Recovering Plant Microfossils from Archaeological and other Palaeoenvironmental Deposits: A Practical Guide Developed from Pacific Region Experience

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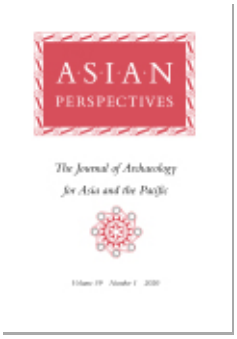

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ABSTRACT

Presented are revised procedures for recovering pollen and spores, phytoliths, and starch and other plant material from archaeological and other palaeoenvironmental deposits for microscopic analysis. The procedures are based on lengthy experience of preparing numerous samples of deposits from Malesia, Melanesia, Micronesia, and Polynesia. The procedures are designed as a simple laboratory guide, outlined in detail and summarized to provide a practical, time-efficient, step-by-step method. The method has been carried out successfully on many types of soils and other deposits from Pacific Islands, including: clays, silts, and sands; waterlogged, porous, peaty, volcanic, and coralline soils; and sediment cores, tools, pot sherds, dental calculus, and coprolites from a range of environmental settings in tropical, sub-tropical, and temperate climates. Also included in the procedures are mounting recovered microfossils on microscope slides and preparing and mounting modern reference samples. Keywords: plant microfossils, density-separation, pollen and spores, phytoliths, starch.

INTRODUCTION

The study of plant microfossils is particularly useful in archaeological contexts for providing information about ancient subsistence plants (Pearsall 2015). Recent decades have seen the development of methods for analysing phytoliths and subsequently starch grains and other microscopic plant remains as valuable additions to the traditional palynological method (Coster and Field 2015; Louderback et al. 2016; Pearsall 2015; Piperno 2006). A recent, comprehensive literature review of methods, including photographs, is given in the work of Pearsall (2015). These advances are important because different types of plants can show different production and preservation of their tissues. This article presents combined methods developed in the Pacific region relating to these differences.

Palynological analysis includes pollen grains of seed plants and spores of ferns and other plants (Cranwell 1953; Large and Braggins 1991; Moar 1993; Moore et al. 1991;

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Orliac 2003; Pearsall 2015). As well as providing insight into past vegetation and environments, in the Pacific region this analysis allows the differentiation of sediments deposited in pre-settlement and pre- and post-European contact times. Using pollen and spores for age determination can be particularly useful if radiocarbon determination is unreliable or not possible. This palynological aging involves pollen of plants introduced intentionally or unintentionally firstly by people prior to European contact, then by people after European contact. For example, the New Zealand pollen record clearly indicates that initial Polynesian forest clearance commenced ca. 650 B.P., as evidenced by the influx of spores of *Pteridium esculentum*, an invasive indigenous ground fern, coincident with charcoal (McGlone et al. 1993). The presence of these spores and charcoal in deposits thus provides that date as a probable maximum age. This initial evidence is followed by pollen of European-introduced trees and herbaceous pasture weeds, providing a probable maximum age of ca. 1830 C.E., when *Pinus* trees were first recorded as naturalized (Hayward et al. 2004; Webb et al. 1988). New Zealand deposits without these palynological indicators are thus likely of pre-settlement age.

Pollen has provided evidence for numerous Pacific Island subsistence plants, including introduced crops, including *Aleurites moluccana*, *Casuarina equisetifolia*, *Cocos nucifera*, *Colocasia esculenta*, *Cordyline fruticosa*, *Cycas* spp., *Cyrtosperma merkusii*, *Ipomoea batatas*, *Lagenaria siceraria*, *Morinda citrifolia*, *Musa* spp., *Pandanus tectorius*, *Rorippa palustris*, *Sonchus* spp., *Tacca leontopetaloides*, and *Zea mays* (Burley et al. 2018; Horrocks 2004; Horrocks, Baisden et al. 2012*a*; Horrocks, Baisden et al. 2012*b*; Horrocks, Smith et al. 2008*b*; Kahn et al. 2014; Kirch et al. 2017; Kirch, Molle et al. 2015; Leopold 1969; Selling 1946, 1948). The recent identification of phenolic inclusions in fossil pollen preparations represents a new microfossil type for *C. esculenta* (Horrocks, Baisden et al. 2017).

Phytoliths are particles of silica formed in inflorescences, stems, leaves, and roots of many plants (Pearsall 2015; Piperno 2006). Phytolith analysis complements pollen analysis, providing many more Poaceae types than pollen. This form of analysis has provided evidence for several Pacific Island subsistence plants, notably *Broussonetia papyrifera*, *Heliconia* spp., *Musa* spp., *Oryza sativa*, and *Zea mays* (Horrocks, Acabado et al. 2018; Horrocks, Baisden et al. 2012*a*; Horrocks, Baisden et al. 2012*b*; Horrocks, Marra et al. 2013; Horrocks, Nieuwoudt et al. 2014; Horrocks and Rechtman 2009; Kahn et al. 2014; Kirch et al. 2017; Kirch, Molle et al. 2015; Lentfer and Green 2004). Other types of microscopic biosilicates, notably diatoms, radiolarians, and sponge spicules, are extracted along with phytoliths during preparation, providing evidence for wet substrate or aquatic association (Dudgeon and Tromp 2014; Foged 1979; Horrocks, Peterson et al. 2015; Kondo et al. 1994; Pearsall 2015; Piperno 2006). Diatoms are unicellular algae and have cell walls composed of silica, radiolarians are a type of amoeboid protozoa with siliceous skeletons, and sponges are multi-cellular animals with skeletons often composed of siliceous spicules. Diatoms are found mostly in aquatic and sub-aquatic environments; radiolarians and sponges are exclusively aquatic. Diatoms and sponges are found in both marine and freshwater environments; radiolarians are exclusively of marine origin.

Ancient starch analysis includes amyloplasts, starch grains, and other plant material such as calcium oxalate crystals, xylem, epidermis, and parenchyma (Burley et al. 2018; Pearsall 2015; Reichert 1913; Seidemann 1966). Starch is the main substance of food storage for plants and is mostly found in high concentrations of microscopic grains in

underground stems such as tubers and corms and in roots and seeds. The grains are synthesized and stored in amyloplasts, sub-cellular units specialized for this function. Calcium oxalate crystals, comprising needle-like raphides and compound druses, are found in both the aerial and underground parts of many plant taxa. Xylem is a vascular tissue comprising elongated cells through which most of the water and minerals of a plant are conducted. Epidermis is the outer layer of tissue in a plant, while parenchyma is the typically soft and succulent cellular tissue found mainly in the softer parts of leaves, pulp of fruits, bark, and pith of stems. This type of analysis has provided evidence for Pacific Island subsistence plants such as *Artocarpus altilus*, *Colocasia esculenta*, *Cyrtosperma merkusii*, *Dioscorea* spp., *Freycinetia arborea*, *Ipomoea batatas*, *Oryza sativa*, *Pteridium esculentum*, *Solanum tuberosum*, and *Zea mays* (Burley et al. 2018; Crowther 2014; Horrocks, Acabado et al. 2018; Horrocks, Baisden et al. 2017; Horrocks, Baisden et al. 2012*a*; Horrocks, Baisden et al. 2012*b*; Horrocks et al. 2007; Horrocks, Nieuwoudt et al. 2014; Horrocks and Rechtman 2009; Horrocks, Smith et al. 2008*a*; Horrocks, Smith et al. 2008*b*; Horrocks and Weisler 2006; Kahn et al. 2014; Loy et al. 1992; Tromp and Dudgeon 2015).

Pollen and phytolith recovery methods are better developed than those for ancient starch, in part because the study of the latter is more recent. A recent study suggested that there are exaggerated expectations in ancient starch research and that new taphonomic and authenticity criteria are required (Mercader et al. 2018). The study specified a lack of explanation for the lengthy survivability of a biochemically degradable polymer and problems with authenticity and taxonomic identification. Another recent study concluded that total recovery of ancient starch grains is currently doubtful, with gelatinized starches strongly under-represented (Henry et al. 2016). Some advances have been made with identifying degraded starch, however. Lamb and Loy (2005) showed that Congo red staining was an effective, easy way to identify cooked and damaged starch grains collected from archaeological deposits. Subsequent research, however, has presented cases of putative ancient starch grains that are so degraded and discolored that staining is ineffective. Progressive stages in this type of starch decay and discoloration are presented in the work of Horrocks, Baisden and colleagues (2012*a*). A possible way around this problem is the use of Fourier Transform InfraRed spectroscopy to positively identify this type of degraded starch (Horrocks, Baisden et al. 2012*b*; Horrocks, Nieuwoudt et al. 2014; Kahn et al. 2014).

Combining the three methods of pollen, phytolith, and starch analysis identifies the greatest diversity of plant taxa, providing the optimal environmental, plant subsistence, and agricultural evidence for archaeological excavations. Published examples of this evidence are given in Table 1. As well as in published articles, results from this combined approach can be accessed from unpublished cultural resource management and government compliance technical reports (Acabado et al. 2012; Gumbley and Hoffmann 2013; Peterson et al. 2012). If analysis is limited to only one of these methods, some plant taxa will remain invisible in the record. For example, *Dioscorea* spp. have negligible phytolith production (Piperno 2006) and the pollen record can be difficult to detect in Pacific Island contexts because the pollen grains tend to be small, thin-walled, and lacking distinguishing surface ornamentation. These species produce abundant starch grains and xylem in their tubers, however. Specific examples come from studies at archaeological sites in Guam, Vanuatu, and Easter Island, where starch grains and xylem of *Dioscorea* spp. were identified with no accompanying *Dioscorea* pollen (Horrocks, Baisden et al. 2012*a*; Horrocks and Bedford 2010; Horrocks et al.

Table 1. Summary Subsistence Plant Results from Combined Analyses of Pollen/Spores, Phytoliths, and Starch and Associated Plant Material from Four Pacific Island Regions

	MALESIA PHILIPPINES, LUZON, IFUGAO, FORMER TERRACE PROFILES 810–200 CAL. B.P.[a]	**MICRONESIA** GUAM/SAIPAN, HEARTHS AND POTSHERDS 1300–300 CAL. B.P.[b]	**MELANESIA** VANUATU, URIPIV IS./VAO IS., HABITATION LAYERS AND POTSHERDS 3100 CAL. B.P.–PRESENT[c]	**POLYNESIA** EASTER ISLAND, RANO KAU, WETLAND SEDIMENT CORES 5980–0 CAL. B.P.[d]
Pollen and spores		*Cocos*, *Pandanus*		*Broussonetia papyrifera*, *Ipomoea batatas*, *Lagenaria siceraria*, *Musa* sp.
Phytoliths	*Musa* sp., *Oryza sativa*	*Musa* sp.	*Musa* sp.	*Broussonetia payrifera*, *Musa* sp.
Starch and other material	*Oryza sativa*	*Dioscorea alata*, *D. nummularia*, starch grain type from other possible spp.	*Dioscorea nummularia*, *D. pentaphylla*, starch grain type from non-*Colocasia* Araceae	*Colocasia esculenta*, *Dioscorea alata*, *Ipomoea batatas*

Data sources:
[a] Horrocks, Acabado et al. 2018.
[b] Horrocks, Peterson et al. 2015.
[c] Horrocks and Bedford 2005, 2010; Horrocks, Bedford et al. 2009; Horrocks, Nieuwoudt et al. 2014.
[d] Horrocks, Baisden et al. 2012*a*.

2015) (Table 1). In a Philippines study, *O. sativa* was identified at an archaeological site using the distinctive phytoliths and starch grains of this species (Horrocks, Acabado et al. 2018) (Table 1). This identification was not possible using pollen preserved at the same site because most taxa in the family (Poaceae) to which the genus *Oryza* belongs, including *O. sativa*, have pollen that cannot be unequivocally differentiated. Similarly, the Araceae have negligible phytolith production (Piperno 2006), but produce sufficient pollen to be visible in the record, and also produce abundant starch and xylem in their starch-producing organs and abundant calcium oxalate crystals in both their above- and below-ground tissues. Many other species produce sufficient pollen for identification in the Pacific Island microfossil record but negligible starch and phytoliths. These effects are also in part a result of differential dispersal, whereby pollen and spores are adapted for dispersal by wind, animals, or water, whereas phytoliths and starch remains are not. Phytoliths and starch can, however, be transported by erosional wind and water.

As well as differential production and dispersal of plant micro-parts, varying types of sediment and other substrates can cause differential preservation of plant material. Phytoliths, being mineral rather than organic, can often be found well preserved in substrates where pollen and spores and starch remains are not. In unsheltered dryland environments, often the case for archaeological sites, phytoliths can be relatively well preserved, whereas pollen and spores are often poorly preserved as a result of aeration and repeated wetting and drying, which increases decomposition of organic material by soil micro-organisms. On the other hand, phytoliths are often poorly preserved in peaty deposits due to chemical dissolution, but this medium is generally highly favourable for pollen and spore preservation. For example, in an Easter Island study, large sections of the peat layers in wetland sediment cores contained negligible phytoliths, whereas the in-washed terrestrial layers contained high concentrations of phytoliths, including those from leaves of *Musa* sp. and *Broussonetia papyrifera*, reflecting horticultural activity in the catchment (Horrocks, Baisden et al. 2012*b*) (Table 1).

Future advances in plant microfossil research could address the current limitations of starch analysis pointed out by some researchers (Henry et al. 2016; Mercader et al. 2018). Also, DNA analysis of archaeological deposits targeting ancient cultigen species is possible and could address the problem of overlapping microfossil morphologies (Haile 2012; Mercader et al. 2018).

The Remote Oceanic part of the Pacific region, encompassing numerous archipelagos across highly variable environments, has advantages for studying ancient plant subsistence and increasing the understanding of human colonization. This part, comprising Polynesia, Micronesia, and island Melanesia southeast of the Solomon Islands, was the last relatively large geographic area to be colonized within the last ca. 3000–650 years (Sheppard 2010; Wilmshurst et al. 2011). As the colonization occurred recently, many archaeological deposits are relatively young and have not been obliterated by lengthy occupation periods. Also, the more than 70 plant species introduced by early people to Polynesia have few or no close relatives in much of the Pacific region, which allows for more confident identifications of plant microfossils and provides tracking evidence for the sequence of colonization of the numerous archipelagos (Whistler 2009).

Presented here is a practical guide for preparing samples from archaeological and other palaeoenvironmental deposits for pollen, phytolith and starch analyses, suitable for the novice as well as the experienced. The purpose is to provide procedures that have been simplified and combined as much as possible, while maximising recovery

from small volume samples. The procedures build on two of the author's earlier works (Horrocks 2004, 2005), with methodological innovations and enhancements by other researchers incorporated when appropriate (e.g., Pearsall 2015). These works are modified, expanded, and combined in the light of approximately 25 years of personal hands-on experience gained during preparation and analysis of over 2000 sedimentary and archaeological samples from throughout the Pacific region, including Malesia, Melanesia, Micronesia, and Polynesia, as well as forensic samples mostly from New Zealand (see this author's and collaborators' works cited above). The procedures have evolved in collaboration with numerous different research groups and been carried out successfully on many types of soils and other deposits, including: clays, silts, and sands; waterlogged, porous, peaty, volcanic, and coralline soils; and sediment cores, dental calculus, coprolites, pot sherds, and tools from a range of environmental settings in tropical, sub-tropical, and temperate climates.

The three different microfossil analyses of pollen and spores, phytoliths, and (more recently) starch grains discussed here each provides valuable information on the presence of specific plant taxa in archaeological and other palaeoenvironmental contexts. The production, dispersal, and preservation of these plant micro-parts are well-researched for phytoliths and especially pollen and are becoming more so for starch. The three analytic methods have largely evolved separately as disparate, stand-alone applications with little overlap, and each has its limitations as outlined above. These limitations can be redressed in large part by combining the three analyses to complement one another. The combined methodology presented here was developed with the complementary nature of the three analyses firmly in mind, depending on the nature of the sampled substrates and the research questions.

The methods for all microfossil types outlined here involve density separation using heavy liquid solutions of sodium polytungstate, which has the advantage of being non-toxic. For pollen preparation, the use of this chemical replaces the traditional step using hydrofluoric acid, which is extremely toxic and increasingly requires installing a specialized fume hood in laboratories (Campbell et al. 2016; Muriale et al. 1996). The procedures are designed for consistency, reliability, simplicity, and time efficiency for both large and small numbers of samples. For example, as many steps as possible are carried out in the same test tubes to minimize transferring samples back and forth between different types of containers.

For phytolith and starch preparation, both are recovered from the same sub-sample. The sample is split for pollen and spore analysis, however, because the respective methods are generally mutually exclusive. Pollen is often present in low concentrations in archaeological deposits; taking extra separation steps with the same sample can exacerbate this problem. The specific gravity of the heavy liquid solution used for starch separation can remove some or many pollen grains and spores, which then require a subsequent treatment (acetolysis) that destroys most starch and associated plant material. Notes on mounting microfossils on slides for microscopy and preparing modern reference samples are also given. The procedures are outlined in detail and summarized in Tables 2 and 3.

For all Analyses

Sample collection for the methodology outlined here is compatible with standard practices of field collection of palaeoenvironmental material for numerous types of subsequent analyses, including plant microfossils. Comprehensive details of this

collection, including sampling deposits by hand from archaeological test pits and features and collecting wetland sediment cores for subsequent sub-sampling, are given in the work of Pearsall (2015).

A convenient maximum sample size for the methodology is about standard matchbox size, although the methods are designed to also maximize the extraction of plant microfossils from samples that are inherently smaller than this, such as dental calculus and scrapings or brushings from potsherds and tools (Hart and Ives 2013; Piperno et al. 2009; Zarillo et al. 2008). Bulk soil and sediment samples that have been sieved to collect macroscopic material, including macroscopic plant material, can also be subjected to the methods outlined here. Dental calculus requires skipping some of the steps or carrying them out in different order, however; such modifications are addressed in the relevant steps below.

When preparation of samples for plant microfossil analysis is underway in the laboratory, risks of sample cross-contamination can be minimized by using the following disposable plastic tools:

(1) Graduated (1 ml) centrifuge tubes (10 ml) with permanently attached push-on/pull-off caps to hold sub-samples. Because handling of tubes and some of the chemicals used may remove ink labels, labels should be scratched onto the side of plastic tubes with a sharp metal point. The scratch marks can then be indelibly inked.
(2) Graduated (0.50 ml and 0.25 ml) transfer pipettes (3.0 ml and 1.0 ml), replaced for each step for each sample. Use 3.0 ml pipettes for transfer and 1.0 ml pipettes for microscope slide preparation.
(3) Drinking straws, narrow rather than wide diameter, for stirring. Narrow diameter straws are more effective at dislodging the very bottom of the pellet after centrifuging. Being hollow, straws displace very little liquid in the tubes. Cocktail stirrers, toothpicks, and the like can also be used and can be snapped to the desired length. Do not use stirrers made of plant material such as wooden or bamboo cooking skewers or toothpicks, as these can contain phytoliths, starch, and other plant material.

A maximum of eight or nine samples is a convenient number to prepare at a time. Nine is the maximum number of test tubes that will fit upright into a 250 ml glass beaker used as a boiling water bath.

Leave the straws in the test tubes for occasional stirring when tubes are in the boiling water bath. The straws will not be damaged.

A swing-out rather than fixed-angle centrifuge is required so that the pellet is not deposited at an angle up the side of the test tube rather than at the base.

All acid work should be carried out in a fume hood with extractor fan.

Use only powder-free gloves, as the powder on disposable rubber gloves often contains starch grains, most commonly of *Zea mays*. Workers carrying out starch preparations should familiarize themselves with potential ambient starch grain contamination on laboratory equipment, in reagents, and on powder-free gloves (Crowther et al. 2014; Mercader et al. 2018). Starch contamination of hands and clothing is possible from handling food, while pollen and phytolith contamination is more likely to be picked up outdoors.

Dispose of the decanted fraction after centrifuging, except where stated otherwise. Most of the centrifuging is done at 2000 revolutions per minute (rpm) for 3 minutes (min). In steps where rpm, time, or specific gravity of solutions differ, the alternatives are underlined and highlighted in bold font.

Label test tubes with sample names or numbers and, for phytoliths, also use the letters 'phy'. Label another set of tubes with sample names or numbers for starch and

associated material and also use the letters 'sta'. Label a third set of tubes with sample names or numbers for pollen and spores, also using the letters 'pol'.

Use a straw for stirring the pellet after centrifuging and having added another liquid. Hold the tube at eye level and run the straw gently around the top of the pellet, working downwards, or gently move the straw up and down around the side of the pellet, working downwards. If done too quickly, some of the pellet might stick in the straw. There will usually be a small section of the pellet at the very bottom of the tube that the straw cannot reach. This remainder can usually be dislodged by inverting the tube with cap firmly on and gently flicking the tip of the upended tube with a forefinger or middle finger while shaking. Note that if this flicking is done too strongly, depending on the tube brand, the tube might be weakened and then split at a later stage of the procedure while centrifuging, releasing most if not all the contents. Do not tap the tube on the work bench to dislodge the pellet, as this will have the same effect. Depending on the type and amount of sample, stirring may not always be required; the test tube can simply be shaken with the cap on. Vortex mixers can be used but are often ineffective at dislodging all the pellet material.

Use 22 × 40 mm cover slips on slides, to allow maximum scanning area.

Tap water can be used for rinsing items such as test tubes and sieves, with a final rinse of distilled water.

Combined Procedure for Recovering Starch and Associated Material and Phytoliths

To avoid the risk of gelatinization of starch grains, avoid the use of very warm or hot (>35 °C) solutions or instruments during starch residue separation. Heating is only required during starch preparation for melting the mounting agent when making up slides for microscopy (see Step 5 below). Table 2 on page 202 summarizes the necessary steps for recovering starch, associated material, and phytoliths, but the complete procedure outlined here continues through to mounting and storing slides.

Step 1. Sub-Sampling

For each sample, add up to 4–5 cm^3 of material to the tubes labelled for phytoliths. If intending to measure microfossil concentrations with exogenous markers (see Step 2), use a 0.5 ml spoon to measure the volume of the sub-sample, then tap or scrape the material into the test tube. To avoid spillage, ensure the spoon diameter is less than the tube diameter. Using a spoon is generally more accurate and precise for gauging volume than using the graduations on the side of the test-tube. Consider using larger amounts of material for samples that appear very sandy and or seem to have a low concentration of organic material.

Step 2. Optional—Addition of Exogenous Markers

Carry out this step only if intending to measure concentrations of microfossils. For starch, *Lycopodium* tablets from Lund University, Sweden, can be used (available from https://www.geology.lu.se/services/pollen-tablets). To avoid dissolution of any calcium oxalate crystals in the samples by the acid used to dissolve the tablets, first dissolve the tablets in empty test tubes. For each sub-sample, place one *Lycopodium*

tablet into an empty test tube and add 6 ml 10% hydrochloric acid, stir, and wait for the fizzing reaction to cease. Add a little at a time to avoid overflowing. Centrifuge at 2000 rpm for 3 min and decant. Add distilled water, stir, centrifuge at 2000 rpm for 3 min, and decant. Add distilled water, stir, and pour into the test tubes containing the sub-sample material. Stir, centrifuge at 2000 rpm for 3 min, and decant.

A recent study statistically tested the suitability of silica microspheres from Merck (LiChrospher® Si 1000) for phytolith application (Aleman et al. 2013). The study found the microspheres to be suitable. Consult the study for details on making up aqueous solutions of different microsphere concentrations to be added directly to phytolith sub-samples. Stir, centrifuge at 2000 rpm for 3 min, and decant.

Step 3. Deflocculation and Removal of Heavy Particles and Clay

For dental calculus, skip this step. Instead, add 3–6 ml of 10% hydrochloric acid and wait for fizzing to cease. This treatment dissolves the calculus, releasing any microfossils therein. The treatment will also dissolve many if not all calcium oxalate crystals present. Centrifuge at 2000 rpm for 3 min and decant. Add distilled water, stir, centrifuge at 2000 rpm for 3 min, and decant. Then proceed to Step 4.

For all other types of samples, this step breaks up the matrix. Sufficient clay is removed in this step for starch analysis. (More clay is removed during the phytolith procedure, see below). Add 6 ml 5% sodium polymetaphosphate solution and leave overnight or for at least 8 h. (Dishwashing powder, which has been used as an alternative, is not recommended as some brands or batches contain starch grains and other microscopic plant material.) With caps firmly on, shake tubes vigorously from time to time during this period. Coprolites may require breaking up with a metal or glass stirring rod if they appear still lumpy after several hours.

When ready to proceed, shake vigorously one final time, top up with distilled water, shake again, and pour the sample into a 50 ml beaker. Rinse tube with distilled water. To remove heavy particles, swirl beaker, pause for 5 seconds to allow the heaviest particles to settle, and decant the liquid fraction back into the tube. Dispose of the settled fraction and thoroughly rinse beaker with distilled water between samples. Centrifuge at 2000 rpm for 3 min and decant. Add distilled water, stir, centrifuge at 2000 rpm for 3 min, and decant. Repeat water wash until the supernatant is clear.

Step 4. Density Separation of Starch and Associated Plant Material

For dental calculus, follow the instructions in this step, but use **2.0 specific gravity** sodium polytungstate solution instead of 1.8 specific gravity. The higher specific gravity will ensure separation of any pollen and spores as well as starch and associated material. Both microfossil types will thus be separated together and can then be mounted as such on the same slide, as per Step 5.

For all other samples, this step separates starch and associated plant material such as xylem and parenchyma tissue from the matrix. Add 2 ml of 1.8 specific gravity sodium polytungstate solution and mix thoroughly. Although temperature affects specific gravity measurements, under normal laboratory conditions the solution can be made by dissolving sufficient chemical in water until 100 ml weighs 180 g.

Centrifuge at **1500 rpm** for 3 min; **do not decant**.

Holding the test tube at eye level in good light, gently and carefully stir the upper part of the top layer (i.e., the layer of liquid and any microfossils above the pellet) with the pipette without disturbing the pellet, then pipette most of this layer, again without disturbing the pellet, and transfer the liquid into the test tube labelled for starch. Top up with distilled water to dilute the heavy liquid so the starch residues can be centrifuged to the base of the tube. With cap firmly on, shake tube vigorously, centrifuge at 2000 rpm for 3 min, and decant. If the sodium polytungstate is going to be recycled (see below), decant into a reagent bottle labelled "used sodium polytungstate." Add distilled water, stir, centrifuge at 2000 rpm for 3 min, and decant. Starch and associated material are now ready to be mounted on slides for microscopy.

As well as amyloplasts and individual starch grains, this procedure also extracts fragments of xylem cells, epidermal and parenchyma tissue, charcoal, and calcium oxalate crystals. The latter, however, are potentially problematic in that their 2.2 specific gravity is heavier than that of the 1.8 specific gravity of the heavy liquid used for the starch separation. This author often finds the crystals in starch separations, however; their presence is explained either by their having been caught up with other plant material extracted or changes in chemical composition because of degradation. The crystals, highly visible in cross-polarized light, can be seen on starch slide preparations embedded in tissue fragments or scattered as individual whole or fragments of raphides and druses, often highly corroded and in abundance. The Araceae have these crystals in their tissues in such high concentrations that if the starchy organs or leaves of this family have been formerly associated with the sampled deposits, the starch separation will include crystals (Sunell and Healey 1979). If more attention to calcium oxalate crystals than this approach is desired, then another density separation, at 2.2 specific gravity, after the starch extraction and before the hydrochloric acid step, can be attempted. Note, however, that this specific gravity is very close to that of phytoliths, which is 2.3. Alternatively, dry or wet untreated sample material can be mounted in glycerol jelly directly onto the microscope slide (see Step 5). If crystals are present in a high concentration, they will be microscopically visible despite the high concentration of substrate. Use a tiny, barely visible amount of sample tapped or smeared onto the slide for this latter method.

Step 5. Mounting and Storage of Starch and Associated Plant Material

Mount starch residues on slides in glycerol jelly, an aqueous agent commonly used for pollen. Major advantages of this mountant are that it is easily handled, as it melts in a warm water bath, is solid at room temperature, and has excellent optical properties (Batten and Morrison 1983; Moore et al. 1991). Place a few cm^3 of jelly into a 50 ml glass beaker in a lukewarm to warm water bath to melt, bearing in mind the warning about gelatinization of heated starch given above. A glass beaker wider than and about the same height as the 50 ml beaker, with about 10 mm water and sitting on a hot plate at very low heat, is ideal for this.

Using a 1 ml pipette, place a drop of melted jelly onto the slide, nearer one side to allow space for a sticky label. Using another 1 ml pipette, place a drop or less of the recovered sub-sample onto the jelly and mix with the pipette tip, spreading to less than cover slip size (22 × 40 mm). If large fragments of plant material are visible to the naked eye, crush them with the pipette tip or remove them before affixing the cover slip, otherwise it may not sit properly on the slide. Affix cover slip and check concentration of plant material on the

slide under 100× and 400× magnification. Ideally, as much material should be in the field of view under 400× magnification without a high concentration of extraneous material, such as charcoal or residual clay particles, potentially obscuring plant material of interest. If necessary, make up another slide with the concentration adjusted.

The glycerol jelly on the slide preparations will set. Unlike for the pollen and phytolith slide preparations outlined below, re-melting the jelly so starch grains and other material can be turned on slides for better viewing is not recommended, or at least should be carried out cautiously, because of the risk of gelatinization.

To prevent gravitational movement of microfossils on prepared slides, permanently store horizontally in trays. Coverslips should face down as these exert pressure on material over time. Store away from direct sunlight as this can melt glycerol jelly. Slides of ancient starch and associated material mounted in glycerol jelly can be stored for many years. Starch preparations remaining in test tubes after slides have been made up can be stored at room temperature. These preparations can remain in their original test tubes with caps firmly on; despite the caps, in the experience of this author, they totally and permanently dry out after several months, which aids preservation. Alternatively, the tubes can be refrigerated at 2–4 °C.

Step 6. Removal of Large Particles, Humic Colloids, and Further Removal of Clay

For dental calculus, skip steps 6–8 and proceed directly to Step 9.

To continue the combined procedure and recover phytoliths from all other types of samples, decant the remainder, if any, of the 1.8 specific gravity solution from the original test tubes (i.e., those labelled for phytoliths). Add distilled water, stir, centrifuge at 2000 rpm for 3 min and decant. Add 3 ml each of a 0.1 molar sodium pyrophosphate solution and 10% potassium hydroxide, and place in a boiling water bath for 8 min, stirring occasionally. Longer than 10 min may etch silica (Piperno 2006). Remove from bath, top up with distilled water, shake with lid firmly on and pour through a 250 μm sieve into a 50 ml beaker. Use a small, flat, hard-rimmed laboratory test sieve sitting on top of the beaker. Although the sieve will overlap the beaker, this does not matter because the liquid will go almost directly into the sieve at the point of pouring and not spread out to the sides. Use no more than 10 ml water so all or most can be poured back into the tube. Dispose of fraction on sieve. Rinse sieve and beaker with distilled water between samples. Centrifuge at 2000 rpm for 3 min and decant. Add distilled water, stir, centrifuge at 2000 rpm for **2 min**, and decant (Lentfer and Boyd 1999). Repeat this water wash at this reduced time until supernatant is clear.

Step 7. Removal of Carbonates

To remove carbonates, add 10% hydrochloric acid, stir, and wait for fizzing reaction, if any, to cease. Add a little at a time because if carbonates are highly concentrated in the samples (e.g., if shelly), excessive fizzing can cause overflowing. Centrifuge and decant. Add distilled water, stir, centrifuge at 2000 rpm for 3 min, and decant.

Step 8. Removal of Organic Material

Organic matter can be removed by either oven combustion or nitric acid digestion. For combustion, add one drop, or three at the most, of water to the sub-sample, stir with

the tip of the pipette, then pipette into small ceramic or stainless-steel crucibles. Place crucibles into an oven, with a ceramic or stainless-steel slab or plate on top of the crucibles to prevent loss of or cross-sample contamination by any initial boiling of the moist sub-sample. Heat at 500 °C for 4 h. (The pollen procedure can be started while waiting for the completion of this step.) When this step is completed, and the crucibles have cooled, add a drop or two of water to the crucible, stir with the pipette to loosen the material, which will be crusty, and pipette back into the test tube. Tap the end of the pipette up and down on the material in the test tube to break it up. Top up with distilled water, stir, centrifuge at 2000 rpm for **2 min**, and decant.

For nitric acid digestion, use only nitric acid compatible test tubes, as some types of plastic are damaged by nitric acid. Use only glass or metal stirring rods as plastic straws will be damaged. A narrow, 4 mm wide stainless-steel spatula, with a rounded end so it will reach the very bottom of the test tube, rinsed between samples is ideal. If nitric acid compatible test tubes are not available, an alternative, albeit more complicated, option is to transfer the sub-samples to labelled glass test tubes for this step. As scratching labels onto glass tubes is difficult or undesirable, use indelible ink and keep an eye on it during this step to make sure it does not become illegible. Add 6 ml concentrated nitric acid, stir, and place in a boiling water bath until the reaction has finished. Occasionally add a few granules of potassium chlorate to the tubes with the narrow spatula and stir. This step usually takes from a few minutes up to approximately 1 h, depending on the amount of organic material in the sample. Keep an eye on the level of the water bath and top up as necessary. If the solution is tinted red or red-orange, organic material is still present. If there is no red tint, organic material has been removed. The solution is usually tinted yellow when the reaction has finished. Seal the glass test tubes with rubber bungs. Centrifuge the glass or plastic tubes at 2000 rpm for 3 min, and decant. Add distilled water, stir, and, for the glass tubes, pour back into the original plastic test tubes. Centrifuge at 2000 rpm for **2 min** and decant.

Step 9. Density Separation of Phytoliths

This step separates phytoliths from the matrix. Add 2 ml sodium polytungstate solution, 2.3 specific gravity, and mix thoroughly. The solution can be made by dissolving sufficient chemical in water so that 100 ml weighs 230 g. Centrifuge at 2000 rpm for **5 min**; **do not decant**.

Holding the test tube at eye level in good light, gently and carefully stir the upper part of the top layer (i.e., the layer of liquid and microfossils above the pellet) with the pipette without disturbing the pellet, then pipette most of this layer in one action, again without disturbing the pellet, and invert the pipette and rest it upside down in the test tube rack directly adjacent to the test tube so you know they belong together and not to any other tubes or pipettes in the rack. Dispose of pellet from test tube, rinse tube in distilled water, and pipette phytolith separation back into tube. Note that for some types of material, such as dental calculus, it could be desirable to retain and store the pellet in case other types of analyses are planned (Hendy et al. 2018; Warinner, Rodrigues et al. 2014; Warinner, Speller et al. 2015; Weyrich et al. 2017).

Top up with distilled water to dilute heavy liquid so the phytoliths can be centrifuged to the bottom of the tube. Hold tube upside down with cap firmly on, shake vigorously, centrifuge at 2000 rpm for 3 min, and decant. If the sodium polytungstate is going to be recycled (see below), decant into a reagent bottle labelled

"used sodium polytungstate." Add distilled water, stir, centrifuge at 2000 rpm for **2 min**, and decant. Phytoliths are now ready to be mounted on slides for microscopy.

Step 10. Mounting and Storage of Phytoliths

Mount phytoliths on slides in Caedex, a synthetic Canada balsam. Other suitable mounting agents are permount, histoclad, benzyl benzoate, and glycerine (Piperno 2006). As phytoliths are often highly concentrated in separations if present, smear a small amount using a 1 ml pipette on a microscope slide and allow to completely dry on a hotplate at very low heat. Remove from the hotplate and use another pipette to add a drop of mounting agent or a smear of agent if not sufficiently viscous, mix using the pipette, and affix a 22 × 40 mm cover slip. If viscosity is low, the mounting agent will spread more effectively under the cover slip if placed back on the hot plate. Check concentration of plant material on the slide by viewing under 100× and 400× magnification. If necessary, adjust concentration and make up another slide. Ideally, as many phytoliths should be in the field of view under 400× magnification as possible without such a high concentration that it overwhelms the eye and makes counting difficult.

Phytolith separations can include other types of biogenic silica, notably diatoms, radiolarians, and sponge spicules. Unlike starch and pollen separations, there is usually negligible extraneous organic plant material in phytolith separations because virtually all organic material is removed during recovery.

Caedex and equivalent resinous mounting agents solidify on prepared slides as their thinning agents evaporate, which may take up to a couple of weeks. During the drying period, store prepared slides horizontally in trays to prevent gravitational movement of phytoliths. During analysis, phytoliths and other biogenic silicates can be turned on slides for better viewing by applying gentle pressure to the cover slip immediately above the item of interest with a fine-tipped battery-operated soldering iron. Do this at 100× magnification rather than greater magnifications to allow adequate room for the iron tip under the shorter objective. Phytoliths mounted on slides in resinous agents can be stored for many years without showing signs of deterioration. Phytolith separations remaining after slides have been made up can be stored at room temperature for possible subsequent use. These can remain in their original test tubes with caps firmly on. Being non-organic, the phytoliths will not degrade.

PROCEDURE FOR RECOVERING POLLEN AND SPORES

The steps for recovering pollen and spores are summarized in Table 3 on page 203, however the guidelines below continue through to mounting and storage of slides.

Step 1. Sub-Sampling

For dental calculus, see Step 4 of the starch separation procedure, in which pollen is extracted along with starch and associated plant material.

For all other types of samples, add 4–5 cm^3 of material per test tube. If intending to measure pollen and spore concentrations (Step 2), use a 0.5 ml spoon to measure the sample, and tap or scrape the material into the test tube. This is more accurate and precise than gauging the amount from the graduations on the side of the test tube. To avoid spillage, ensure the spoon diameter is smaller than the tube diameter. Consider

using larger amounts for samples that appear very sandy or seem to have a low concentration of organic matter.

Step 2. Optional—Addition of Exogenous Markers

Carry out this step only if intending to measure pollen and spore and/or charcoal concentrations. Place one *Lycopodium* tablet (from Lund University) in each test tube with sub-sample and add 6 ml 10% hydrochloric acid, stir, and wait for fizzing reaction to cease. Add a little at a time to avoid overflowing. Centrifuge and decant. Add distilled water, stir, centrifuge at 2000 rpm for 3 min, and decant.

Step 3. Deflocculation and Removal of Macroscopic Material and Clay

Add 3 ml each of a 0.1 molar solution of sodium pyrophosphate and 10% potassium hydroxide, and place in a boiling water bath for 10–20 min, stirring occasionally. Top up with distilled water, shake with cap firmly on, then pour through a 130–150 μm sieve into a 50 ml glass beaker. Cut the sieve from a roll of steel mesh into an approximately 6 × 6 cm square, slightly indent it, and rest it on the beaker. Squirt distilled water through the sieve to wash in pollen trapped within the larger particles, ensuring that the sample volume does not exceed 10 ml. Between samples, rinse the sieve with water (need not be distilled) and hold over a flame with tweezers until red hot to ensure no plant material remains caught in the mesh. The sieve square can be used for many samples, but will need replacing when the first fine hole or split appears. Pour back into test tube, centrifuge at 2000 rpm for 3 min, and decant. Add distilled water, stir, centrifuge at 2000 rpm for 3 min, and decant. Repeat water wash until supernatant is clear.

Step 4. Density Separation of Pollen and Spores

This step separates pollen, spores, and some other types of plant material from the matrix. Add 2 ml sodium polytungstate solution, 2.0 specific gravity, and mix thoroughly. The solution can be made by dissolving sufficient chemical in water so that 100 ml weighs 200 g. Centrifuge at 2000 rpm for 3 min; **do not decant**.

Holding the test tube at eye level in good light, gently and carefully stir the upper part of the top layer (i.e., the layer of liquid and microfossils above the pellet) with the pipette without disturbing the pellet, then pipette most of this layer, again without disturbing the pellet, then invert the pipette and rest it upside down in the test tube rack directly adjacent to the test tube so you know they belong together and not to any other tubes or pipettes in the rack. Dispose of pellet from test tube, rinse tube in distilled water, and pipette pollen separation back into tube. Top up with distilled water to dilute heavy liquid so the microfossils can be centrifuged to the bottom of the tube. Hold tube upside down with cap firmly on, shake vigorously, centrifuge at 2000 rpm for 3 min, and decant. If the sodium polytungstate is going to be recycled (see below), decant into a reagent bottle labelled "used sodium polytungstate." Add distilled water, stir, centrifuge at 2000 rpm for 3 min, and decant.

Step 5. Acetolysis

Acetolysis removes cellulose, a common plant polysaccharide, and enhances the visibility of the pollen and spore sculpturing, aiding in identification. The reagents

react vigorously with water so materials with which they come into contact must be dehydrated. To dehydrate the sample, add 3 ml glacial acetic acid, stir, centrifuge at 2000 rpm for 3 min and decant. Then with test tubes facing towards the back of the fume hood, add 3 ml 9:1 ratio of acetic anhydride and concentrated sulfuric acid. The solution must have been made up the same day. Place in boiling water bath for 4.00–4.25 min. Avoid less than 4 min; longer than 4.5 min will darken and can damage pollen grains, making identification difficult. Centrifuge at 2000 rpm for 3 min and decant. Add 3 ml glacial acetic acid, stir, centrifuge at 2000 rpm for 3 min, and decant. Add distilled water, stir, centrifuge at 2000 rpm for 3 min, and decant. Pollen and spores are now ready to be mounted on slides for microscopy.

As well as pollen and spores, this procedure can be used to extract fragments of xylem cells and charcoal and phenolic inclusions (Horrocks, Baisedn et al. 2017). In addition, degraded starch grains (discolored, expanded, disintegrated) of *Ipomoea batatas* and fragments of disintegrating amyloplasts of *Colocasia esculenta* can sometimes be found on pollen slides, despite the harsh chemical and heating treatment. Presumably the degraded starch has survived this treatment because it has undergone chemical changes associated with decay or there has been some protection from enclosing tissue during the procedure.

Step 6. Staining

For staining to make pollen grains more conspicuous, use a 1 ml pipette to add a drop of 0.1% basic fuchsin solution. This concentration is usually adequate and should avoid overstaining, although samples with high concentrations of microscopic charcoal fragments, often encountered in archaeological deposits, may require a stronger solution (0.2%). To make 100 ml of 0.1% solution, add 0.1 g of basic fuchsin crystals to 100 ml of distilled water. Add 0.2 g to 100 ml water for 0.2% solution. Leave for a few minutes, stirring or shaking vigorously several times, then pour through a 130–150 μm sieve to remove undissolved crystals.

Step 7. Mounting and Storage of Pollen and Spores

Mount pollen and spore extractions on slides in glycerol jelly. Place a few cm^3 of jelly into a 50 ml glass beaker in a lukewarm to warm water bath to melt. A glass beaker wider than and about the same height as the 50 ml beaker, holding about 10 mm water and sitting on a hot plate at very low heat, is ideal for this.

Using a 1 ml pipette, place a drop of melted jelly onto the microscope slide, more to one side to allow a sticky label to be attached after the cover slip is attached. Using another 1 ml pipette, place up to a drop of the recovered sub-sample onto the drop of jelly and mix with the pipette tip, spreading to less than cover slip size (22 × 40 mm). Check concentration of plant material on the slide by viewing under 100× and 400× magnification. If necessary, adjust concentration and make up another slide. Ideally, as much material should be in the field of view under 400× magnification as possible without a high concentration of extraneous material, such as charcoal or residual clay particles, that would potentially obscure plant material of interest.

The glycerol jelly on the slide preparations will set. During analysis, pollen grains, spores, and other microscopic plant material can be turned on slides for better viewing by applying gentle pressure to the cover slip immediately above the item of interest

with a fine-tipped battery-operated soldering iron. Do this at 100× magnification, as opposed to greater magnifications, to allow adequate room to manipulate the iron tip under the shorter objective.

To prevent gravitational movement of material on prepared slides, permanently store horizontally in trays. Coverslips should face down as these exert pressure on material over time. Store away from direct sunlight as this can melt glycerol jelly. Slides of pollen and associated material mounted in glycerol jelly can be stored for many years. Swelling of pollen grains will start to occur after about a decade, however. Pollen preparations remaining in test tubes after slides have been made up can be stored at room temperature. These preparations can remain in their original test tubes with caps firmly on; despite the caps, in the experience of this author, they permanently dry out after several months, which aids in their preservation. Alternatively, the tubes can be refrigerated at 2–4 °C.

Recycling Sodium Polytungstate

Sodium polytungstate is an expensive chemical. This expense can be reduced by recycling the saved, diluted sodium polytungstate solution. Reduce the solution by bringing it to a boil, then simmering. A small stainless-steel saucepan with a heat-proof handle and without a lid is ideal for this.

After several days most of the extraneous material in the used sodium polytungstate solution will have sunk to the base of the bottle, forming a visible thin layer. In this case, slowly pour the solution into the saucepan and stop when the bulk of the extraneous material starts to enter the saucepan.

Reducing will reach a point where crystals become visible. If this occurs, keep adding small amounts of distilled water to re-dilute without over-diluting. After reducing, allow to cool, then pour through Grade CF/C 0.7 μm (to no more than 1.0 μm) filter paper, 9 cm diameter. Place the filter paper in a 10 cm funnel and rest this in the neck of a reagent bottle labelled "clean sodium polytungstate." The more the solution is reduced, the slower the filtration will be.

PREPARING MODERN REFERENCE SAMPLES OF PLANT MICROFOSSILS

Plant material selected for modern reference can be selected from provenanced collections, such as herbariums, or from growing plants. If the latter, only experts qualified in taxonomic identification should carry this out.

Starch and Associated Plant Material

For mounting starch residues from modern reference material, simply mount directly on slides in the same manner as in Step 5 for starch sub-samples outlined in the procedure above. If the material is desiccated, place into distilled water for up to several hours beforehand to hydrate.

Phytoliths

Start at the combustion/nitric acid step as outlined in Step 8 for phytolith sub-samples above by placing small fragments of the plant tissue into a crucible and combusting.

After this combustion step, carry out the hydrochloric acid Step 7, and proceed directly to Step 10 for mounting.

Pollen and Spores

Start at Step 3 in the procedure outlined above for pollen and spore sub-samples, using 6 ml 10% potassium hydroxide solution and excluding the sodium pyrophosphate. Then continue as per the procedure.

TABLE 2. PROCEDURE FOR COMBINED RECOVERY OF STARCH AND ASSOCIATED PLANT MATERIAL AND PHYTOLITHS[a]

STEP ORDER	PROCEDURE
1	Add 4.0–5.0 cm^3 material to 10 ml phytolith tube.
2	Add 6 ml 5% sodium polymetaphosphate solution, leave overnight or at least 8 hr, shaking occasionally. Top up with water, shake, pour sample into 50 ml beaker, swirl, wait 5 seconds, pour liquid back into tube, centrifuge at 2000 rpm for 3 min, decant. Dispose of settled fraction in beaker. Add water, stir, centrifuge at 2000 rpm for 3 min, decant. Repeat until clear.
3	Add 2 ml sodium polytungstate solution, specific gravity 1.8, shake, centrifuge at **1500 rpm** for 3 min, pipette top layer into starch tube, top up with water, shake, centrifuge at 2000 rpm for 3 min, decant. Add water to starch tube, stir, centrifuge at 2000 rpm for 3 min, decant. Starch separation complete.
4	Add water to phytolith tube, stir, centrifuge at 2000 rpm for 3 min, decant. Add 3 ml each of 0.1 molar sodium pyrophosphate solution and 10% potassium hydroxide, place in boiling water bath for 8 min, stirring occasionally. Remove from bath, top up with water, stir, wash through 250 μm sieve into 50 ml beaker. Centrifuge at 2000 rpm for 3 min, decant. Add water, stir, centrifuge at 2000 rpm for **2 min**, decant. Repeat at reduced time until clear.
5	Add 10% hydrochloric acid, stir and wait for fizzing reaction to cease. Centrifuge at 2000 rpm for 3 min, decant. Add water, stir, centrifuge at 2000 rpm for **2 min**, decant.
6	Add pellet to crucible, heat at 500 °C for 4 hr. Transfer back into tube, top up with water, stir, centrifuge at 2000 rpm for **2 min**, decant. If using nitric acid digestion option, see text.
7	Add 3 ml sodium polytungstate solution, 2.3 specific gravity, shake, centrifuge at 2000 rpm for **5 min**, decant. Pipette top layer, invert pipette and rest in test tube rack. Rinse tube, pipette layer back into tube, top up with water, shake, centrifuge at 2000 rpm for 3 min, decant. Add water, stir, centrifuge at 2000 rpm for **2 min,** decant. Phytolith separation complete.

[a] Different procedure applies for dental calculus.

Table 3. Procedure for Recovery of Pollen and Spores[a]

STEP ORDER	PROCEDURE
1	Add 4–5 cm^3 material to 10 ml test tube. Add 3 ml each of 0.1 molar sodium pyrophosphate solution and 10% potassium hydroxide, place in boiling water bath for 10–20 min, stirring occasionally. Top up with water, pour through 130–150 μm sieve. Centrifuge at 2000 rpm for 3 min, decant. Add water, stir, centrifuge at 2000 rpm for 3 min, decant. Repeat wash until clear.
2	Add 2 ml sodium polytungstate solution, 2.0 specific gravity, shake, centrifuge at 2000 rpm for 3 min Pipette top layer, invert pipette and rest in test tube rack. Rinse tube, pipette layer back into tube, top up with water, shake, centrifuge at 2000 rpm for 3 min, decant. Add water, stir, centrifuge at 2000 rpm for 3 min, decant.
3	Add 3 ml glacial acetic acid, stir, centrifuge at 2000 rpm for 3 min, decant. Add 3 ml 9:1 ratio acetic anhydride and concentrated sulfuric acid. Place in boiling water bath for 4.00–4.25 min, stirring occasionally. Centrifuge at 2000 rpm for 3 min, decant. Add water, stir, centrifuge at 2000 rpm for 3 min, decant. Add 3 ml glacial acetic acid, stir, centrifuge at 2000 rpm for 3 min, decant. Pollen and spore separation complete.

[a] Different procedure applies for dental calculus.

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