Phytolith Sample Collection & Processing Procedure
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PHYTOLITH SAMPLE COLLECTION

Phytolith samples were collected either from the profiles of excavated archaeological units, the profiles of "offsite" soil testing units, or from discrete archaeological contexts from site CA-SMA-113. Discrete contexts sampled for phytoliths were generally ash deposits. Phytolith samples collected from unit profiles were collected as column samples, i.e. at regular vertical intervals. For both onsite and offsite columns, phytolith samples were collected from the vertical center of each 10cm arbitrary level. For example, the first sample was collected at a depth of 5+/- 2cm (the approximate center of the first 10cm level), the second sample was collected at 15+/-2cm and so on.

When soil samples were collected from archaeological profiles, columns were placed judgmentally to represent the overall stratigraphy and to avoid disturbed areas such as rodent burrows. Since offsite soil sampling units were only 50x50cm and up to 130cm deep, it was difficult to determine whether there were meaningful differences in the profile faces, so soil columns on offsite units were placed on the most geometrically regular face. Prior to collection, profile faces were scraped with a clean, sharpened trowel to expose an uncontaminated surface. Soil samples were collected by making two horizontal cuts into the face of the profile at the approximate center of each 10cm level, and then cutting vertically on either side of the soil at an angle to create a wedge of soil 4-5cm high and 8-10cm wide at the profile face. The wedge of soil and any additional soil to be collected from the level were placed in a cleaned dustpan, broken up into small pieces with a trowel, and placed in labeled 4mil plastic bags for storage. For samples collected from discrete archaeological contexts, the feature to be sampled was first scraped with a clean trowel to expose a fresh surface. Soil was removed from the scraped area and placed in labeled bags for storage. The amount of soil collected for phytolith sampling was generally 50-100g dry weight.

PHYTOLITH SAMPLE PROCESSING

General Notes

Throughout the phytolith processing procedure, powder-free latex gloves were worn to prevent contamination and to provide protection from chemicals. Lab supplies used (e.g. beakers, glass rods) were washed with Contrex and tap water after each use to prevent cross-contamination. Distilled water was not used to rinse all washed glassware because 10L of sampled tapwater was found to contain no phytoliths. All hazardous chemicals were manipulated under a fume hood according to recommended laboratory safety protocols. Distilled water, produced using a MegaPure still, was used in all cases where water was to come in direct contact with soil samples.

Initial Preparation

This step ensures that a consistent and homogenous sample of soil is collected for processing. From each sample, ca. 20-30g of soil was removed from the sample bag, placed in a labeled weigh boat, and dried in an oven at 60* Celsius for 8-12 hours. Samples were transferred from weigh boats into a mortar and pestle and gently crushed to break up aggregated portions of the soil. Samples were transferred from the mortar and pestle into a 500 micron geological sieve (USGS Standard Sieve Series #35) and shaken. The portion of the sample less than 500 microns was collected for additional processing, and the portion greater than 500 microns was returned to the sample bag. After each use, the sieve was washed with tap water and Contrex and dried with compressed air.

Deflocculation

Deflocculation neutralizes electrical charges that cause small particles to aggregate. These charges prevent the removal of clay-sized particles.

The fraction of each sample less than 500 microns was collected and 10.000+/- 0.010g of soil was transferred into a 50ml polypropylene tube and 30ml of 10% sodium bicarbonate solution was added to each sample. Each 50ml tube was labeled both on the side of the tube and on the removable cap to prevent accidental cross-contamination. Tubes were vortexed until well mixed and clamped horizontally in

a shaker. Horizontal placement of tubes prevented any portion of the soil sample from settling into the tapered lower end of the tubes during shaking, which might prevent particles from being exposed to the sodium bicarbonate solution. Samples were agitated gently in the shaker for 12-16 hours. Tubes were removed from the shaker and centrifuged 5 minutes at 3000 rpm. Supernatant was discarded and 40ml distilled water was added to dilute the remaining sodium bicarbonate solution.

First Clay Removal

The purpose of the first clay removal step is to eliminate the majority of clay-sized particles in each sample. Since the later "carbonate dissolution and organic digestion" step liberates additional clay-sized particles from the soil matrix, attempting to remove all clay-sized particles prior to digestion would be futile. However, eliminating the majority of clay-sized particles at this time has several benefits. First, it prevents samples from forming a "sticky" pellet after centrifugation, which requires extended vortexing (sometimes 5-10 minutes) to disaggregate. Second, it prevents clay from forming a "cap" on top of the sample while in pressurized microwave vessels during the "carbonate dissolution and organic digestion" step. The author has observed that if a clay layer "cap" forms during digestion, it can prevent the larger particles in a sample from being exposed to sufficient chemicals to digest all organic material. Third, removing clay at this point reduces the sample volume ca. 25-50% (of course, this will vary with soil type), which makes digestion more efficient by removing material that would react with the chemicals employed.

Sample tubes were vortexed until no visible clumps of aggregated sediment remained and centrifuged (Centra CL3; Rotor #243; fast spin up; fast spin down) for 75 seconds at 1000rpm ("clay spin"). This treatment caused particles greater than ca. 3 microns to settle out of the ca. 12cm water column in each tube. Determination of centrifuge settings was based on CITATION and modified by successive experiments checking different speeds and times to ensure that only clay-sized particles remained in supernatant after centrifugation. Sample tubes were gently (so as not to disturb the pellet) transferred from the centrifuge into tube racks. For each sample, a labeled 20ml syringe with no needle attached was used to remove the supernatant. These syringes are small enough to fit into the 50ml tubes, allowing continuous removal of supernatant from the top of the water column only. This method was used instead of pouring off supernatant because it avoids disturbing the pellet, which is not well-consolidated after the "clay spin." Supernatant was removed by syringe until the water column was ca. 2cm above the pellet. Distilled water was added to the 50ml tube to fill it to 45ml. These steps were repeated until the supernatant was no longer opaque (i.e. until the gradation lines on the opposite side of the tube were just visible through the water column). Generally 6-12 repetitions were necessary to attain this level of supernatant clarity.

Dessiccation

Water must be removed from each sample to prevent dilution of the chemicals used during the "carbonate dissolution and organic digestion" step. Neglecting this step could prevent efficient digestion.

After the final repetition of the "first clay removal" step, each 50ml sample tube contained a loosely consolidated pellet and a ca. 2cm column (~10ml) of distilled water. Each tube was vortexed to disaggregate the pellet and poured into a labeled 50ml Pyrex beaker. While holding each tube inverted over the beaker, a squeeze bottle of distilled water was used to rinse all remaining sediment from the tube into the beaker. The cap of each tube was likewise rinsed to remove any remaining sediment. Each 50ml beaker was placed in a oven at 60*C until completely dry (12-24 hours).

Carbonate Dissolution and Organic Digestion

This step removes HCl-soluble (i.e. nonsilicate) minerals and organic materials from each sample. Eliminating these materials assists in isolating phytoliths, producing less cluttered slides. It also liberates any phytoliths that may be held in nonsilicate mineral or organic matrices.

After drying, a pellet of dried sediment remained in the bottom of each 50ml Pyrex beaker. This was broken into small pieces (less than ca. 5mm) using a glass rod, which was also used to liberate any material adhering to the sides or bottom of the beaker. The disaggregated sediment was poured into a clean 50ml Teflon microwave pressure vessel. To each vessel, 3ml of hydrochloric acid, 5ml of nitric acid, and 2ml of 30% hydrogen peroxide was added using a 20ml syringe. For each chemical, the portion to be used was transferred into a Pyrex beaker under a fume hood. The chemical was drawn into the empty syringe and added to each microwave pressure vessel successively. A beaker of distilled water was kept on hand to rinse excess chemical from the syringe and from beakers.

In highly organic samples, a vigorous reaction may occur when adding the hydrogen peroxide. To prevent the reaction from causing solution to overflow out of the microwave vessel, a supply of glass rods was kept on hand during addition of the hydrogen peroxide. Hydrogen peroxide was added slowly, about one drop per second. If a vigorous reaction began to occur (a layer of foam that began rising quickly towards the top of the tube), the glass rod was used to agitate the foam layer until it subsided. Each microwave pressure vessel was sealed. The microwave system used in processing has a carousel of 12 pressure vessels, one of which has a pressure sensor that is used to monitor the sample during processing. The pressure sensor was placed on the sample that had the most vigorous reaction after the addition of hydrogen peroxide.

Each microwave pressure vessel is equipped with a "rupture membrane" that is designed to burst if pressure in the tube reaches a critical level. In initial microwave digestions, the author found that digestion at 100psi would occasionally cause rupture membranes to burst, necessitating reprocessing of the affected samples. To prevent this, the microwave digestion protocol employed here used a lower pressure setting. Samples were placed in the microwave and heated until pressure inside the moitored vessel reached 80psi. Pressure was held at 80psi for 60 minutes, and then the samples were cooled for 15 minutes or until the pressure sensor read less than 20psi.

Vessels were removed from the microwave and opened under the fume hood. A glass rod was used to disaggregate the sample at the bottom of each pressure vessel. After 20-30 seconds of stirring, an additional 2ml of hydrogen peroxide was added to each sample and the glass rod was used to break up any foam layer that formed. Samples were again sealed and placed back in microwave for a second digestion with the same settings as the first (80psi for 60 mins and a 15-minute cooldown).

After the second digestion, samples were removed from the microwave to the fume hood and uncapped. For each sample, the microwave pressure vessel was removed from the carousel, swirled gently for 20-30 seconds to mix the contents, and poured off into a labeled 15ml polypropylene tube. Since these tubes have narrow openings, small funnels can be used to prevent spills. While holding the pressure vessel inverted, a squeeze bottle filled with distilled water was used to rinse any remaining sediment out of the pressure vessel and into the 15ml tube. Occasionally it was not possible to transfer all of the material from the microwave pressure vessel into the 15ml tube at once. In this case, the remaining material was rinsed into the 15ml tube after the first centrifugation.

The 15ml tubes containing sediments and digestion chemicals were centrifuged for 5 minutes at 3000rpm to form a well-consolidated pellet. The supernatant was poured off into a beaker for later neutralization with sodium bicarbonate. If any material remained in the microwave pressure vessel, it was rinsed into the 15ml tube at this time. If not, tubes were filled to 14ml with distilled water, vortexed well, and centrifuged a second time for 5 minutes at 3000 rpm. This is the first rinse with distilled water. After pouring off the supernatant, a second rinse was performed in the same way.

Heavy Liquid Flotation

This step separates phytoliths and other light particles from denser minerals by floating them in a liquid with density greater than that of the phytoliths but less than that of most minerals. At the beginning of this step, each 15ml sample tube contains a sediment pellet with as little remaining distilled water supernatant as possible. When the heavy liquid is added to the tube, it mixes with the distilled water remaining in the pellet, diluting the heavy liquid. Since the aim of this procedure is to recover phytoliths as completely as possible, the heavy flotation step is carried out three times. With each repetition, there is less of the original distilled water remaining to dilute the heavy liquid. It is undesirable to dry the samples prior to adding heavy liquid because doing so may cause aggregation of particles, preventing phytoliths from floating.

A 2.3g/ml solution of sodium polytungstate solution was prepared and drawn through a 1 micron filter two times with a vacuum pump. Five milliliters sodium polytungstate was added to each 15ml tube (hereafter the "heavy fraction tube") and the contents vortexed until fully disaggregated. Samples were then centrifuged for 3 minutes at 3000rpm. Since there is so much sediment in the solution after vortexing, it will tend to stick to the sides of the 15ml tube. This is undesirable, since this sediment would be rinsed out along with the floating "light fraction," contaminating the light fraction with heavy sediment particles. After the preceding centrifugation step, to reduce the amount of sediment adhering to the sides of the tube, the supernatant is used to rinse these adhering particles into the supernatant, which will not contain many sediment particles after the 3-minute spin. Once sediment particles were rinsed from the sides of each tube, they were centrifuged for 5 minutes at 3000rpm. After centrifugation, a layer of floating

particles was present at the top of the supernatant column. Sometimes this layer of particles was relatively thick and well-consolidated, causing it to cap the supernatant and adhere to the walls of the tube. A glass rod was used to stir the top ca. 2cm of the supernatant, disaggregating the light fraction. The supernatant was then poured off into a separate labeled 15ml tube (the "light fraction tube"). The pellet at the bottom of the heavy fraction tube was usually well consolidated and did not appear to contaminate the light fraction while pouring off. Since the samples still contained some clay at this time, the clay usually formed a cap over the heavy fraction pellet that prevented it from becoming disaggregated.

After the first flotation, 5ml sodium polytungstate was added to each heavy fraction tube and the samples vortexed well. A second flotation step was carried out exactly as before. After the second flotation step, the 15ml light fraction tube contained 10ml of partially diluted sodium polytungstate solution and all light fraction particles. To consolidate the light fraction particles and to remove the heavy liquid, 5ml of distilled water was added to each of the light fraction tubes. Tubes were vortexed well and centrifuged for 5 minutes at 3000rpm, and then the supernatant was poured off and collected for recycling. Dilution of the supernatant in the light fraction tubes in this way reduced the density from 2.3g/ml to ca. 1.5-1.6g/ml, so that any particles less dense than this would be poured off with the supernatant.

After removing supernatant from the light fraction tubes, 5ml of sodium polytungstate was again added to the heavy fraction tubes, these were vortexed, and the third flotation was carried out as before. After the third flotation, supernatant in the light fraction tube was again diluted by filling the tube to 14ml with distilled water, vortexing well, and centrifuging for 5 minutes at 3000rpm. Supernatant was collected for recycling. To recover any remaining sodium polytungstate solution in the heavy fraction tube, distilled water was added to each tube to fill it to 14ml, the samples were vortexed well, centrifuged for 5 minutes at 3000rpm, and the supernatant collected for recycling. After this, all material in the 15ml tubes was transferred back into the original 50ml tube for storage. This was done by adding distilled water to each tube, vortexing, and pouring off into the 50ml tubes. An inverted squeeze bottle was used to rinse any sediment adhering to the sides of the 15ml tube into the 50ml tube.

Second Clay Removal

After flotation, the light fraction tube will contain clay particles that were not removed during the first clay removal step and clays liberated during carbonate dissolution and organic digestion. To remove these particles as completely as possible, a second clay removal step similar to the first is necessary.

To each 15ml tube, distilled water was added, filling the tube to 14ml. Each tube was vortexed until the pellet was fully disaggregated and centrifuged for 75 seconds at 1000rpm as in the first clay removal step. After centrifugation, tubes were gently removed from the centrifuged, placed in a tube rack, and a labeled 5ml syringe with a 5cm needle attached was used to remove the supernatant. As before, removing the supernatant in this way prevents any disturbance to the loosely consolidated pellet at the bottom of the tube. The 5ml syringes were small enough to fit inside the 15ml tubes, allowing removal of up to 10cm of the 12-13cm water column. Supernatant was removed until the water column was 1cm above the pellet. To avoid disturbing the pellet, supernatant was removed from the top of the water column only. This clay removal procedure was repeated until the water column appeared completely transparent after centrifugation

Size Fractionation of Particles

At this stage, the 15ml sample tubes contained light particles greater than ca. 3 microns in size. This fraction usually included particles that were too large to be phytoliths, greater than ca. 75 microns in size. It was desirable to eliminate particles of this size from the samples because the vast majority of them were not phytoliths and because they increase the depth of the immersion oil layer during slide mounting, causing problems in the digital imaging procedure described below. To separate "large" from "small" particles, Stokes' equation was used to determine the settling time of a 75 micron particle of 2.3 g/cm^3 density through a 10cm water column. Settling time was estimated to be 25 seconds. Settling time was increased to 30 seconds for the purposes of this procedure to account for instability in the water column at the beginning of each settling phase.

Distilled water was added to each 15ml tube, filling it to 13ml. This created a ca. 10cm water column above the pellet. The tube containing the water column and pellet will be referred to as the "large particle tube." The large particle tube was vortexed and shaken vigorously, then placed upright in a tube

rack and left undisturbed for 30 seconds, after which it was poured off into a second labeled 15ml tube (the "small particle tube"). The water column in the large particle tube was poured off until ca. 2ml of liquid remained or until the settled portion of particles at the bottom of the tube began flowing towards the end of the tube. After this, the small particle tube was centrifuged for 5 minutes at 3000rpm and the supernatant discarded. Since the samples contained few particles smaller than 3 microns at this stage, the pellet was usually not well consolidated after centrifugation. Decantation of the samples was discontinued if the pellet became visibly disaggregated. In these cases, the small particle tubes were centrifuged again for 5 minutes at 3000rpm and decantation was attempted again.

After this, distilled water was again added to each large particle tube and the procedure carried out two more times. By the end of the third fractionation step, the portion of solution decanted from the large particle tube appeared almost completely transparent, indicating that the overwhelming majority of small particles had been transferred into the small particle tube. Both tubes were centrifuged for 5 minutes at 3000rpm and decanted, leaving ca. 1ml of solution in each tube. A glass pipet was used to transfer particles from the 15ml tubes into 2ml polypropylene centrifuge tubes. The two size fractions were labeled "LP" for "large particles" and "SP" for "small particles." The computer-assisted phytolith imaging technique described below makes use of only the "SP" samples. Before phytolith extract was added to each tube, it was weighed to a precision of +/-0.1mg and the tube weight recorded. After transfer into 2ml tubes, samples were placed in an oven at 60*C and dried for 12-16 hours. Tubes were weighed again and the weight of both "SP" and "LP" phytolith extract was calculated and recorded.

Particle Staining

In order to distinguish phytoliths from other silicate particles, two dyes were used to stain non-phytolith particles remaining in the small particle portion of the phytolith extract. Each "SP" tube was vortexed (dry) for ca. 30 seconds to mix the contents of the tube. From each "SP" phytolith extract sample, a portion of phytolith extract 5.0+/-0.5mg was removed (using a flat toothpick as a spatula) and added to a new labeled 2ml tube, the "SPE" or "standardized phytolith extract" tube. As with "SP" tubes, each "SPE" tube was weighted to +/-0.1mg precision and the weight recorded prior to use. To each SPE tube, 1.0ml of a saturated solution of methylene blue and crystal violet was added. This solution was prepared by adding 3.5g methylene blue and 1.7g crystal violet to 100ml of distilled water and stirring the solution for an hour using a magnetic pellet. After adding the staining solution to each "SPE" tube, the tubes were capped, placed in a 3.75L container half full of water, and shaken for 12 hours.

"SPE" tubes were removed from the shaking container and centrifuged for 5 minutes at 3000rpm. Samples were gently removed from the centrifuge and placed in a tube rack. A labeled pipet was used to remove the supernatant column to 1cm above the bottom of the tube. As in the clay removal steps, the supernatant column was removed from the top only. Supernatant removed from the "SPE" tubes was discarded. "SPE" tubes were topped off with distilled water vortexed until well mixed, then centrifuged again for 5 minutes at 3000rpm. These rinsing steps were repeated until the supernatant appeared very faintly blue after centrifugation. After the final rinsing cycle, "SPE" tubes were placed in an oven at 60*C for 12-16 hours to dry. After drying, "SPE" tubes were weighed again and the weight of stained phytolith extract was calculated to +/-0.1mg precision. For each 1.0mg of phytolith extract, 0.05ml (0.046g) of Cargille "Type B" immersion oil (0.923g/ml) was added to each "SPE" tube. This produced a standardized-density phytolith extract in each tube.

Mounting "SPE" Slides

To facilitate computer-assisted image analysis, it was desirable for each slide to have a layer of standardized phytolith extract as thin as possible. Using a round toothpick, each "SPE" tube was stirred vigorously for at least 60 seconds or until the contents appeared to be homogenized. A small portion of standardized phytolith extract (ca. 0.1ml) was drawn into a disposable glass pipet and one drop (holding the pipet at a 45* angle) was expelled onto the center of a 25x75x1.0mm glass slide. A round toothpick was used to mix and spread the expelled extract into a rectangle roughly 20x30mm in size. The toothpick was used to pop any bubbles that formed during mixing, and the phytolith extract was covered with a 24x30mm cover slip. Sally Hansen "Extreme Wear" fingernail polish was used to seal the edges of the cover slip just prior to the standardized phytolith extract reaching the edge. Slides were allowed to dry overnight, and any leaks were sealed by cleaning the area around the leak and adding additional fingernail polish. After drying, slides were placed in storage boxes and stored horizontally until use.

Mounting "LP" Slides

"LP" portions of each phytolith sample were not stained. These were transferred from 15ml tubes into 2ml tubes after the "size fractionation of particle" step, placed in an oven at 60*C and dried for 12-16 hours. After drying, "LP" tubes were weighed and the weight of "LP" extract was recorded. Unlike "SPE" samples, standardized extracts were not made for "LP" particles. This is because the large particles tend to hold the cover slip high above the surface of the microscope slide, requiring the addition of more immersion oil to the extract in order to prevent large voids from forming under the cover slip. Immersion oil was judgmentally added to each "LP" sample. These were mixed well with a round toothpick, drawn into a disposable glass pipet, and 3 drops (holding pipet at a 45* angle) of extract were expelled onto the center of a 25x75x1.0mm glass slide. A round toothpick was used to spread the extract into a rectangle roughly 20x30mm in size, and a 24x30mm cover slip was placed over it. Sally Hansen "Extreme Wear" fingernail polish was used to seal the edges of the cover slip just prior to the standardized phytolith extract reaching the edge. Slides were allowed to dry overnight, and any leaks were sealed by cleaning the area around the leak and adding additional fingernail polish. After drying, slides were gently cleaned with a dampened Kim Wipe, allowed to dry, and placed in storage boxes and stored horizontally.