montford Shrewsbury SY4 1 HW England E-mail: fsc.publications@ukonline.co.uk www.field-studies-council.org
A guide to the morphology of the diatom frustule. HG Barber and EY Haworth (1981)	The Librarian The Ferry House Far Sawrey Ambelside Cumbria LA22 0LP England E-mail : EHaworth@fba.org.uk
An illustrated key to common diatom genera from Southern Australia. PA Gell, JA Sonneman, M Reid, MA Illman and AJ Sincock (1999)	Murray Darling Freshwater Research Centre PO Box 921 Albury NSW 2640 Australia
An illustrated guide to common stream diatom species from temperate Australia. JA Sonneman, A Sincock, J Fluin, M Ried, P Newall, J Tibby and P Gell. (1999)	Murray Darling Freshwater Research Centre PO Box 921 Albury NSW 2640 Australia

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