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Micropaleontological preparation techniques and analyses

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Micropaleontological preparation techniques and analyses

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CAUTION

Many of the laboratory techniques described in this manual implement the use of toxic chemicals that are dangerous for your health. Acids and organic solvents are the two types of chemicals are currently used. The most frequently used acids are hydrochloric (**HCl**) and hydrofluoric (**HF**), and organic solvents are carbon tetrachloride (**CCl_4**) which produces harmful vapours. This manual contains warnings for these products when they are used during a procedure. Also, these dangerous chemicals are written in bold throughout the text.

INTRODUCTION

The microfossils contained in sediments can provide a large amount of information on past environments. Microfossil assemblages are indicators of the physical and chemical conditions of their habitat, providing access to qualitative or quantitative reconstructions of environmental parameters. The microfaunal and microfloral inventory and concentration calculations can also provide biogenic fluxes and sedimentary input. Finally, microfossils can incorporate the geochemical signature from the environment in which they form. For example, the carbonate shells of ostracods and foraminifera can be used for geochemical and isotopic analyses.

All samples in a sediment core or stratigraphic sequence are unique and invaluable. Each sample is susceptible to multiple analyses, not only micropaleontological, but also sedimentological and geochemical. Special care is taken to properly manage the various subsamples and analytical residues in order to optimise the whole sample. The first objective of this document is to state the subsampling procedures, sediment storage and management, that have been implemented in our laboratory at GEOTOP in order to maximise access to the samples.

Different sample preparation techniques for micropaleontological analysis can be used depending on the required result. The techniques described in this document were adopted or developed with the goal to proceed with quantitative analyses of microfaunal or microfloral populations (counts, concentrations, flux, percentages). The laboratory protocols were also established to maximise the number of micropaleontological and/or geochemical analyses within the same sample.

1. SAMPLE MANAGEMENT

1.1 Sediment core subsampling

Sampling is performed either during the research cruise or in the laboratory. After measurement of physical properties (density and magnetic susceptibility) with multi sensor-track core logger (MSCL), the sediment cores are split longitudinally in half: one is described and then archived, and the other is subsampled. Ideally, the subsampling is conducted immediately after splitting the cores, before dehydration of the sediment.

Wherever the subsampling takes place, strict precautions must be respected. Teflon tools are normally used in order to avoid contamination that would bias trace element analyses. Prior to subsampling, the surface of the working half of the core, which may have been contaminated during splitting, is cleaned by removing a thin (<1 mm) layer of sediment with a spatula. U-Channel subsampling in the center of the core is often done for further analyses of magnetic properties. Subsampling for micropaleontological and geochemical analyses is done afterward. Special care should be made to avoid subsampling the sediment touching the core sleeve (~ 2 mm), which consists of a zone of smeared sediment that is therefore contaminated. The voids created during subsampling are filled with pieces of styrofoam to avoid the movement of sediment within the core. The working half is then sealed with plastic wrap and inserted into a hard plastic tube that is identified with the core name and section at each end.

The sampling protocols and the list of all samples and their storage requirements are provided in the cruise report at sea or in the sediment management notebook.

1.2 Notebook for sediment management

The volume of sediment is often limited, for example subsampling at 1 cm intervals from the working half of a 10 cm diameter core provides approximately 30 cm^3 of sediment. Considering the limited quantity of material, a maximum volume of sediment is allotted to the various types of micropaleontological analyses such as:

- microfauna (mainly benthic foraminifera): 10 cm^3
- palynomorphs (pollen, dinocysts, etc.): 5 cm^3
- calcareous nannofossils and diatoms: 1 cm^3

The geochemical (C, $CaCO_3$, C/N, U/Th, ^{210}Pb, ^{137}Cs, notably) and sedimentological analyses are performed on an approximate volume of 10 cm^3.

In order to have proper sediment management, each user must document the quantity of material and its destined analysis. This information must be added to the sediment management notebook

along with the other documentation for the sediment core.

2. SAMPLE PREPARATION TECHNIQUES FOR CARBONATE MICROFOSSIL ANALYSIS (FORAMINIFERA, OSTACODS, AND PTEROPODS)

2.1. General information

The sediments may contain abundant microfauna that can be observed and analysed with a binocular microscope. This microfauna contains multiple types of microfossils, including benthic and planktonic foraminifera (protozoans in the class rhyzopods), ostracods (subphylum crustacea), and pteropods (class gastropoda). Although the ecology of these organisms is different, they are all characterised by a carbonate shell (or an agglutinated one like some thecamoebians) with dimensions on the order of a hundred micrometres. The samples destined for microfaunal analyses are prepared following the same protocol, justifying their grouping here.

The sample preparation for microfaunal analyses consist of relatively simple techniques, relying essentially on sieving the sediment.

The observation, sorting and counting of foraminifera, ostracods, and pteropods are performed mainly on a dried fraction at relatively small magnification (x20 to x500). The microfossils can be manipulated with a moist paint brush to avoid static. Their identification often requires the observation of their different sides (e.g. dorsal or ventral) after manipulation with a paint brush. The structure of the calcareous shell (e.g., ornamentation, pore density) can be viewed with an electron scanning microscope.

2.1.1. Foraminifera

Foraminifera are the microfossils most commonly used for paleoecology and marine biostratigraphy, due to their abundance in continental margin marine sediments (mostly benthic species) or in deep ocean sediments (dominant planktonic species) and their relative large dimensions, which greatly facilitate their manipulation and observation. Foraminifera are exclusively marine and can occupy different habitats: pelagic (planktonic species), epibenthic or endobenthic (benthic species). Their test or fossilised shell consists of multiple connecting chambers, with those of adult forms reaching up to 10^2 µm. Planktonic foraminifera are good stratigraphic indicators of the interval covering the Jurassic to present, while benthic foraminifera are found since the Cambrian (Ordovician to present for calcareous species). The foraminiferal carbonate tests are privileged to be used for geochemical analyses (trace elements, Mg/Ca, $\delta^{18}O$ and $\delta^{13}C$, ^{14}C).

In abyssal sediments, planktonic foraminiferal concentrations may range up to 10^5 tests/cm^3. They are particularly abundant in the low latitude environments where they produce

"foraminiferal calcareous oozes". Within deep environments, below the lysocline, the preservation of planktonic and other calcareous microfossils can be affected by dissolution either selectively or completely.

2.1.2. Ostracods

The fossilisable part of the ostracod is a shell consisting of two calcified valves from 10^2 to 10^4 µm in size. Ostracods may be present in all sedimentary environments, including lacustrine, marine, and terrestrial. They represent a large taxonomic diversity and consist of stratigraphic markers from the Cambrian to present. Ostracod valves can also be analysed for the isotopic composition of oxygen and carbon.

2.1.3. Pteropods

Pteropods have an aragonitic shell ranging from 10^2 to 10^4 µm in size. Pteropods are exclusively marine and occupy the mesopelagic zone of the oceans. They can be abundant and produce ooze in marine sediments of mid to low latitudes. Due to their aragonitic shell, they are susceptible to dissolution. The stratigraphic distribution of pteropods is up for debate: their presence since the Cambrian is proposed, however the taxa undisputedly appear in the Cretaceous.

2.2. Sample preparation

2.2.1 Routine techniques

Equipment: precision balance
Other materials: 50 ml graduated cylinder, 250 ml beaker, funnel, n°4 Whatman filter, 106 µm mesh sieve (previously 63 µm and 125 µm mesh sieves), wash bottle with distilled water, 250 ml plastic containers, 12 ml Nalgene containers, labels

1- Fill a graduated cylinder with 20 cm^3 of distilled water, place it on a balance and then tare it (make the value equal 0.0 g).
2- Measure 5 cm^3 of wet sediment by displacement in the graduated cylinder. Record the volume and weight of the wet sediment that was subsampled.
3- Fold the n°4 Whatman filter into four and place it in the funnel, then place the funnel onto a 250 ml plastic container that has been labelled with the sample number. Pour the sample into the funnel, and rinse the graduated cylinder with the wash bottle of distilled water in order to recuperate all of the sediment.
4- Store the container, funnel and filter containing the sample on a shelf so that the sample can dry at room temperature for 24 to 72 hours depending on the consistency of the sample.

5- Weigh the dry sample and record its weight. This value allows for the calculation of the percent moisture in the sediment.
6- Transfer the sediment into a 250 ml beaker containing approximately 100 ml of distilled water, and allow the sample to disintegrate for about 30 minutes.
7- Empty the contents of the beaker onto the 106 µm sieve over the sink and properly wash the sample with warm tap water. The less than 106 µm fraction is collected for other analyses, notably palynological analyses.
8- Proceed with a final rinse with distilled water and pour the two larger size fraction onto n°4 Whatman filters to dry at room temperature.
9- Record the dry weight of the larger size fractions and transfer the sample into a 12 cm^3 Nalgene container labelled with a sticky label marked with the sample identification number.

2.2.2. Heavy liquid separation (rarely used)

*Attention: this technique requires the use of carbon tetrachloride (**CCl_4**). This solvent is very harmful, not only by contact with the skin but also by the vapours. Always work with this solvent under the fume hood and wear protective gloves.*

Equipment: fume hood Other materials: 250 ml beaker, funnel with a n°4 Whatman filter, 12 ml Nalgene containers, labels Chemicals: **CCl_4** in solution (density ~ 2)

When the samples are very sandy, it is possible to proceed with the separation of microfauna and the mineral fraction by using heavy liquids (e.g. carbon tetrachloride, **CCl_4**). The dry sieved sample is mixed with a heavy liquid (density ~ 2) in a beaker and the supernatant fraction is sieved into a filter as described above (the heavy liquid is collected for use with the subsequent fractions after filtration). Several rinses are required in order to recuperate all of the microfauna. The mineral fraction, which decants in the beaker, is separated from the microfauna. After drying, the various fractions are transferred into labelled Nalgene containers.
The heavy liquid separation technique must be performed under the fume hood due to the toxicity and volatility of the chemicals. Since the chemicals can contaminate the sample for further chemical analyses, this should be a technique used as a last resort.

2.2.3. Staining living foraminifera

Chemicals: rose bengal in powder, formalin or ethanol.

The surface sediment may contain living microfauna. The endobenthic foraminifera live in the sediment, up to a few centimetres depth. In order to establish the habitat of certain foraminiferal species and study the populations, it is useful to distinguish the living foraminifera from the fossil tests. To accomplish this task, we add a solution of rose bengal to the sediment as soon as it has been sampled to selectively stain the living cells.

The solution used is a mix of 2 g of powdered rose bengal in 1 L of formalin or ethanol. The solution, prepared in advance, is mixed with the sediment immediately after sampling. We generally add 15 ml of the solution per 10 cm^3 of sediment. The sample containers must be well sealed. The coloured sample can be stored for many years. The living foraminifera at the time of sampling will retain pigmentation unlike the fossil tests.

2.3. Subsampling and sieving

Equipment: splitter, series of sieves (63, 125, 150, 250, 500 μm), counting plate, paint brush.

The microfaunal richness of the sediment can be quite variable. Concerning benthic foraminifera and ostracods, the number of individuals per unit volume is generally low: the extraction of all specimens present in the whole sample is often necessary for a statistically representative sample for a population analysis (N > 200). As for the planktonic foraminifera, the number of individuals per unit volume can be considerable. When the density or the concentration of microfossils is high, observation and counting on the plate can't be performed on the whole sample. We then proceed on extracting from a representative fraction of the sedimentological, geochemical, or micropaleontological facies of the sample (an aliquot) with the help of a splitter that can separate the sample into two equal fractions. The sample can be split into as many fractions as necessary (x2, x4, x8, x16, x32...) to obtain an aliquot containing a population with a reasonable density for analysis. It is important to note the final fraction of the sample (1/2, 1/4, 1/8, 1/16, 1/32...) represented by the aliquot in order to calculate the subsequent concentration.

Other than the splitting, granulometric separation can or must be performed prior to the observation, identification and countingn. The granulometric separation is dependent on the type of microfossil to be analysed. With the routine technique (see 2.2.1) implying sieving at the >106 μm, the small fraction is kept for palynological analyses and the coarse > 106 μm is available for the microfaunal analyses. For planktonic foraminiferal analyses, we proceed by sieving at 150 μm and only the ≥ 150 μm are used for systematic counting. The smaller fraction and juvenile forms are therefore excluded. This is a convention adopted by most micropaleontologists analysing planktonic foraminiferal populations since the databases destined for transfer functions, have been established using this fraction. However, there now many researchers

claiming that many subpolar species such as *Turborotalia quinqueloba* are underrepresented in the >150 μm fraction.

There is no formal convention for benthic foraminiferal analyses, and a certain disagreement exists within the micropaleontological community. Given the small size of some species (e.g. *Stetsonia* which is characteristic of polar environments), many micropaleontologists have supported analyses on microfauna > 63 μm. However, most micropaleontologists only identify microfauna that are > 125 μm to avoid counting the juvenile forms which are often abundant and difficult to identify. At GEOTOP, we have adopted an intermediate position: the counting is performed on three fractions; the ≥ 250 μm, 150-250 μm and 106-150 μm size fractions can be used for species identification and countings. The results are usually reported from the overall >106μm fraction. The >63 μm size fraction is used when required for the purpose of the research project.

No convention exists for ostracods, however counting the ≥ 63 μm fraction is performed most often. Pteropods are generally analysed within the ≥ 150 μm size fraction, similarly to planktonic foraminifera.

2.4. Counting and concentration calculations

After splitting and sieving, the microfauna are spread evenly for observation under a binocular microscope. The microfaunal taxa identified are systematically enumerated. However, in spite of the earlier splitting, the number of individuals in the aliquot may still be too high to justify systematic counting. It is then possible to proceed with the analysis on a portion of the aliquot by counting the individuals on a section of the observation plate. The plate is divided into a quadrate of equally sized squares; counting in randomly assigned squares produces a systematic counting. In this case, it is necessary to note the number of squares counted with respect to the number of total squares to calculate the fraction of the plate that is counted. A random distribution of the analysed squares is important since the microfossils tend to selectively distribute themselves on the counting plate due to their form (which is more or less round), their dimensions or their weight.
The enumerations performed by systematic counting on the total or partial area allows for calculation, by extrapolation, the concentration of microfauna in the sediment, either the number of individuals per unit of weight or volume of initial sediment.

The concentration $\mathbf{C = \frac{n \times a \times s}{pse}}$

where "**n**" represents the total number of counted microfossils
"**a**" is the number of splits, or 1/the aliquot fraction

"**s**" is the ratio of the plate area over the area analysed
"**pse**" is the initial weight or volume of the sample.

2.5. Extraction of foraminifera for stable isotope analysis

The extraction of carbonate tests from the benthic or planktonic foraminifera for isotopic analyses ($\delta^{13}C$ and $\delta^{18}O$) is usually performed from the palynological residues (> 106 or > 120 μm; see chapter 5). However, if the residues do not contain a sufficient amount of foraminifera (which is often the case for benthic species), it is possible to use the foraminifera from the microfaunal analyses.

The foraminiferal isotopic analyses are performed on monospecific populations, due to species specific fractionation or habitat. Otherwise, in order to avoid contamination, foraminifera containing clay despite washing during sieving or that have been pyritised must be discarded.

Isotopic analyses of planktonic foraminifera are performed on approximately 10 tests of the same species and size (e.g., *Neogloboquadrina pachyderma* sinistral from the 150 to 250 μm size fraction). The species to extract depends on the assemblage present at various intervals in the core. Technically, it is possible to obtain an isotopic measurement from the analysis of 2 to 3 planktonic foraminiferal tests. However, it is preferable to analyse a constant quantity to simplify the operations of the mass spectrometer. It is of note that larger populations of foraminifera (~ 50 shells) had to be measured on the previous generation of mass spectrometers (in the 1980s and 1990s).

The tests are picked and placed onto micropaleontological slides with one or two holes identified with the sample number (cruise, core, depth). Also written on the slide as well as in the notebook is the number of foraminifera picked and on the slide.

The number of benthic foraminifera necessary for isotopic analysis varies with the size of the species to be analysed (between 2 and 30 tests). The species analysed, preferably epibenthic, is dependent on the assemblage which can vary from one core to another. In general, 4 to 5 species are extracted simultaneously in order to obtain a composite series of analyses. It is fairly rare that one single benthic species is present throughout the whole sedimentary sequence, particularly when the core site is situated in a location with large amplitude variations of environmental parameters.

2.6. Extraction of foraminifera for ^{14}C analysis

The analysis of foraminifera for accelerator mass spectrometry (AMS) requires approximately 10 mg of carbonate. It is possible however to obtain a measurement on lower quantities (1 mg is a minimum). The analyses are generally performed on monospecific populations of planktonic foraminifera from the > 106 μm size fraction. The foraminifera are extracted from the palynological residues or from the microfaunal preparations (and after counts and stable isotope analysis). A laboratory notebook is present for recording any extractions for ^{14}C analyses, indicating the sample number, the extracted species name and total weight of the foraminiferal sample.

3. SAMPLE PREPARATION TECHNIQUES FOR THE ANALYSIS OF COCCOLITHS AND OTHER CALCAREOUS NANNOFOSSILS

3.1. General information

Calcareous nannofossils are very small microfossils (2 to 50 μm) composed of calcium carbonate. They are very good biostratigraphic markers within marine sediments by covering the Jurassic to present.

Among the calcareous nannofossils, coccoliths are the dominant group. Coccoliths are plates that form the backbone of coccolithophores, unicellular algal biflagellates belonging in the division of crysophytes. Calcareous nannofossils also include certain species of dinoflagellate cysts that are unicellular algal biflagellates belonging to the division of Dinoflagellata. Other calcareous nannofossils are observed in Mesozoic sediments (e.g. *Nannoconus, Schizophaerella*). However, their biological affinities are not known.

Coccoliths may be present in very large numbers in pelagic sediments (in the order of million to billion individuals per cm^3) and they may form oozes. In abyssal environments, below the lysocline, the preservation of coccoliths can be affected by the dissolution of calcium carbonate.

The objective of the preparation of a sample for nannofossil analysis is simple. Smear slides are used in many cases. However, it is desirable to defloculate and homogenise the sample to produce a slide containing a uniform sample of nannofossils.

The observation and counting of calcareous nannofossils is most commonly performed at a polarising optical transmitted light microscope at high magnification (1000x). The rotation of the slide on a rotating plane is useful for observing certain structures whose visibility depends on the angle of reflection. A phase contrast is occasionally used. The quality of observation is better when using a scanning electron microscope rather than an optical microscope. The scanning electron microscope is more time consuming than optical microscope. Thus, is not commonly used for routing counting but it is very helpful for taxonomic identification and the observation of certain microstructures (for example, dissolution features).

The preparations for observation under an optical or electron microscope are identical, all except for mounting the slide.

3.2. Sample preparation

Equipment: precision balance, incubator, ultrasonic bath
Materials: glass Petri dishes (60 mm), 100 ml beakers, slides, cover slides (22 x 22 mm), wash bottle containing distilled water, synthetic resin (Hyrax), 8 ml plastic containers, labels

1- Subsample 1 cm^3 of fresh sediment.
2- Place the sample in a pre-labelled glass Petri dish and weight it, then dry the sample in the incubator (40°C) for 12 hours.
3- Record the dry weight and transfer the sediment into a pre-labelled 8 ml plastic container (the volume and dry weight are essential for calculating the concentration per unit of volume. It is also good to note that the residue can be used for geochemical analyses).
4- Remove approximately 0.01 g of dry sediment by weighing it on the precision balance, and transfer it to a 100 ml beaker (the exact weight of the subsample to be treated is used in the concentration calculation).
5- Add 2.0 ml of distilled water to the sediment.
6- Place the beaker into the ultrasonic bath and sonicate for 30 seconds to 1 minute. This step disintegrates and deflocculates the sediment.
7- Glue two 22 x 22 mm cover slides to the bottom of a 60 mm diameter glass Petri dish.
8- Transfer the deflocculated sediment from the beaker into the Petri dish and rinse the remaining sediment from the beaker with 1-2 ml of distilled water into the Petri dish.
9- Shake the Petri dish a few times to disperse the sediment evenly across the surface of the dish.
10- Dry the Petri dish in the incubator (40°C) for a minimum of 8 hours.
11- Once the sample is dry, the two cover slides are transferred with tweezers to two small pre-labelled plastic Petri dishes. They are stored until time of analysis.
12- One of the slides is permanently mounted with synthetic resin (Hydrax) for counting the nannofossils with a polarising microscope. The other slide is reserved for observations with a scanning electron microscope (SEM).

3.3. Coccolith counting using a polarising microscope

The observation and counting of coccoliths is performed with a polarising microscope with a magnification of 1000x to 1200x. Since coccoliths are generally very abundant, the identification and counting of all individuals present on the slide would be a difficult task. The counting is therefore performed on a certain number of optical fields randomly distributed on the slide. In principle, when the preparation is adequate, the coccoliths are evenly distributed on the slide. Reproducibility tests on this method of counting produces a variation coefficient of approximately 10 % from one optical field to the next.

The coccoliths are counted in a minimum of 10 optical fields. When the concentrations are lower, a larger number of optical fields are counted until a minimum of 300 individuals have been counted. In the case of a sample with very few coccoliths, the whole surface of the slide is examined under the scanning electron microscope.

After calculating the number of individuals in the optical fields, the diameter of the optical field must be measured. The dimensions of the optical field can vary from one microscope to another, and the diameter can be measured with a micrometric slide.

3.4. Concentration calculations

The enumeration allows for the calculation of the concentration of coccoliths in the sediment by extrapolation, in numbers of individuals per unit weight or volume. The optical fields must be considered as an aliquot of prepared subsample. Therefore, the ratio of the optical field surface area and the Petri dish surface area must be known to calculate the number of coccoliths in the treated subsample:

$$N = \frac{n \times \pi(rp)^2}{nc \times \pi(rc)^2} = \frac{n \times (rp)^2}{nc \times (rc)^2}$$

where "**N**" represents the number of coccoliths in the subsample
"**n**" represents the total number of counted coccoliths
"**rp**" is the radius of the Petri dish
"**nc**" corresponds to the number of counted optical fields
"**rc**" is the radius of the optical field.

The weight of the subsample ($\mathbf{p_{se}}$) is known, as well as the corresponding initial weight ($\mathbf{p_e}$) of the 1 cm^3 sample, therefore a simple cross multiplication allows for the calculation of the concentration (**C**),

where $C = \frac{N \times p_e}{p_{se}}$

4. SAMPLE PREPARATION TECHNIQUES FOR THE ANALYSIS OF DIATOMS AND OTHER SILICEOUS MICROFOSSILS

The lake and marine sediments may contain abundant siliceous microfossils. In the marine environment, siliceous microfauna is represented by radiolarian endoskeletons (protists of the division sarcodina and class actinopoda) and ebridiens (protists of the division dinoflagellata), and by silicisponge spicules. Many marine algae produce siliceous microfossils: diatoms (class bacillariophyceae) with resistant frustules and cysts that fossilise, several representatives from the class of chrysophytes (chrysomonad cysts and silicoflagellate endoskeletons), and rare dinoflagellates (in particular the endoskeleton of *Actiniscus*). In lake sediments, siliceous microflora consists mainly of diatoms and chrysophyte cysts.

The most common siliceous microfossils in Quaternary deposits are the frustules of diatoms whose dimensions are of the order of 10 to 100 micrometers. Diatoms are indeed the dominant component of primary productivity in most marine and lacustrine environments. Their concentrations can reach millions of individuals per litre in the water column, and hundreds of millions of frustules per cubic centimetre in the sediment. Diatoms can build up and produce oozes or diatomite. Diatoms are good stratigraphic markers covering the Cretaceous to present, but are mostly used in Neogene biostratigraphy. The distribution of diatoms depends upon temperature, salinity and chemical characteristics of water such as pH and nutrients. Diatoms are useful in paleolimnology.

The abundance of siliceous microfossils in the sediment depends on the production of siliceous microfauna and microflora, but can be strongly affected by dissolution. The silica saturation of the water column and sedimentary environments is extremely variable and a determining factor. In general, low pH (< 7) and rapid accumulation rate promotes the preservation of biogenic silica. In alkaline conditions, often characterized by an under-saturation of silica, dissolution is frequent. This may be selective, if not total. The radiolarian or diatom assemblages, whose frustules and skeletons are made of relatively fragile opal, are often affected by dissolution. The solubility of silica increases with temperature. For this reason, better preservation occurs in cold environments. In paleoceanography., diatom analyses are useful mostly for the study of polar and subpolar environments and upwelling regions.

Preparation techniques presented below are intended primarily for the analysis of diatoms. However, these preparations allow the observation of other siliceous microfossils.

4.2. Sample preparation

There are many techniques for sample preparation for the analysis of siliceous microflora. Sample preparation for the analysis of nannofossils may also be used to make quantitative enumeration (see 3.2). However, better species identification is performed by additional treatments to eliminate carbonates and organic matter. The preparation techniques described below are suitable for a systematic analysis of siliceous microflora.

Equipment: precision balance, incubator, centrifuge, heating block for centrifuge tubes
Materials: 50 ml centrifuge tubes, 10 ml beakers, 10 μm Nitex mesh sieves, wash bottle containing distilled water, labels
Chemicals: hydrochloric acid (10% HCl), hydrogen peroxide (30% H_2O_2), phenol in solution

1- Subsample 1 to 2 cm^3 of sediment and record the volume and weight.
2- Dry the subsample in an incubator (60°C) for 24 hours or at room temperature until completely dry, then record the dry weight.
3- Remove and transfer 1.0 g of dry sediment to a 50 ml centrifuge tube.
4- Add 15 ml of hydrochloric acid (10% **HCl**) and allow it to react for a few minutes.
5- Add 15 ml of hydrogen peroxide (30% H_2O_2) and gently heat to 95°C until reaction is complete (approximately 20 minutes, must watch the reaction and stir sample to avoid it from overflowing).
6- Allow the sample to cool for a few minutes. Add 15 ml of distilled water. Centrifuge for 10 minutes at 2000 rpm.
7- Eliminate the supernatant. Refill with 45 ml of distilled water. Re-centrifuge and repeat the rinsing process three times.
8- Sieve the sample through the 10 μm Nitex mesh and collect both size fractions (< 10 μm and > 10 μm).
9- Dilute each fraction in 25 ml of distilled water in a pre-labelled glass container. Add a few drops of phenol and mix well.

4.3. Preparation of thin sections

Equipment: hot plate, micropipette (0.5 ml)
Materials: micropipette tips (0.5 ml), slides and slide covers (22 x 22 mm), synthetic resin (Hyrax), labels

1- Prepare two slide covers (22 x 22 mm) and place them on the hot plate at low temperature.
2- Pipette 0.5 ml of the > 10 μm fraction onto one of the slide covers and 0.2 ml of the < 10 μm fraction on the other. Make sure that the cells are homogeneously distributed on the slide cover (note: the pipette volume depends on the concentration of the diatom cells in the sample).

3- Allow the slide covers to dry.
4- Place one drop of synthetic resin (Hyrax) onto two slides and place a slide cover onto each of the slides.
5- Heat the slides until the resin has evaporated from the toluene (note: take care not to overheat, must avoid premature hardening and yellowing of the resin).
6- Remove the slides from the hot plate. Adjust the cover slides on the slides and eliminate the air bubbles and excess resin.
7- Prepare a second set of slides for each sample.

4.4. Diatom counting under optical microscope

Observation and counting of diatoms are generally made with an optical transmitted light microscope with magnification ranging from 250x to 1250x. To increase the contrast and facilitate observation of some structures, phase contrast or color filters are frequently used.

Based on the density of diatoms on the slide, counts are made on an aliquot of the total area. In general, we produce the counts on a number of lines distributed evenly over the slide. The ratio between the counted area and the total area of the cover slide must be known for the subsequent calculation of concentrations.

The enumeration of diatoms is usually based on the number of valves because whole frustules are rarely preserved (2 valves fit into the other to produce a frustule). Particular attention should be paid to the potential stacking of the two frustule valves, or of several frustules as in the case of colonial diatoms. Fragmentation of valves is common. It may be due to a mechanical syndepositional or postdepositional disturbance, to rough handling of the samples, or to partial dissolution of the opal which can weaken the valve structure. In the case of fragmentation, fragments of centric diatoms are counted (N = 1) when the central node can be seen, diatoms fragments are counted (N = 12) only once one is observed. The counting results are presented in numbers of valves, if not in numbers of frustules (number of valves / 2) per unit weight or volume.

4.5. Concentration calculation

Counts made it possible to calculate, by extrapolation, the concentration of diatoms in the sediment in number of valves or frustules per unit weight or volume.

A cross multiplication allows for the calculation of concentrations as follows:
1. Number of valves per pipette volume (VP) = number of counted valves * (analysed area / total area).
   Note: VP must be calculated in each of the prepared size fractions (> 10 μm and < 10 μm)

2. Number of valves > 10 μm per gram of dry sediment (Vg > 10 μm) = VP * (pipette volume / total volume of the suspended fraction > 10 μm).

2'. Number of valves < 10 μm per gram of dry sediment (Vg < 10 μm) = Vp * (pipette volume / total volume of the suspended fraction < 10 μm).

3. Number of valves per gram = Vg > 10 μm + Vg < 10 μm.

Note: if the weight of the dry sample is not equal to 1.0 g, an additional cross multiplication is needed to calculate the concentration per unit weight. Moreover, if the volume per dry weight is known, it is possible to calculate the concentrations per unit volume.

5. SAMPLE PREPARATION TECHNIQUES FOR PALYNOLOGICAL ANALYSIS (POLLEN AND SPORES, DINOFLAGELLATE CYSTS, AND OTHER PALYNOMORPHS)

5.1. General information

Originally, the term palynology applied mainly to the study of pollen (palynos = dust). By extension, palynology now consists of all microfossils with a refractory organic membrane formed of chitin or sporopollenin resistant to hydrochloric and hydrofluoric acids. The microfossils include algal cysts and organic linings of various protists: they are grouped under the term of palynomorphs.

The pollen analysis requires a pre-treatment of the sediment to concentrate the palynomorphs and promote their microscopic observation. The technical preparation of the sediment consists of mechanical separation (sieving and/or heavy liquid separation) and chemical treatment (hydrochloric and hydrofluoric acids, potassium ...). Laboratory protocols are different depending on the type of sediment studied (organic or terrigenous) and the objective of the analysis (enumeration or taxonomy). After treatment, the residue contains palynomorphs that are mounted between a slide and coverslip for microscopic analysis. The observation of palynomorphs, whose dimensions are generally between 5 and 150 μm, is made with high magnification (> 250x) under a transmitted light optical microscope or a scanning electron microscope. Different techniques of optical microscopy can be used, including interference contrast and fluorescence.

Palynology is certainly one of the most important micropaleontological disciplines: it allows the study of all types of deposits from the Precambrian to present in land, lake or sea. The advantage of the palynomorphs over other microfossils is their ability for preservation, despite the dissolution of silicates or carbonates. Preservation of palynomorphs may however be affected by advanced sub-aerial oxidation of organic matter, or a very basic environment.

Palynology is a key tool in paleoecology because it allows the reconstruction of marine or lacustrine paleoenvironments. Since palynomorphs constitute the bulk of the refractory organic matter, palynology can be used as a tracer of the origin and nature of organic carbon. Moreover, the palynofacies and altered state of palynomorphs can be used as tracers of diagenesis in sedimentary and petroleum geology.

In the field of Quaternary palaeoecology, palynology can be divided into two major disciplines: terrestrial palynology which mainly concerns the study of pollen and spores, and marine palynology based mainly on the study of dinoflagellate cysts.

5.1.1. Pollen, spores and other continental palynomorphs

Lake sediments generally contain a large number of organic microfossils. The most common among them are pollen and spores, which are the reproductive bodies of vascular plants (of the division spermatophytes and pteridophytes, respectively). The spores of mosses (bryophytes) and fungi (mycophytes) are also composed of chitin and are fossilised. In continental aquatic environments, several algae produce organic microfossils: such as certain chlorococcales and zygnematales (of the division chlorophyta), and some dinophyceae (of the division dinoflagellata). In soil and lake environments, the organic linings of thecamoebians (protozoa of the class rhizopoda) are fossilised.

Pollen and spore microfossils are most used for paleoenvironmental and paleoclimate reconstructions. Produced in large numbers by vascular plants and well preserved, they are very abundant in lake sediments (10^3 to 10^6/cm^3). Their morphology most often allows identification at the genus level. The pollen assemblages provide reconstructions of past vegetation and plant landscapes, and to trace the evolution of climate.
The spores and pollen are used to trace the phylogeny of vascular plants since the Silurian. These microfossils provide good stratigraphic markers and paleogeography.

5.1.2. Dinoflagellate cysts and other marine palynomorphs

Marine dinoflagellate cysts (of the class dinophyceae) are the dominant palynomorph assemblages. The spores of some prasinophytes (of the division chlorophyta), organic linings of tintinnids (of the class ciliate) and benthic foraminifera (of the class rhyzopoda) and chitinozoans and acritarchs (extinct groups whose affinities are uncertain) are organic microfossils that can be observed in marine sediments.

In paleoceanography and paleoclimatology, the dinoflagellate cysts prove to be valuable indicators. The cysts, which are hypnozoites related to reproduction (diploid phase of the life cycle of dinoflagellates), provide a picture of productivity in the photic zone. The current distribution of dinoflagellate cyst assemblages appears to be closely related to their physico-chemical environment: nutrients, temperature, salinity, seasonality, and sea ice cover. Dinoflagellate cysts are particularly abundant in marine sediment environments such as continental, epicontinental, and estuarine margins (10^2 to 10^5 cysts/cm^3).

Dinoflagellate cysts are good indicators for the biostratigraphic interval from the Jurassic to present their optimum marking the Cretaceous. The acritarchs, some of whom are the ancestors of dinoflagellates, are excellent biostratigraphic markers during the Proterozoic, Paleozoic and Mesozoic. The chitinozoans are also widely used in biostratigraphy of the Paleozoic.

5.2. Sample treatment and preparation

The density of palynomorphs in the sediment is relatively low, around 10^1 to 10^5 individuals per unit volume. The volume of treated sediment depends on the type of sediment studied. The pollen analysis of lake deposits can be made from 1 cm^3. The palynological analysis of marine deposits generally requires the treatment of 5 cm^3 of sediment. Laboratory preparations include concentrating the palynomorphs using mechanical manipulations (multiple sieving) and chemical treatments. A minimum of two days is required to prepare the samples.

The samples are preferentially treated in pairs to balance the tubes during centrifugations. Series of 6 or 12 samples are prepared simultaneously. The treatment routinely uses distilled water for rinsing and various other manipulations. Any centrifugation is preceded by tube equilibration with distilled water on a scale designed for this purpose.

5.2.1. Sample pre-treatment

Previous method (< 2000)

Equipment: magnetic stir plate, hot plate, centrifuge, micropipette (0.5 ml)
Materials: magnetic stir bars, 25 ml graduated cylinder, 50 ml centrifuge tubes, 250 ml beakers, micropipette tips (0.5 ml), 10 µm Nitex mesh and 120 µm sieves, wash bottle containing distilled water, labels
Chemicals: sodium metaphosphate (10% $Na(PO_4)_6$), phenol in solution
Other: marker pollen suspended in an Erlenmeyer flask

1- At least one hour prior to the start of pre-treatment, suspend the marker (reference) pollen (*Eucalyptus globulus*) by homogenising it with a stir bar in an Erlenmeyer flask.
2- Assign the series numbers to the samples and record the information in the laboratory notebook.
3- Subsample 5 cm^3 of sediment (e.g. in general for marine sediments), measured by displacement in a 25 ml graduated cylinder.
4- Transfer each sample into a prelabelled beaker. At this step, a defloculant (a few drops of sodium metaphosphate in solution (10% $Na(PO_4)_6$)) can be used to disintegrate the clays.
5- Boil the sediment for 4 to 6 minutes to disintegrate the sample.
6- Add 0.5 ml of the marker pollen solution with the micropipette (the precision of the micropipette should be regularly calibrated), replacing the micropipette tip with every addition.

7- Filter each sample through the sieves by stacking the 120 µm sieve over the 10 µm one. Sieving through the 10 µm sieve is accelerated by placing a magnetic stir bar on the Nitex mesh, and placing the sieves on a magnetic stir plate. The >120 µm and < 10 µm fractions are collected in labelled plastic containers. Dry the < 10 µm fraction in an incubator (40°C), and transfer the dried sediment to a labelled plastic bag. This fraction is kept for clay analysis.
8- Transfer the 10 to 120 µm fraction to into a labelled conical centrifuge tube.
9- Centrifuge the tube at 2000 rpm for 10 minutes and remove the supernatant.

Current method:

Equipment: magnetic stir plate, centrifuge
Materials: magnetic stir bars, 25 ml graduated cylinder, 50 ml centrifuge tubes, 250 ml beakers, 10 µm Nitex mesh and 106 µm sieves, wash bottle containing distilled water, labels
Chemicals: phenol in solution
Other: Stockmar *Lycopodium clavatum* tablets

1 - Assign the series numbers to the samples and record the information in the laboratory notebook.
2 - Make subsampling, weight and dry the sediment as indicated in section 2.2.1 (steps 1-5).
3 - Transfer each sample into a prelabelled beaker.
4 - At this step, warm water is added to help sediment to deflocculate
5 - Add one or two tablets of *Lycopodium* (each tabletcontains of the order of 10 000, with the mean number being indicated on the Stockmar flask; the number of tablet added depends upon the expected concentrations of palynomorphs).
6- Filter each sample through the sieves by stacking the 106 µm sieve over the 10 µm one. Sieving through the 10 µm sieve is accelerated by placing a magnetic stir bar on the Nitex mesh, and placing the sieves on a magnetic stir plate. The >106 µm and < 10 µm fractions are collected in labelled plastic containers. Decant the < 10 µm fraction. This fraction is kept for clay analysis.
7- Transfer the 10 to 106 µm fraction to into a labelled conical centrifuge tube.
8- Centrifuge the tube at 2000 rpm for 10 minutes and remove the supernatant. Add a drop of phenol is the residue is stored.

5.2.2. Chemical treatments

*Caution: the chemical treatments consist of attacking the sediment with hydrochloric (**HCl**) and hydrofluoric (**HF**) acids to eliminate carbonate and silica minerals respectively. The*

*hydrofluoric acid, in particular, is very dangerous: all contact or inhalation must be avoided. Wearing gloves, a smock, and protective eyewear is essential. The acidifications are exclusively performed under an acid resistant fume hood. After use, the acids are disposed of in containers for toxic chemicals following the strict guidelines provided by the province. Prior to each hydrofluoric acidification, the application of a protective cream is suggested. At the end of all chemical treatments, washing your hands with soap and water is advised: experience has shown us that laboratory gloves are not always an effective precaution. Absorbent pads are used for large acid spills, except for **HF**. If there is an **HF** spill in the fume hood, neutralise it with NaOH. In the case of skin contact with **HF**, rinse and wash with soap and cold water for 15 minutes and then apply the antidote cream (found in the laboratory) and follow the directions (contact with **HF** is not accompanied by an immediate burning sensation, but will be felt deeper in the skin after a few minutes, so even in doubt, it is recommended to proceed with washing and cream immediately).*

Equipment: centrifuge, heating block for centrifuge tubes, vortex mixer
Materials: 25 ml graduated cylinder, 50 ml centrifuge tubes, 8 ml conical tubes, metal spatulas, 10 µm Nitex mesh and 120 µm sieves, wash bottle containing distilled water, labels
Chemicals: hydrochloric acid (10% **HCl**), hydrofluoric acid (48% **HF**), potassium hydroxide (10% KOH)

1- Homogenise the pellet of sample and add a few millilitres of 10% **HCl**. Mix the pellet with a metal spatula gradually adding **HCl** (the **HCl** solution is poured gradually to avoid a violent reaction causing the acid to overflow). Note the intensity of the reaction in the laboratory notebook. Place the tubes in the heating block for about twenty minutes to complete the reaction. Centrifuge at 2000 rpm for 10 minutes. Pour the supernatant liquid into the appropriate waste container and mix the pellet with a spatula or the vortex mixer.
   Note: when the reaction is complete, the acid is greenish or brownish in colour. In the first case, the bulk of the pellet is probably mineral. In the second case, it is likely that the sediment contains a lot of organic matter. An attack of potassium hydroxide (10% KOH) will be required to complete the chemical treatment.
2- Add a few millilitres of hydrofluoric acid (49% **HF**). Mix the pellet using a metal spatula, gradually adding the acid (**HF** is poured over time to avoid a violent reaction causing the acid to overflow). Note the intensity of the reaction in the laboratory notebook. Place the tubes in the heating block for about twenty minutes to complete the reaction. Centrifuge at 2000 rpm for 10 minutes. Pour the supernatant liquid into the appropriate waste container and mix the pellet with a spatula (the use of the vortex mixer is not recommended because small drops of **HF** can be projected).
3- Proceed to treatment with hot **HCl** for twenty minutes (see step 10) in order to eliminate fluorosilicates gels formed during the reaction with **HF**. Centrifuge at 2000 rpm for 10

minutes. Drain the supernatant liquid and homogenise the pellet.

4- The previous steps (11 and 12) are repeated one, two or three times until the silicates and fluorosilicates are completely dissolved. The **HF** treatment can be done at night, leaving the sample remain in the acid (see step 11).
5- When samples contain a lot of organic matter, it is desirable to proceed with a treatment potassium hydroxide (10% KOH) for a maximum of 10 minutes, followed by a centrifugation. Such treatment is intended to deflocculate the organic matter and should not be prolonged because it can alter the organic membrane of some dinoflagellate cysts (peridinials in particular).
6- At the end of chemical treatments, wash the pellet with distilled water to remove residual acid. Centrifuge the supernatant and drain.
7- A final sieving at 120 and 10 μm is achieved.
8- Recover the 10 to 120 μm fraction and centrifuge for 10 minutes. Remove supernatant and transfer the pellet to 8 ml conical tubes and centrifuge again for 10 minutes. The pellet will is then ready to be mounted on a slide for subsequent observation under an optical microscope.

5.2.3. Optional treatments

The preparation techniques described above constitute an established protocol for the treatment of samples rich in inorganic particles (silicates and carbonates) and in view of systematic counts. Other techniques can be used depending on the type of sediment or analytical purpose. The most common techniques are listed below for guidance:

5.2.3.1. Separation by heavy liquid: heavy liquids can be used at the start or end of treatment for better separation of organic microfossils (density < 1.2) and the mineral fraction. Heavy liquids of common use include bromoform-acetone, zinc chloride or zinc bromide with density > 1.4, and sodium polytungstate with density of 2.0 after mixing with water (initial density of 2.89; density calibration with a pycnometer). Amongst heavy liquids, sodium polytungstate is particularly useful because it is nontoxic, water soluble and can be recycled. Onboard ship, it allows palynological preparation and separation of silica when the use of **HF** is forbidden for safety reason. The use of heavy liquid preparation provides proper preparations, perfect for taxonomic observations, photographing specimens and systematic descriptions. It does not, however, recover all palynomorphs because a loss on the tube walls can occur, some palynomorphs containing pyrite or other mineral particles in the cavity or on their processes can settle out, palynomorphs diagenetically mineralized or very mature can imperfectly separate. The heavy liquid separation is therefore to be avoided in the context of systematic counts.

5.2.3.2 Potassium hydroxide : when samples contain high amounts of organic matter, it is useful to do a treatment with potassium hydroxyde (KOH - 10%) during 10 minutes maximum. The treatment is followed by centrifugation, decantation and sieving with water on 10µm mesh sieve. Such a treatment helps to defloculate organic matter. It should not be applied more than a 10 minutes because it may alter the wall of some dinocyst taxa, the Protoperidinioids notably

5.2.3.3. Acetolysis and other oxidation techniques: in conventional palynology (the study of pollen and spores), oxidation techniques are frequently used to destroy the intine of pollen grains or eliminate the maximum organic matter in the gyttja or peat. Acetolysis is a technique conventionally used in aeropalynology, melissopalynology or continental palynology. It is a treatment in a solution of sulfuric acid and acetic anhydride, which would not affect the exine of pollen grains. This method of oxidation of organic matter, however, causes the dissolution of some aquatic palynomorphs, especially certain dinoflagellate cysts (peridinals and gymnodinials groups in particular). The acetolysis is therefore banned at GEOTOP. The fact that little information exists on dinoflagellate cysts in lake sediments may be because continental palynologists systematically use acetolysis destroying the dinoflagellate cysts belonging to the families of peridinaceae, gymnodiniaceae or ceratiaceae or who are generally abundant in freshwater environments. In addition to acetolysis, various oxidation techniques are used in other laboratories, "Luber" for example, which consists of a treatment with a combination of nitric acid and hydrochloric acid. These techniques are quite corrosive and affect all palynomorphs, including some pollen grains.

5.3. Slide preparation

Mounting the pellet (residue containing palynomorphs) is delicate work that requires some attention. The materials used include gelatinised glycerin (see 5.7), toothpicks, slides and coverslips (22 x 22 mm or 22 x 75 mm), and a hotplate. The main steps of the assembly are as follows:

1- Immediately after centrifugation of the 8 ml conical tube, empty the supernatant using the hand pump to remove as much water without creating turbulence or pumping the residue.
2- Place a small cube of gelatinised glycerin on a pre-labelled slide and placed the slide on the hot plate and wait until the cube melts.
3- Homogenise the pellet using the vortex mixer, then take a drop of the pellet with a disposable Pasteur pipette, place the drop onto the gelatinised glycerin.
4- Mix the pellet and glycerin with a toothpick gently extending the solution on the slide and let the excess water evaporate.
5- Place the coverslip avoiding the formation of air bubbles.
6- Allow the slide to heat for a few minutes so that the glycerin spreads evenly under the

coverslip.

In the tube containing the residual pellet, add a few drops of phenol solution to prevent the development of bacteria. The tubes are kept refrigerated in labelled containers.

Other media for mounting can also be used. Gelatinised glycerin has the advantage of being a semi-permanent medium and easy to manipulate. Silicone oil and liquid glycerin media are commonly used in conventional palynology: they can turn the grains, but they are limited and must not be used if taxonomic observations are required. The existing permanent media (hyrax and other polymers) are used exclusively by taxonomists, but are difficult to handle.

Dyes can be added to the mounting medium to increase the contrast of the structures of palynomorphs. The dyes most commonly used are neutral red and basic fushine. These dyes can affect the fluorescence of palynomorphs and limit other observations at the microscope. Moreover, the natural pigment of some palynomorphs, covered by the dyes, can be useful in determining taxonomy. Thus it seems preferable to avoid artificial colouring.

5.4. Observation and counting

Observation and counting of palynomorphs is performed with a transmitted light optical microscope at a magnification of 250x to 1250x. Based on the density of palynomorphs on the slide, you can continuously scan the whole slide or a scan of a few lines distributed randomly on the surface of the slide. Normally, when the slide was mounted with a properly homogenised pellet and glycerin jelly, palynomorphs are distributed evenly over the slide. Depending on the viscosity of the glycerin during mounting, it is possible that the palynomorphs are selectively distributed on the slide, where larger specimens are focused either at the centre or along the margins of the coverslip. If partially scanning the slide is required, it is thus necessary to select lines (3 in minimum) randomly distributed.

The minimum count to be achieved is ideally 300 for pollen grains and dinoflagellate cysts. Obviously, in some samples palynomorphs are too scarce for such amounts, even after a complete scan of the slide. Fewer counts are therefore acceptable, at least for the calculation of concentrations. A minimum of 100 individuals counted may eventually be used to calculate percentages in an assemblage. The counting rules depend on the purpose of analysis (calculation of concentrations or population analysis), the richness and diversity of the species of microfossil.

Different techniques can be used at the microscope. The counts are generally routine using transmitted light, with or without a filter. The interference contrast can be useful for observing semi-transparent organic microfossils, and is recommended for observation and photography of

dinoflagellate cysts. Fluorescent lighting is also useful, since it allows for better visualisation of certain structures. Moreover, the degree of fluorescence of organic microfossils varies by diagenetic alteration of chitin or sporo-pollenin.

5.5. Concentration calculations

The simultaneous counting of palynomorphs and marker grains or spores (*Eucalyptus globulus* or *Lycopodium clavatum*) can be calculated by extrapolation for the concentration of pollen and dinoflagellate cysts in the original sample as the number of individuals per unit weight or volume. The concentration of grains in the suspended marker pollen is known after multiple calibrations with a hematocytometer (see annex), and the volume of the marker added to the sample during the pre-treatment is also known. We are thus able to calculate the number of marker pollen grains added to the sample:

$$Ne = Ce \times Ve$$

where "**Ne**" represents the number of marker grains added to the sample
"**Ce**" represents the concentration of marker grains in suspension (in grains/ml)
"**ve**" is the volume of the suspended marker grains added to the sample (in ml).

The proportion of marker grains and dinoflagellates counted then allows for the calculation of the number of palynomorphs in the sample using cross multiplication:

$$Np = \frac{Ne \times np}{ne}$$

where "**Np**" represents the number of palynomorphs in the initial sample
"**Ne**" represents the number of marker grains added to the sample
"**np**" represents the total number of counted palynomorphs
"**ne**" represents the total number of counted marker grains.

To evaluate the concentration of palynomorphs per unit volume (e.g. grains/cm^3) divide the number of palynomorphs (Np) by the initial volume of the sample.

The marker grain method used in at GEOTOP provides results whose reproducibility has been estimated at 10% with a 95% confidence interval. Marker grains used must be distinct from those present in the sample. The grains of *Eucalyptus globulus* are not native to eastern Canada or the North Atlantic, and they can be used in samples from most of the Pacific. A suspension of *Lycopodium clavatum* is prepared for the analysis of samples collected off Australia. The

calculation of concentrations can be done using different methods. The method of aliquots of weight or volume (using the same principles as the methods used for diatoms) is frequently used. However, the results show a lower reproducibility partly due to inevitable losses during the many operations to concentrate the palynomorphs.

5.6. Preparation and calibration of marker pollen grains in suspension

Palynomorph concentrations are evaluated using a tracer consisting of a calibrated suspension of exotic pollen grains (*Eucalyptus globulus* and *Lycopodium clavatum* are commonly used) with a fixed volume is added to samples before treatments. The marker pollen grains are mixed with a viscous solution to ensure long-term suspension (several hours) and to promote a uniform distribution of grains. Corn syrup is an adequate medium.

5.6.1. Preparation of the suspension of the marker grains

Equipment: magnetic stir plate, centrifuge
Materials: Erlenmeyer flask, magnetic stir bar, 8 ml centrifuge tubes
Chemicals: acetone, corn syrup, phenol
Other: fresh pollen grains

1- Place two pinches of *Eucalyptus globulus* pollen in the 8 ml centrifuge tube.
2- Rinse the pollen several times with acetone and remove the supernatant after each centrifugation.
3- Mix the pellet of pollen with a solution of 120 ml of corn syrup and 80 ml of distilled water in an Erlenmeyer flask and add a stir bar.
4- Add 1 g of phenol to prevent bacterial growth (the odour of phenol must be pronounced).
5- Stir on the magnetic plate for several hours prior to the calibration.

N.B. The tube in which the grains were centrifuged must be thrown out.

5.6.2. Calibration of the suspended *Eucalyptus globulus*

The evaluation of marker grain density in suspension is a very important step because it will serve to calculate the concentration of palynomorphs. The calibration of the suspension is made from a series of measurements (N > 50) on the concentration of marker grains using a hematocytometer.

The calibration requires a microscope with a 10x objective, pipettes pastor, hemacytometer, and a hematocytometer counting slide. Each measurement is carried out according to the following steps:

1- Place the slide on the hemacytometer.
2- Pipette a small quantity of the suspension (preferably from the centre of the Erlenmeyer flask) and place one drop in each cell of the slide which are at the extremities of the hemacytometer. A new pipette tip is used for each sampling of the suspension.
3- Wait a few seconds so that the suspension spreads evenly and stabilises under the coverslip.
4- Place the hemacytometer under the microscope and count the marker grains in two determined series of cells. On both sides of the central cell, the hemacytometer is lined with a checkerboard composed of nine squares. In each of the squares, marker grains are present in 5 sections: the four corner sections and the central section (see attached counting sheet). Ten sections of the hemacytometer square correspond to a 1 mm^3 volume.
5- After counting, clean and dry the coverslip and hemacytometer.

<u>Repeat procedures 1 to 5 a minimum of 25 times to obtain adequate counting statistics.</u>

The concentration of suspended marker pollen grains is evaluated from estimates made in 5 mm^3 (i.e. 5 sets of counting 1 mm^3). Counts in 5 mm^3 of the suspension are of the order of one hundred grains. Such counts are compatible with the pollen counts on the slides.

Proceed with the estimation of the concentration at least five times. The average score is then considered as a representative concentration of marker grains in the suspension: the average is used for calculating concentrations of palynomorphs in palynological slides by a simple cross multiplication (see 5.5). The standard deviation around the average should be at least 10%. If this is not the case, a non-homogeneous suspension was probably the cause and we must conduct additional counts.

It is worth noting that the concentration of marker grains in suspension should be adjusted according to the density of palynomorphs in the samples. Statistically valid concentration calculations normally require a few hundred palynomorphs and an equivalent number of marker grains. For the analysis of samples for palynomorphs (e.g. with concentrations of approximately 10^2 to $10^4/cm^3$ in deep marine sediments), the suspension is prepared so that the concentration of marker grains will be about 30 000 grains / ml (adding ~ 15 000 marker grains per 5 cm^3 of sediment, or ~ 3000 marker grains per cm^3). For the analysis of sediments containing a rich palynoflora (e.g. of the order of the $10^5/cm^3$ in gyttja), it is desirable to have a suspension in which the concentration is high, approximately 200 000 grains/ml (adding 100 000 grains per 1 cm^3 of sediment).

5.7. Preparation of the "Kaiser" gelatinised glycerin for mounting slides

The gelatinised glycerin is prepared following the recipe below:

1- In a beaker, mix 8 g of "Knox" gelatin with 32 ml of distilled water.
2- Add 56 g of glycerin and 1 g of crystalline phenol.
3- Heat for 15 minutes on a hot plate and filter if necessary.
4- Transfer the gelatinised glycerin into a covered plastic container.

It is important to not over mix to avoid producing air bubbles. If needed, colour may be added. The gelatinised glycerin is stored in a closed container at room temperature.