POLLEN PREPARATION PROCEDURE

SAFETY: See associated COSSH form:

CHEMICALS REQUIRED:

Lycopodium	7% Hydrochloric acid (HCl)
Ethanol	Distilled water
10% Sodium hydroxide (NaOH)	Hydrofluoric acid (HF)
Acetic anhydride	Concentrated sulphuric acid (H_2SO_4)
Acetic acid (glacial)	TBA (tert-butanol)
Calcium gluconate gel	Sodium carbonate (or equivalent)
0.2% aqueous safranin	Silicone oil

EQUIPMENT REQUIRED:

40x 50ml centrifuge tubes	40x 15ml centrifuge tubes
50ml test tube racks	15ml test tube racks
Fume cupboard	Water bath
Pop-top plastic storage pots	Vortex /whirli mixer
Glass rods	Teflon rods
1L plastic beakers	150 µm mesh
20 sieve holders	20 funnels
50ml tube caps	15ml tube caps
Cloth	HF safety kit (gloves, apron, face shield)
Waste bottle for HF	Safety pack for HF
DRY graduated cylinder	Gradated pipette and bulb
Glass vials with lids	Nail polish
Tissues	Tooth pick
Disposable glass pipettes and bulbs	Drying oven

NOTES: This procedure is for organic sediments and will need to be adapted to deal with clayey or arid site sediments.

PROCEDURE:

1. Set up stages a bit in advance (but think stepwise!) and keep tidy.

2. Prepare Workspace
    a. Make sure all surfaces are clean and free from dirt and dust.
    b. Turn on lights of fume cupboard, start fan, turn on water bath to 90˚ C (around the blue mark), turn on centrifuge and place 50ml tube holders inside.
    c. Get 40 labeled 50ml tubes (2 sets of 20) and fill out chart with tube numbers, sample references etc.
3. Transfer samples to 50ml tubes.
    a. If subsample is stored in distilled water, rinse into 50ml tubes not using too much water. Add 2-3 drops of ethanol to wet down floating particles. Centrifuge at 3500 rpm for 5 mins, decant. (Decanting tip: do it over the edge of a bucket to steady the tube.)
    b. If subsample is dry, wash with a little HCl into 50ml tubes.
    c. Keep subsample bottles for macrofossils (if you haven't subsampled to bottles, label a set of subsample pots for storing macrofossils).
4. Lycopodium
    a. For each ½ to 3 cm^3 of sediment, add 1 tablet of Lycopodium, depending on how abundant your pollen is – check they are not chipped (as a guide, use 2 per $1cm^3$).
    b. Keep a careful record of sample size, Lycopodium batch number, and Lycopodium density on prep. sheet.
5. HCl – cold overnight or 90˚ C water bath 30min
    a. Add small amount 7% HCl (cautiously if samples are calcareous) and whirlimix. Fill up in stages to the second bar of the rack, keeping levels even. This removes carbonates.
    b. Occasionally stir samples with glass rods (save Teflon for HF).
    c. Balance samples for the centrifuge (weigh each tube holder using the small battery powered scales), adding ethanol if necessary to de-float particles. Centrifuge and decant into fume cupboard sink with running water.
6. Wash samples
    a. Whirlimix to mix sediment, wash in distilled water, add some ethanol, centrifuge and decant. Repeat until supernatant is no longer yellow.
    b. If sediments are not mixing well, stir gently with Teflon or glass rods. Rinse down rods with distilled water and store in second set of 50ml tubes for next use.
    c. If leaving overnight at this stage, wash twice. It's OK to leave it in an acid environment overnight, but not alkali (next step).

[Set up 150 µm sieves and funnels]

7. NaOH step
    a. NaOH removes humic acids (unsaturated organic soil colloids) and disaggregates the sediment.
    b. Whirlimix samples to mix sediment. In fume cupboard, with goggles, fill tubes with small amount of 10% NaOH (just the bottom curved part of the tube). Put samples in water bath at 90˚ C for 3-7 mins. They will whistle a little.
    c. When removing, take care not to drip water from bath into other tubes in rack.
    d. Immediately add distilled water to 2nd line to slow the reaction (NaOH can damage pollen). Pour through sieves into second set of tubes, and keep sieving at least three times or until all the sediment is washed through. (Balance samples, add ethanol, centrifuge and decant.)
    e. Record darkness of the supernatant after first centrifuge. This indicates the relative amount of humic substance in the sediment.
    f. 'Drip' or squirt distilled water to break up clumps. Do not use glass rods to break up sediment as it will crush charcoal (and pollen), making it difficult to distinguish between micro and macrocharcoal. Be careful not to overfill bottom tubes.
    g. Wash macros to one side of sieve (on last sieve) and then into sub-sample bottles and retain (make sure to label with original amount of sediment, depth, site, date etc).

ALTERNATIVELY, if sediments are very clayey, use sodium pyrophosphate ($Na_4P_2O_7$). Add 20 cm^3 (or to 2nd line of the tube rack) of 10% sodium pyrophosphate ($Na_4P_2O_7$), stir well and place in a hot water bath for 10-20 mins.

8. Wash samples
    a. Whirlimix or gently stir sediment and repeat wash (adding ethanol) until supernatant is clear (at least 5 times). Record number of rinses.
9. HCl
    a. Whirlimix, add 7% HCl to 2nd line, balance, centrifuge and decant (and briefly whirlimix).
    b. This step acidifies the sediment and removes residual calcium carbonates.
10. Prepare lab for HF.
    a. This removes silica and silicates.
    b. Make sure someone else is around for first aid and notify them that they need to stay in the area for the whole time you are working with HF. Locate calcium gluconate gel, spill kit and grab card.

c. Make up a large beaker of sodium carbonate solution (about 2 cm of sodium carbonate in bottom of bucket and fill with tap water). Sodium carbonate neutralizes HF, so this is for wash down/safety purposes. Make it saturated but no need to measure. Swish while adding water or it will clump. Wet a cloth with the solution for wiping small drips.

d. Put on BOTH fume cupboard fans

e. Wear apron, visor, and long blue gloves with nitrile ones underneath.

f. Put warning signs on the doors.

g. Get HF waste bottle ready, labelling with expected composition of the waste.

h. Use Teflon or plastic stirring rods (NOT glass!).

HF

i. Carefully decant HF into a small plastic beaker, pouring no more than 50mL at any time.

j. Use the small beaker to pour HF into each sample tube (between 1mL and 10mL depending on the amount of silica in the samples (usually paler samples have more inorganic sediment).

k. Dip tubes in sodium carbonate and put into 90˚ C water bath with a stirring rod in each tube. Leave for 30-90 min (depending on composition).

l. Make sure HF stock bottle is firmly closed. Rinse plastic beaker with sodium carbonate and water.

m. Wipe down everything with sodium carbonate, including gloves.

n. Stir occasionally, leaving rods in. Check that the ventilation is still working at least once every 20 minutes.

o. Remove from bath, dip tubes in sodium carbonate, and stir gently again. Rinse stirring rod with ethanol and put in carbonate bucket.

p. Fill up so that more than 50% of volume is ethanol to slow down reaction.

q. Put red plastic caps on tubes, load into centrifuge bucket in the fume hood and cap with plastic caps. Centrifuge.

r. Wipe down everything with sodium carbonate, including gloves.

s. Remove caps and place in carbonate bucket. Decant HF very slowly into disposal jar. Don't pour out any sample. Wipe top of tube with sodium carbonate cloth then dip in carb bucket before returning to rack.

t. Leave both fans on for at least 30 more mins.

u. Put clearly labelled waste HF jar into a safe pak, leave in fume cupboard in outer lab and notify lab representative in charge of HF disposal.

v. Wash out centrifuge buckets and wash all surfaces thoroughly with water as alkali (carbonate) can damage plastics.

HCl

w. This removes colloidal silica and silicofluorides created by the HF reaction, as well as the last traces of HF.

x. If necessary stir samples with new Teflon or plastic rods.

y. Add HCl to 2nd line, stir again, and put in hot water bath for same amount of time as HF bath or leave cold overnight.

z. Balance with ethanol if necessary, centrifuge, and decant into sink with running water.

aa. Wash with distilled water, add ethanol, centrifuge, and decant.

bb. Examine the sediment. If there is a small separate gray layer or area, there is still clay in the sample and the HF/HCl steps need to be repeated. Or stir and listen for grittiness.

cc. Whirlimix and repeat wash (adding ethanol) until supernatant loses yellow colour.

dd. On final wash, pour into 15 ml tubes. Add small amounts of water at a time, whirli-mix to clean sides of 50ml tube and pour into 15ml tube.

ee. Balance with ethanol, centrifuge, and decant.

[Change over test tube rack in water bath]

11. Acetolysis

a. Get out small tube caps and face shield. Put whirlimix in fume cupboard. Water bath has to be around 90°C. Wear 2 pairs of gloves (remove outer ones inside out).

b. In the fume cupboard wearing face shield, whirlimix, then wash with glacial acetic acid to 2nd line (no ethanol). Cap, centrifuge, and decant into bucket in fume cupboard.

c. This dehydrates the sample. If you have a lot of organic matter, wash twice because if samples are not quite dry they sputter, get lost, and contaminate other samples at the next stage.

d. Make up acetolysis mixture by adding 45 ml acetic anhydride with 5 ml concentrated sulfuric acid (9:1 mixture – make up 100ml if necessary for very organic samples) in a DRY

graduated cylinder. Add sulfuric acid slowly with a graduated pipette (red bulb and separate tube) as it reacts with the acetic anhydride and gives off heat.

e. Sputters violently with water. Keep your gloves dry.

f. Function is to digest cellulose. It may also damage poorly preserved pollen. Time depends on balance between dirty slides and destroyed pollen (between 1-5 mins).

g. Whirlimix.

h. Pour all of acetolysis mixture from graduated cylinder into sample tubes up to 1st line (again, more for very organic samples). Put in hot water bath for 1-5 minutes. Remove from bath and top up with glacial acetic acid (no ethanol).

i. Cap, centrifuge and decant into bucket.

j. Whirlimix and add glacial acetic acid to 2nd line. Cap, centrifuge and decant into same bucket.

k. After one wash with acetic acid it safe to wash with water (very little acetic anhydride will remain).

l. Clean the graduated cylinder with glacial acetic acid then distilled water. Replace in drying cupboard. Do not wash with detergent as traces react with acetolysis mixture.

m. Pour carefully from now on as sediments are less cohesive.

[Start preparing glass tubes and lids with labels and polish]

[Start preparing TBA – loosen lid, warm gently in warm water (TBA Freezes at 25˚C and evaporates (forming explosive fumes) at 80˚C).]

12. Wash samples
    a. Whirlimix and wash with water to 2nd line, adding ethanol. Centrifuge and decant. Repeat 2 or 3 times until pH is neutral-ish. Can add dilute NaOH if samples are still very acid as long as you then rinse again after use.

13. Staining
    a. Add 2-4 drops of 0.2% aqueous safranin to each sample, whirlimix, wash with distilled water, balance with ethanol, centrifuge and decant.

14. TBA
    a. TBA should be used in fume cupboard.

    b. Whirlimix and add TBA to 2nd line. To start pouring release and tighten screw top of bottle, point away as often squirts due to change in temp/pressure.

c. Centrifuge and decant, leaving some TBA in tube (about 1.5 cm) – can use pipette to remove TBA.

d. Transfer samples into glass tubes with disposable pipettes, adding more TBA if necessary. Do not overfill tubes or they will break in the centrifuge.

e. Balance and cap tubes. Wrap in tissue paper, leaving tail at the top for retrieving, centrifuge and decant.

15. Silicone oil
    a. Add as much silicone oil as residue, with toothpick or dropper, and mix. Not more or the pollen will be too dilute on the slide.

    b. Leave open tubes (covered with paper towel) in cupboard to allow TBA to evaporate for at least 24 hours.

16. Tidy up
    a. Refill all bottles used, and note consumables that are finished or close to finishing on the lab board. Make sure there is enough for at least 2 more pollen preps.

    b. Leave all surfaces clean and tidy

    c. Ensure all electrical items are switched off

    d. Wash up tubes and sieves etc. with detergent to remove dirt and sand. Rinse with tap water, then leave soaking in Neutracon solution overnight (about 1 capful of Neutraon in a bucket of water).

    e. Rinse off Neutracon in fast running water multiple times. Rinse everything well in distilled water as there can be pollen in tap water.

    f. Ensure equipment is dry and return all equipment to correct cupboards.

Department of Zoology

Codes of Practice for the Storage, Handling & Disposal of Hydrofluoric Acid

1. Hazards of Using Hydrofluoric Acid (HF)

- **Extremely toxic**. Readily absorbed through the skin - **skin contact may be fatal**. May be fatal **if** inhaled or ingested.
- **Corrosive**. Causes severe burns.
- Any contact with this material, even minor, requires immediate medical attention.
- **Incompatible** with glass and metal.

2. General Information

Only authorized workers may handle HF. The list of authorized users is held by the Head of Laboratory, the Safety Officer and by the Laboratory Technician.

All users of HF must:

- Receive copies of the relevant HF COSHH assessment and these Codes and the date of this should be recorded. They should be instructed to comply with the requirements of these documents at all times. They should be questioned by their supervisor to ensure they understand the requirements of these documents and must sign the COSHH form.
- Receive formal practical training in the procedure they are to carry out and a record of this should be kept using the training record template.
- Be given a copy of the HF Grab Card. This card must be available at all times when working with HF and be provided to any emergency health care worker in case of exposure.
- Be fitted with a full face mask (with 3M 6038 filters) and be trained to test for HF vapour levels in an emergency. The mask should be used to make safe the area if there are low levels of vapour present.

All laboratory activities involving HF must take place within a fume cupboard designated for HF work. The fume cupboard will be inspected annually through an external contractor.

Procedures in the event of a spillage and location of spill kits, calcium gluconate gel and sodium carbonate etc. should be brought to the attention of all users. Stocks of calcium gluconate gel should be renewed by the Laboratory Technician and/or by the Laboratory Safety Officer as per manufacturer's guidance.

HF may only be used during regular working hours. Always ensure that knowledgeable laboratory personnel have been alerted and at least one is in the general vicinity when working with HF.

A notice stating "DANGER – HF IN USE" should be put on the entrance of the laboratory in which HF is being used.

The user is responsible for making sure the area is thoroughly cleaned after every use.

3. Personal Protective Equipment

HF may not be used unless the correct personal protective equipment (PPE) is worn. This should consist of a laboratory coat, full face shield, reusable long rubber gloves, rubber apron, long trousers and shoes which completely cover the feet. The user should also wear a pair of disposable nitrile gloves under the reusable gloves as long as this does not interfere with their ability to handle laboratory equipment.

All gloves must be inspected for tears and pin-holes prior to donning. Reusable gloves must be rinsed with sodium carbonate solution after use before cleaning in warm water with a mild detergent.
Presence and integrity of PPE will be checked during Laboratory Technician's monthly lab check.
Full face masks and gas testing kit should be kept at the entrance to F3b (outer lab) during HF work.

4. HF Procurement

All HF will be ordered by the Laboratory Technician or designated laboratory user. The Laboratory Technician will ensure that stocks held do not exceed 2L.

5. HF Storage

- All open bottles containing HF must be stored in a fume cupboard or in safe packs in the designated acid store. Sealed bottles must be stored in the designated acid store.
- HF must not, under any circumstances, be stored in glass containers. No more than 2L waste HF may be held in the laboratory at any time.

6. HF Protocols (See Pollen Prep Protocol for details about the HF process)

• All preparation of HF must take place within a fume cupboard designated for HF use.

• Always check that the fume cupboard extraction is working to full capacity and that the sash is kept as low as possible (The maximum height the sash should be raised to is indicated on each cupboard).

• Fume-generating material should be placed at least 15cm into the fume cupboard.

• Fume cupboard fans should stay on for at least 30 minutes after using HF.

• If sample tubes are transported, they must be placed in a sample rack. Tubes are only removed from the fume cupboard with caps in place and contained in a sealed centrifuge bucket.

• Before starting work with HF, make up a beaker of sodium carbonate solution. This should be placed inside the fume cupboard with a clean dishcloth.

• Gloves and contaminated equipment should be rinsed in the sodium carbonate solution before washing up.

• Sodium carbonate is an irritant, especially to the eyes, so use it only as needed.

• The sodium fluoride formed during neutralisation is toxic and mutagenic. Make sure that all sodium carbonate is cleaned up from the fume cupboard once HF work is completed and that traces from any spillage are thoroughly washed away.

7. HF Disposal

The Laboratory Technician is responsible for ensuring the correct and timely disposal of waste HF.

- No more than 2 L HF waste may be held in the lab at any one time.
- For larger amounts of waste generated during individual procedures this should be disposed of as it is produced.

The Laboratory Technician will contact the University Safety Office to arrange for delivery of waste HF to the University collection point.

- The Laboratory Technician will complete form TW2/10, available from Sharepoint, and will email this to the Hazardous Waste Technical Officer (Ms Frances Russell frances.russell@safetv.ox.ac.uk).
- A date and time will be given in which to deliver the waste to the University hazardous waste store.
- On receipt of delivery instructions from Safety Office, the Laboratory Technician or appointed member of the lab will label each HF waste bottles with full name/volume/concentration of the chemicals and also specify materials that the waste HF/EtOH contains (e.g. 60 ml Hydrofluoric acid (concentration 40%) and 40 ml Ethanol (concentration 100%) with sediments (sand/silt/clay).
- The bottles in safe packs will be put in a bottle carrier and delivered on foot by the appointed member of the lab to the collection point.
- Only 2L (4 bottles) may be carried by any one person.

8. **Use of Gas Testing Kit**

For use in emergencies (spillage or loss of ventilation)

- Extension may be fitted to make readings from a distance (unscrew tip and replace with tube)
- Break off both ends of glass tube (score by gently rotating in tip breaker and then snap downwards)
- Fit tube so air will flow in the direction of arrow.
- Use one pump stroke, if any HF is measured (especially if the yellow material turns pink), multiply by 4 for a reading in ppm.
- Use 4 pump strokes, with 45 seconds gap between each to read off a concentration between 0 and 20ppm. The yellow material will turn brown to indicate presence of HF, and pink if there is a very strong concentration.
- Check the humidity (using meter on fume hood) - if the humidity is high, the concentration may be overestimated so check in the manual for a correction table.

- Tubes may be disposed of in glass waste bin. If any HF was detected, pump air through in a working fume hood prior to disposal.

9. Hydrofluoric Acid Emergency Plans

Contacts

Department Safety Officer	71266
Area Safety Officer	71445
Administrator	71204
University Safety Office	70811
University Security Services	89999

First Aiders

Sharon Cornwell (E150)	71259
Karl Heilbron (E17a)	71265
Stuart Wigby (D25)	81842
Patricia Faria Shayler (D5)	71156
Malcom Ryder (D27)	71181
Nick Hawkins (B7)	71126

First Aid Advice

All casualties must be sent immediately to the nearest A&E department (i.e. John Radcliffe Hospital), even where exposure is minor, as a precautionary measure. All users who may potentially have received exposure to HF but who do not require immediate hospital treatment should be referred to University Occupational Health on ext 82676.

Skin Contact

- Remove all contaminated clothing while wearing two layers of protective nitrile gloves.
- Drench with fast running cold water for at least 5-10 minutes.
- Rub calcium gluconate gel into all affected areas of skin and massage until at least 15 minutes after the pain goes or the ambulance arrives.
- Cover the area with a dressing soaked in the gel and lightly bandage.
- Wearing butyl rubber protective gloves, laboratory coat and eye protection to at least

EN166F, any contaminated clothing should be placed in a bucket of water or equivalent placed in the fume cupboard.

- After ensuring the clothing is totally immersed and rinsed several times it can be put in the normal waste for disposal.

Eye Contact

Irrigate with running water for at least 20 minutes or until the ambulance arrives. Do NOT use calcium gluconate gel in the eyes.

Inhalation

Remove the casualty from exposure without putting yourself or others at risk, rest and keep warm until the ambulance arrives.

Ingestion

Wash out mouth thoroughly with water if casualty is conscious, ensuring mouthwash is not swallowed.

Spillage Procedures & Other Emergencies

Spillage Contained within Fume Cupboard

- Ensure that nobody approaches the spillage area and the fume cupboard continues to operate.
- Wearing full PPE, spread calcium carbonate liberally over the spillage.
- Working slowly and carefully, mix the chemicals to a slurry and carefully scoop up to containers in a fume cupboard.
- The neutralized mix should then be washed down the fume cupboard drain with plenty of running water.
- Any remaining slurry should be cleaned up using absorbent sheeting provided in the laboratory spill kit.
- Wash site of spillage thoroughly with water.

- Cleaning materials/cloths should be bagged up and kept in a fume cupboard and disposed of through the University Safety Office using the procedure described in Section 7.

Spillage Outside of Fume Cupboard

If spillage is significant, **do not** attempt to clean up due to inhalation risk.

- If deemed necessary, evacuate the lab, summon the Fire Brigade by telephone on (9)999 and evacuate the building by breaking one of the fire alarm call points.
- Ensure ventilation continues to run, open fume cupboard sashes if possible before leaving.
- Contact the University Safety Office on ext. (2)70811. They may be able to arrange for a person trained in dealing with dangerous spillages to come to the department.
  Very small spillages:
- If spill kit is to hand, spread calcium carbonate on the spillage.
- Evacuate any other laboratory users and don face mask and full PPE before cleaning up as for spillage in fume cupboard (detailed above).

Loss of ventilation during HF procedure

- Try to quickly determine the reason for the loss of performance (e.g. sash too high, baffle blocked) and correct it.
- If there is any doubt about the level of ventilation, evacuate the area, leaving other fume cupboards running where possible.
- Test the air (section 8)
- If HF is detected above 30ppm, or no one trained to deal with HF is available, summon the Fire Brigade by telephone (9)999 and evacuate the building by breaking one of the fire alarm call points.
- If any HF detected in the air up to 30ppm, wear full PPE and full face mask to make safe all procedures by removing samples from heat and ensure that all sample tubes and stock bottles are closed.
- No one may enter the lab without mask and PPE until the air has been retested and shown less than 1ppm HF.
- Ensure that ventilation is working before continuing work.

Evaluation of risk with control measures in place:

- Skin contact (large area): extremely serious harm, very unlikely, affecting user only
- Skin contact (small area): moderate harm, unlikely, affecting user only
- Inhalation: extremely serious harm, very unlikely, affecting anyone in the lab (usually no more than 3 people)
- Ingestion: extremely serious harm, extremely unlikely, affecting user only

This Code may be subject to revision by the *DSO,* ASO or Head of Department on the advice of the University Safety Office, or to respond to changing requirements.