Protocol for extraction of pollen and dinoflagellate cysts from marine sediments

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1. Preparation of sediment: take approximately 5 g of wet sediment and place it in the oven overnight at 40°C until the sediment is dry. Sediments with a lot of water may need to be kept in the oven for longer.

2. Estimate volume of sediment: put 15cc of distilled water in a 25cc polypropylene graduated cylinder. Place the tube on a digital scale and reset it to zero (tare weight). Then add the sediment to the water and record the weight and displaced volume.

3. Sediment separation: this separation allows extracting several proxies from the same sample set. The top fraction is recovered for the study of foraminifera, and the bottom fraction for the study of pollen and dinoflagellate cysts. Take a 150µm sieve of 10cm diameter and place it on a 1000ml beaker. Put the sample on the sieve and gently wash it with water. Rinse the sample until the residue on the sieve is clean and the water coming through is clear. Transfer the upper fraction to a separate vial. Leave the lower fraction in the beaker and let it settle for a minimum of 48 hours. Cover the beaker to avoid contamination.

Cold hydrochloric (HCl) treatment: it is important to carefully follow this treatment to eliminate calcium carbonate particles.

4. First, use a vacuum pump to extract excess water from the beakers. Use a tube with a 90^{o} angle tip (Photo #1) and place on the wall of the beaker to prevent accidental aspiration of the sample. Once the majority of water is removed, transfer the sample to a 100ml polypropylene centrifuge tube with round or conical bottom and caps.

5. Centrifuge the samples at 2500 rpm for 7 minutes and decant the supernatant. Stir pellets up again with a test tube agitator (Photo #2). Add 1 or 2 *Lycopodium* tablets (Appendix 1) to each sample and record the number of tablets added and the number of spores per tablet.

6. The cold HCl treatment consists of three steps: First, add a few ml of HCl at 10% to each tube and stir with a glass rod. This first step is done with HCl at low concentration to prevent an intense reaction and overflowing of the tubes. Second, add a few ml of HCl at 25% to each tube and wait a few minutes while stirring. Finish by adding HCl at 50% to each tube and wait a few minutes while stirring. Add more HCl at 50% until the sample has stopped reacting (no longer fizzing or effervescing).

Hydrofluoric acid (HF) treatment (cold): this treatment eliminates silicates and silica.

7. Centrifuge the 100 ml tubes for 7 minutes at 2500 rpm and decant the supernatant. Stir pellets up again with a test tube agitator. Add between 40 and 50 ml of **cold HF at 45%** to each tube.

Cap tubes and place them on a shaker (Photo # 3) for 4 to 5 hours. Avoid changing the concentration of HF added to the samples because higher concentrations may produce effervescence and the risk of losing the sample.

8. Centrifuge the samples again at 2500 rpm for 7 minutes and decant the supernatant. Stir pellets up again with a test tube agitator. Add between 40 and 50 ml of **cold HF at 70%** to each tube. Cap tubes and place them on a shaker for 28 to 30 hours.

   Warning: Be careful during this operation. It is imperative to follow all security guidelines for the safe use of hydrofluoric acid (HF): use long gloves and goggles, and always open and handle only in a closed chemical hood. Avoid leaving tubes or bottles open during treatment.

Second HCl treatment: this treatment eliminates fluorosilicates.

9. Centrifuge the tubes for 7 minutes at 2500 rpm and decant the supernatant. Stir pellets up with a test tube agitator. Add between 40 and 50 ml of **cold HCl at 25%** to each tube. Cap tubes and place them on a shaker for 15 minutes.

   Note: It is very important to this do this HCl right after treating with HF and before rinsing with distilled water. There might be a risk fluorides forming and hamper the rest of the treatment.

Rinsing

10. Centrifuge the tubes for 7 minutes at 2500 rpm and decant the supernatant. Stir pellets up with a test tube agitator. Fill tubes with distilled water. Centrifuge the tubes for 7 minutes at 2500 rpm and decant the supernatant. The samples are ready for filtration.

Filtration. This requires the implementation of a filtration system (Photo # 4) and the help of a vacuum.

11. Stir pellets up with a test tube agitator and place the sample on a nylon filter mess of 10 µm (Photo # 4). Rinse with distilled water using a squeeze bottle of 50 ml. The size of the squeeze bottle is essential because it allows producing a jet powerful enough for filtration. If the filter gets obstructed, place the ultrasound tip for 20 to 40 seconds (Photo #5) and continue to rinse with abundant water using the squeeze bottle. The success of this operation depends on the coordination between the ultrasound and the squeeze bottle. It is important to measure well the time of the ultrasound, because if applied for too long it can break microfossils.

12. Once the residue is clear and the water coming through the filter is clear, transfer the samples from the filter to a 50ml polypropylene test tube using the squeeze bottle and distilled water. Rinse the filter well to detach any microfossils that may be stuck in the mesh.

13. Centrifuge the tubes for 7 minutes at 2500 rpm and decant the supernatant. Use a vacuum pump and a tube with the 90^{o} angle tip to extract excess water from the beakers. Transfer the residue with distilled water in a plastic 8ml conical tube. Centrifuge the tubes for 7 minutes at 2500 rpm and decant the supernatant using the vacuum pump and the 90^{o} angle tube. The residue is ready for mounting.

Microscope slide mounting. Two kinds of mounts can be done depending on the microfossils to be studied.

Fixed slides with glycerin jelly. This mounting technique is used for the study of dinoflagellates.

14a. Place a microscope slide (76 x 26mm) on the hot plate (temperature 200°C-250°C). Put a drop of glycerin jelly mixture (see annex) onto the slide and add a few drops of sample, dosing according to the desired concentration. Evaporate the water and then put a cover slip (24x32mm) over the sample. Remove the slide from the hot plate and fix edges with *histolaque*.

Moving slides with bidistilled glycerin with Phenol. This mounting technique is used for the study of pollen grains.

14b. Place a microscope slide (76 x 26mm) on the hot plate (temperature 200°C-250°C). Put a drop of glycerol (see annex) onto the slide and add a few drops of sample, dosing according to the desired concentration. Evaporate the water. Meanwhile add a line of *histolaque* on each side of a cover slip (24x32mm) lengthwise. Once evaporation is complete, place the coverslip over the sample. Remove the slide from the hot plate and fix the remaining two sides with more *histolaque*.

Conservation:

15. To preserve the prepared samples, fill the 8ml tubes with distilled water, and add a few drops of glycerol (see annex).

Photos:

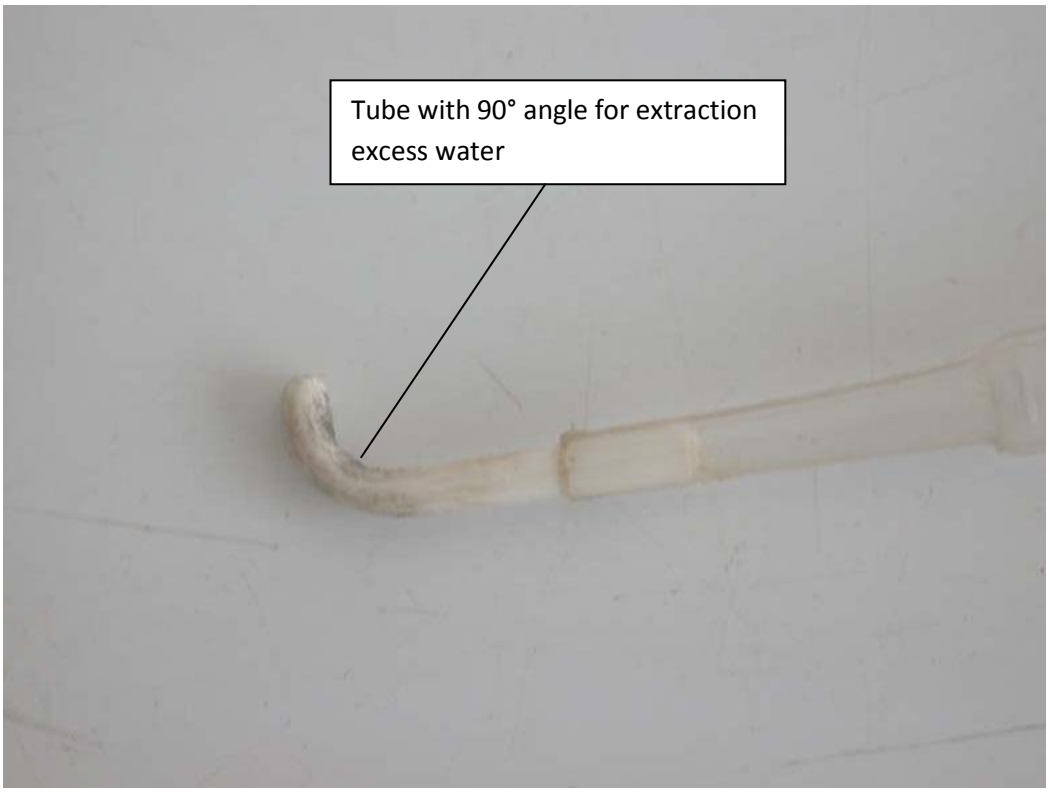

Photo n°1

Photo n°2

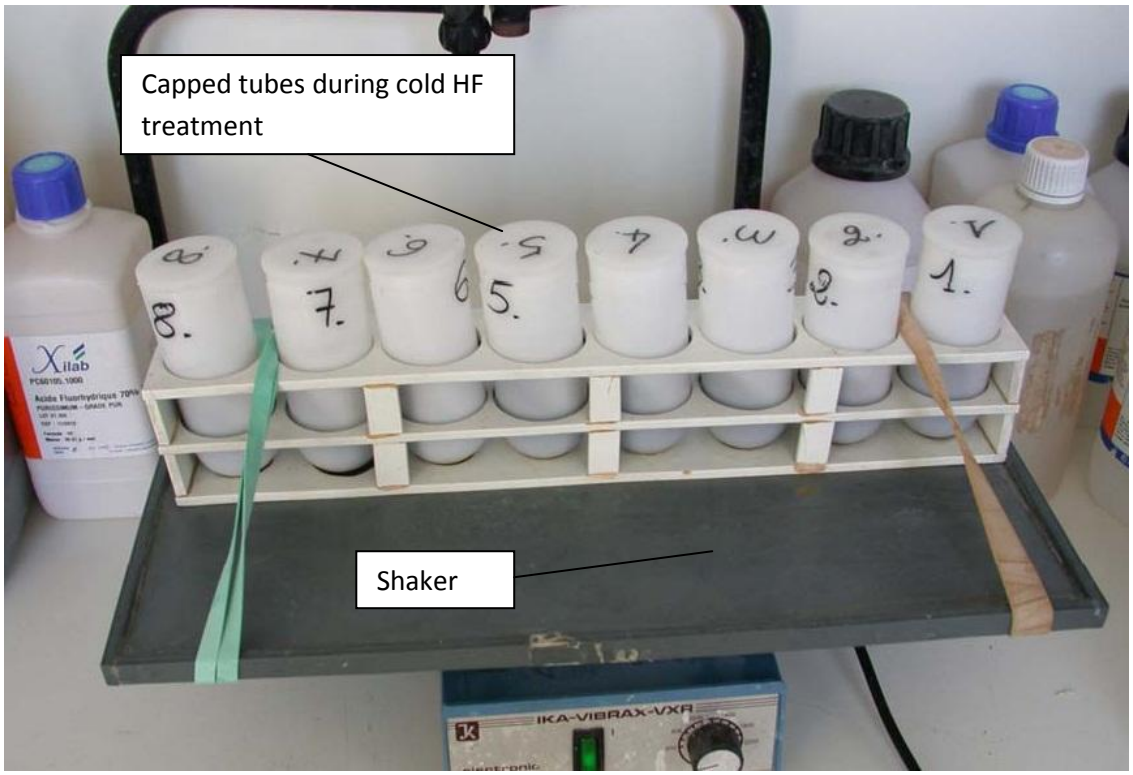

Photo n° 3

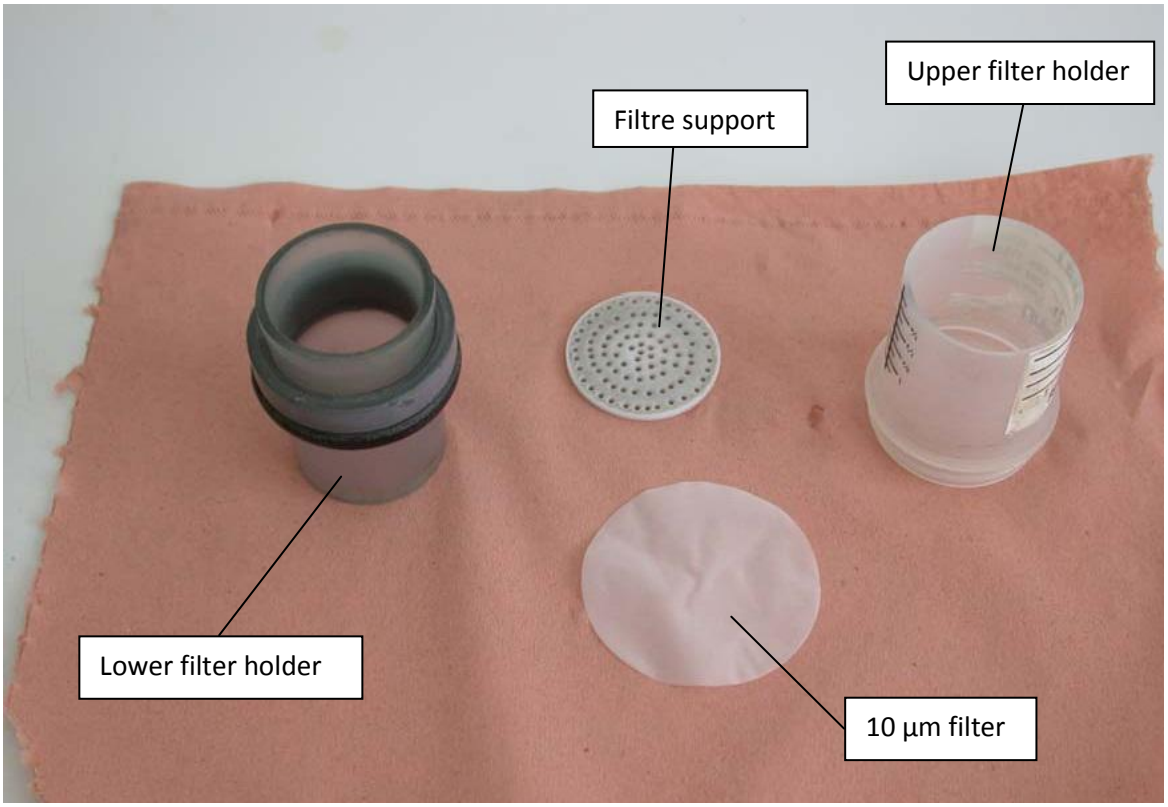

Photo n° 4

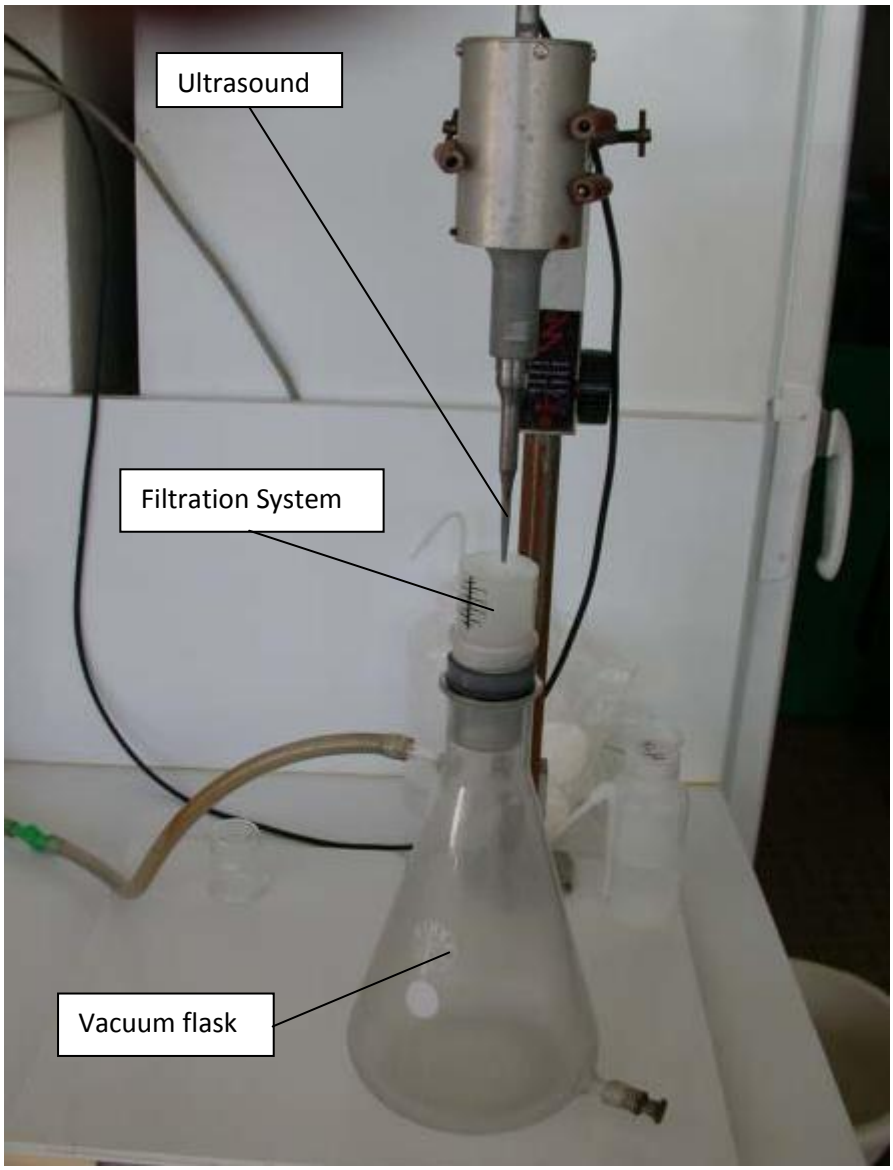

Photo n° 5

Annexes:

Supplier for *Lycopodium* tablets

Lund University
Department of Geology
Quaternary Sciences
Sölvegatan 12
SE-223 62 Lund
Sweden
Fax: 46-46-2224830

Supplier for 10 micron filters:

Saulas and Co.
5, rue des spruce
BP 20
10160 Paisy Cosdon
France
Fax: 03 25 40 74 87

Preparation of glycerin jelly:

Place 10g of gelatin in a beaker and 34.2 cc of distilled water. Let stand cold for 2h without stirring. Then add 51 cc of 98% glycerol and 1g of phenol. Heat until complete dissolution. The solution solidifies as it cools down. The beaker on the hot plate to liquefy the gelatin for during slide mounting.

Preparation of Phenol bidistilled glycerin for conservation:

Put 1% phenol in 1 liter of 98% glycerol.