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Evaluating microfossil content of dental calculus from Brazilian sambaquis

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Abstract
To date, limited numbers of dental calculus samples have been analyzed by researchers in diverse parts of the world. The combined analyses of these have provided some general guidelines for the analysis of calculus that is non-destructive to archaeological teeth. There is still a need for a quantitative study of large numbers of calculus samples to establish protocols, assess the level of contamination, evaluate the quantity of microfossils in dental calculus, and to compare analysis results with the literature concerning the biology of calculus formation. We analyzed dental calculus from 53 teeth from four Brazilian sambaquis. Sambaquis are the shell-mounds that were established prehistorically along the Brazilian coast. The analysis of sambaqui dental calculi shows that there are relatively high concentrations of microfossils (phytoliths and starch), mineral fragments, and charcoal in dental calculus. Mineral fragments and charcoal are possibly contaminants. The largest dental calculi have the lowest concentrations of microfossils. Biologically, this is explained by individual variation in calculus formation between people. Importantly, starch is ubiquitous in dental calculus. The starch and phytoliths show that certainly *Dioscorea* (yam) and *Araucaria angustifolia* (Paraná pine) were eaten by sambaqui people. Araceae (arum family), *Ipomoea batatas* (sweet potato) and *Zea mays* (maize) were probably in their diet.

Keywords: Sambaquis, Starch, Phytolith, Calculus, Dental, Dental Anthropology, Paleonutrition, Archaeobotany, Bioarchaeology.

The recovery and analysis of microresidues from dental calculus is a relatively new line of investigation. Calculus analysis has the potential of revealing the genera and species of dietary plants, patterns of cultivation, methods of food preparation, and, in comparison with dental pathology, the relation of diet to dental disease (Babot, 2006; Beck and Torrence, 2006; Hardy et al., 2009; Henry and Piperno, 2008; Loy, 1994; Loy et al., 1992; Reinhard et al., 2001; Walshaw, 1999; Wesolowski et al., 2007).

The first methods were described by Fox et al., 1996 and Nelson, 1997a and Reinhard et al. (2001). These researchers developed parallel methods. In general, they collected the dental calculus, noted the characteristics such as size and weight, extracted the microfossils using acid, and then identified the recovered microfossils. Nelson (1997a) and Reinhard et al. (2001) used dental picks or scalpel blades to flake dental calculus off of the enamel surfaces of teeth. Reinhard et al. (2001) specified that their methods were good for samples of 0.1 g or larger but were not consistently useful with smaller samples.

Nelson (1997a) based her published work on her more detailed thesis (1997b). She focused her work on determining the cause of severe dental wear in a prehistoric Peruvian population using a combination of SEM analysis of microwear, macroscopic examination of dental pathology and recovery of plant microfossils from dental calculi and plant reference materials. She was able to show that women used their teeth to prepare fibers for textiles from cotton and totora leaves. It was this non-dietary mastication that caused severe dental wear. She also reported the finding of dietary starch and phytoliths.

More recently Boyadjian et al. (2007) presented the results of an experimental "dental wash" method for recovering microfossils from extremely small deposits of dental calculus based on her thesis (Boyadjian, 2007). This method differed from Reinhard et al. (2001) and Fox et al. (1996) in that the dental calculus was dissolved by briefly immersing teeth crowns directly in dilute hydrochloric acid. Although microfossils were recovered, the enamel surfaces of the teeth appeared to be eroded. Therefore, Boyadjian et al. (2007) recommended that this method be avoided in future research.

Table 1. Uncalibrated dates for the sites under study with source of material dated.

Site	Date	Ceramic	# studied individuals/ # studied teeth	Dating method
Morro do Ouro	3870 ± 40 BP (burial 80, unpublished)	No	14/14	C14, collagen from human bone
	4300 ± 50 BP (burial 31, unpublished)			C14, collagen from human bone
Forte Marechal Luz	1110 ± 100 BP, 850 ± 100 BP (Bryan and Gruhn, 1993)	No	4/7	C14, charcoal and carbonized seeds associated with burials
	1550 ± 40 BP (burial H22, unpublished)			C14, collagen from human bone
Enseada 1	1390 ± 40 BP, (De Masi, 2001)	Yes	17/21	C14, collagen from human bone
Itacoara	1250 ± 30 BP (burial 02, unpublished))	Yes	11/11	C14, collagen from human bone
	1570 ± 20 BP (Bandeira, 2004)	Yes		Charcoal

Most recently, Henry and Piperno (2008) presented the analysis of five teeth from Tell al-Raqa'i, Syria. The teeth come from three burials dating to the third millennium BC and two others less securely dated between the third millennium BC to the Islamic period. By ESEM analysis, these authors demonstrated that removal of dental calculus using dental tools does not harm the surface of the teeth. They introduce a Calgin wash stage into the chemical extraction procedure. They present a comment based on unpublished experimentation that "modern starch samples from *Z. mays* have shown that weak solutions of HCl and room temperature reactions over a 24 h period do not damage the starch grains" (Henry and Piperno, 2008, P. 1945). They also detail procedures for making starch reference collections from modern plants. Hardy et al. (2009) proposed that the protection of starch in dental calculus hinders diagenesis. They also introduce chemical tests to verify the starch origin of microfossils in dental calculus.

It is our purpose in this paper to detail a non-destructive method for safe analysis of dental calculus that can recover microresidues from extremely small dental calculus deposits. It is also our purpose to present some basic principles of dental calculus analysis based on observations of variation among the 53 teeth included in our study. We present herein refinements that resolved the problems encountered by Reinhard et al. (2001) and Boyadjian et al. (2007) from the work of Wesolowski (2007). We refer to Wesolowski (2007) for a detailed presentation of the pathological significance of microresidues for understanding dental disease.

We analyzed dental calculus from four Brazilian shell-mounds, or "Sambaquis". Sambaquis are dispersed along most of the length of Brazil's seashore and can be monumental in size. Some sambaquis are 40 m high, often reaching thousands of square meters in area. Most researchers consider them as evidence of prehistoric sedentary fisher-gatherers, heavily specialized on seashore exploitation. Some sambaquis show evidence of multiple uses, including activity areas, shelters and burials. Other sambaquis seem to be exclusively funerary and ritual sites (Gaspar, 1998).

1. Materials and methods

Dental calculi were collected from two non-ceramic and two ceramic sambaquis (Table 1). Morro do Ouro and Forte Marechal Luz are the non-ceramic sites and Enseada 1 and Itacoara are the ceramic sites (Figure 1). The calculi samples from 14 individuals

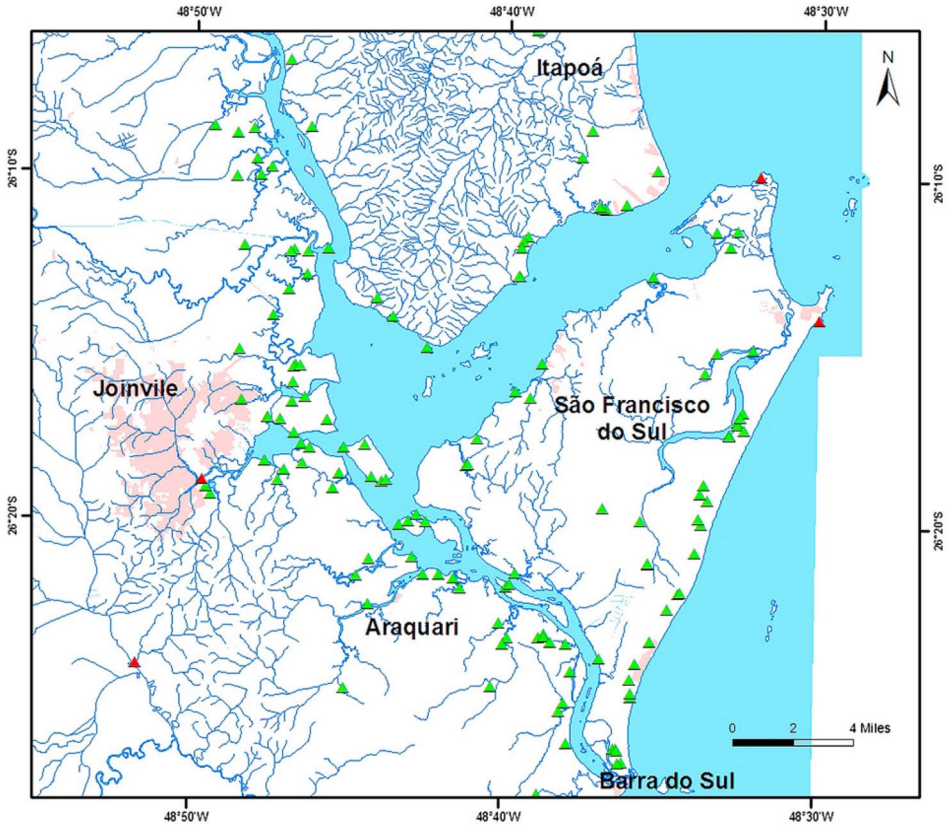

Figure 1. Map of research area. Sambaquis are represented by gray triangles. The sambaquis analyzed for this paper are represented by black triangles.

Table 2. The provenience for each sample is presented below. ULM = upper left molar, URM = upper right molar, LRM = lower right molar, LLM = lower left molar, ULPM = upper left premolar, URPM = upper right premolar, LRPM = lower right premolar, LLPM = lower left premolar, LLC = lower left canine, URC = upper right canine, LRLI = lower right lateral incisor, LRCI = lower right central incisor, LLCI = lower left central incisor. Numbers indicate placement, for example 1ULM = the first upper left molar, 2 ULM = the second upper left molar, etc.

Burial number	Tooth Sampled	Sex	Age
MO 8	1 ULM	♀	18–22
MO 2	3 ULM	♀?	40–44
MO 80	1 LRPM	♂	35–39
MO 60A	3 LRM	♂	45–49
MO 60B	3 URM	♂	40–49
MO 10	2 LLM	♂?	20–30
MO 44B	1 URM	♀	45–49
MO 29	1 ULM	♀	30–34
MO 50	1 LRM	♀	+50
MO 51	1 ULM	♀?	16–18
MO 23	2 URM	♂	35–44
MO 28	LLC	♂	45–49
MO 25	2 URPM	♂?	20–24
MO 22	1 ULM	♂?	30–34
Enseada 1 8529	2 ULM	♀	+55
Enseada 1 8530	1 LLM	♀	+50
Enseada 1 8532	LRLI	♀	35–39
Enseada 1 8636	1 URM	♀	25–29
Enseada 1 8693	LRCI	♀	+50
Enseada 1 8693	1 ULM	♀	+50
Enseada 1 8703	1 LRM	♀	25–29
Enseada 1 8704	2 LRM	♀	35–39
Enseada 1 8533	1 LLM	♂	35–39
Enseada 1 8534	LLC	♂	45–49
Enseada 1 8623	3 LLM	♂	20–24
Enseada 1 8634	3 LRM	♂	35–39
Enseada 1 8634	LRLI	♂	35–39
Enseada 1 8647	1 LLM	♂	40–44
Enseada 1 8647	2 URM	♂	40–44
Enseada 1 8681	2 ULM	♂	40–44
Enseada 1 8681	1 LRPM	♂	40–44
Enseada 1 8638	1 LRM	?	Sub adult
Enseada 1 Sn2	1 ULM	?	Infant
Enseada 1 8680	2 LLPM	?	Infant
Enseada 1 8638	1LRM	?	Infant
FML-SCR 6	1 ULM	♀	15–19
FML-SCR 10	1 ULM	♂	35–44
FML-SCR 10	3 LRM	♂	35–44
FML-SCR 22	3 URM	♂	20–29
FML-SCR 23	LRC	♂	20–34
FML-SCR 23	2 LLM	♂	20–34
FML-SCR 23	3 LLM	♂	20–34
Itacoara 11	1 LLM	♀	20–24
Itacoara 21	2 LRPM	♀	25–29
Itacoara 27	1 LRPM	♀	20–29
Itacoara 13	LRCI/LRLI	♀	30–34
Itacoara 34	1 LRPM	♀	50+
Itacoara 25A	URC	♂	45–49
Itacoara 12	3 LLM	♂	45–49
Itacoara 29	LLCI	♂?	15
Itacoara 26	1 LRM	?	18–20
Itacoara 25B	1 LLM	?	18–20
Itacoara 31	3 LRM	?	25–34

were analyzed for Morro do Ouro, 21 samples from 17 individuals were analyzed for Enseada 1, 7 samples from 4 individuals were analyzed for Forte Marechal Luz, and 11 samples from 11 individuals were analyzed for Itacoara (Table 2). The weights of the samples ranged from less than 0.001 to 0.075 g. Fifty of the 53 dental calculus samples were smaller that 0.05 g, 43 were smaller that 0.02 g, and 19 were smaller that 0.005 g. The largest dental calculi were extracted from Forte Marechal Luz teeth,

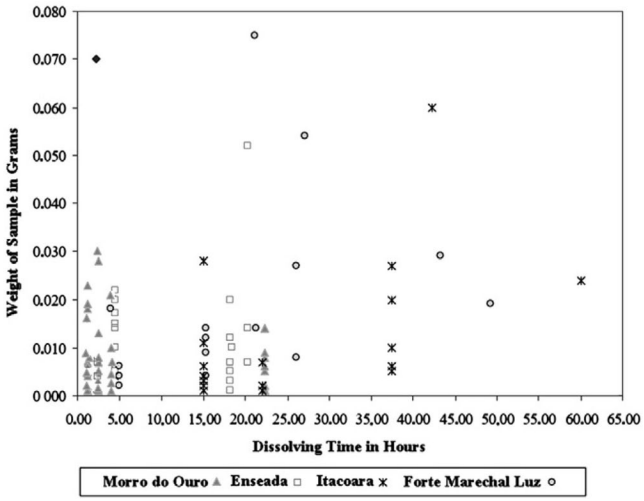

Figure 2. Graph showing wide variation in the time needed for the samples to dissolve, ranging from 1 h and 15 min to 60 h.

with an average calculus size of 0.021 g. The calculi obtained from samples of the other sites had the following average size: Enseada 1 was 0.012 g, Morro do Ouro was 0.011 g and Itacoara was 0.009 g.

In order to investigate the variation in calculus contents in the same mouth we chose some skeletons to analyzed more than one tooth from the same individual. Two teeth were compared from each of Enseada skeletons 8634, 8647, 8681, 8693, and Forte Marechal Luz' skeleton 10. Three teeth were compared from Forte Marechal Luz skeleton 23.

The collections were excavated more than 30 years ago and had been curated since excavation. They are housed at the Museu Arqueológico de Sambaqui de Joinville and at the Museu Nacional do Rio de Janeiro. The excavations did not include the collection of soil control samples.

Before the dental calculi were collected, the teeth were examined for dental pathology. These analyses were done in the laboratories at the institutions housing the remains.

Neither laboratory had ideal conditions for the prevention of microfossil contamination. For this reason, we employed methods to limit contamination with modern microstructures from the laboratory environment. Within each laboratory, a surface was isolated for dental work. This area was separated from the rest of the lab by suspending sheets of plastic to enclose a square work area. This area was cleaned daily with bleach. A synthetic sponge was used to prevent contamination with plant fibers from paper towels. The work area was cleaned after each analysis to prevent contamination between samples. The doors and windows in the labs were kept closed, and at the Museu Arqueológico de Sambaqui de Joinville, window cracks were sealed with adhesive tape. Disposable powder-free latex gloves were used to handle the archaeological material.

The dentitions were examined for dental wear, caries, abscesses, and other pathological processes. After analysis, teeth were selected for dental calculus removal. The teeth and the calculus surface were cleaned with dental brushes. Before use, the brushes were washed in bleach and then in distilled water. To avoid contamination between samples, each brush was disposed of after cleaning a single tooth. By using gentle pressure applied to the junction line of the calculus and tooth with a dental pick, the dental calculus was dislocated. Each calculus fragment was collected within a Petri plate, the bottom of which was covered with a cotton blotter paper disc.

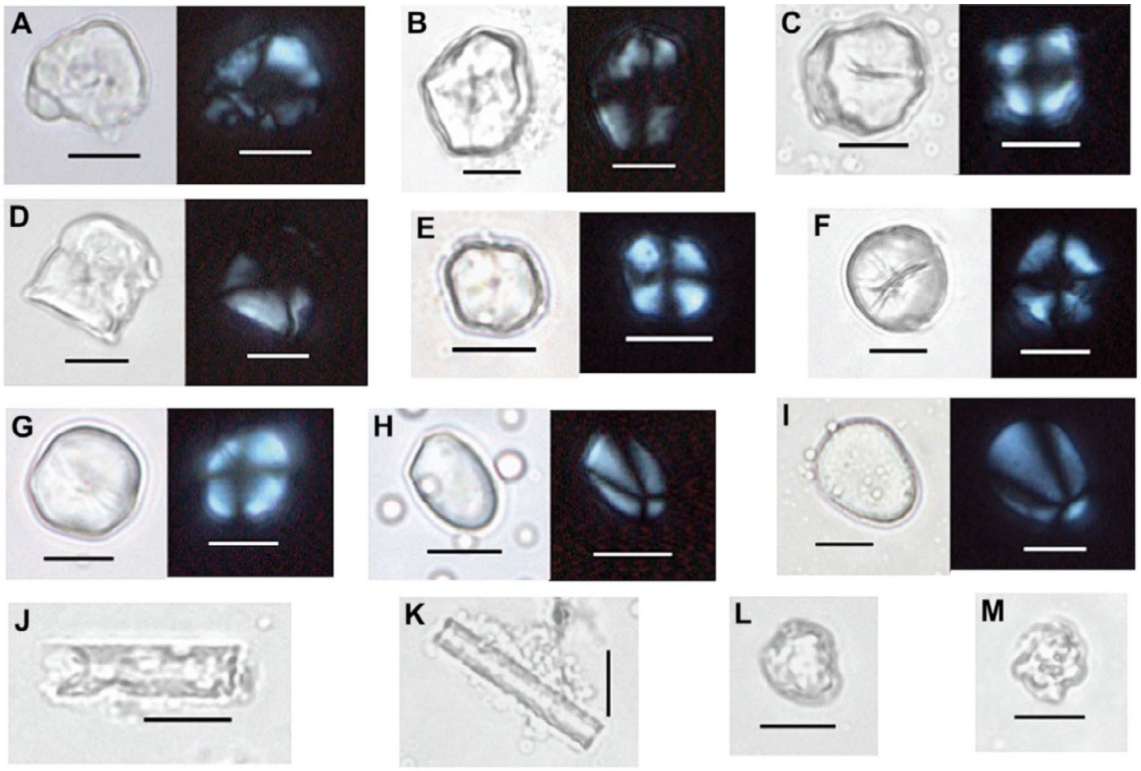

Figure 3. Microfossils from Morro do Ouro area "R". Starch grains are represented in images A–I, and phytoliths are represented in images J–M. The starch grains in images A–D are altered by unknown taphonomic processes. D is unidentifiable, but A–C are possibly *Ipomoea batatas* (sweet potato). E–G are well preserved but can not be definitively identified although G may be Araceae (arum family). H and I are well preserved and are consistent with the genus *Dioscorea* (yam). The phytoliths have not been identified to any specific taxon. The bar scales equal 20 µm. Areas R and A were stratigraphically distinct but contemporaneous.

After removal, each calculus sample was immediately transferred from the filter paper to a microcentrifuge tube. The tube was labeled with an identification number and the filter paper was discarded. Conical, sterile, 1.5 ml micro centrifuge tubes with attached caps were used. The caps provided an efficient and simple means to prevent contamination.

Each calculus sample received a sequential number that was recorded in a laboratory book. Relevant observations such as provenience, color, dental characteristics of the source tooth, the anatomical placement of the source tooth, photograph number, etc. were recorded. Therefore, for each tooth there was a notation of the curatorial institution, name of collection, site name, tomb number, skeleton number, anatomical tooth origin, where on the tooth the calculus formed, pathology observed on the tooth, estimated sex and age at death of the source skeleton, and sample weight. About 1/3 of each sample was saved for future analysis. The remainder was weighed with a scale precise to 3 decimal places. In cases in which the sample weighed less than 0.001 g, the sample weight was recorded as <0.001 g.

The extraction of microfossils from the calculus samples was done in the Microfossil Analysis Laboratory at the School of Geosciences, University of Nebraska – Lincoln. This laboratory was designed to eliminate the potential of contamination. It is a basement lab without windows. It is a positive pressure facility and the air blown into the lab is filtered. Thus, the air entering the lab is free of environmental particles larger than 5 µm. During processing, the lab was used exclusively for dental calculus and no other material was processed. An analysis area within the lab was isolated and treated in the same way as the laboratories in Brazil with the exception that a plastic partition was not made. To limit contamination more, glassware used in this analysis was immersed in 50% hydrogen peroxide for 3 h to dissolve organic microstructures. Then the glassware was cleaned with distilled water. Vinyl powder-free disposable gloves were used to manipulate the material in the lab.

To assess the potential of contamination during chemical processing control samples were prepared. Every eleven dental calculus samples were accompanied by one control. The control sample consisted in one *Lycopodium* tablet (batch 212761) added to a centrifuge tube. All samples, either control or dental calculus, were processed in the same way, and only one site was processed at each time, to avoid cross-contamination.

The centrifuge capabilities of the Microfossil Analysis Facility were for 12, 15, and 50 ml centrifuge tubes. Therefore, we had to adapt a centrifuge for our 1.5 ml micro centrifuge tubes. We found that the micro centrifuge tubes fit well into 12 ml glass tubes. So, for centrifugation we inserted the 1.5 ml tubes into the 12 ml tubes. We chose to keep the samples in the micro centrifuge tube because we found in prior experiments that samples smaller than 1.0 g could result in microfossil loss when 12 ml tubes were decanted. Also, Reinhard et al. (2001) found that 12 ml tubes were inefficient for processing samples less than 1.0 g. The micro centrifuge tubes were opened only long enough to add and decant reagents.

In palynology, exotic spores are added to a sample before processing to allow calculate the concentration of recovered pollen (Maher, 1981; Pearsall, 2000; Reinhard et al., 2006; Warnock and Reinhard, 1992). Reinhard et al. (2001) proposed that it is possible to estimate the quantities of microfossils in dental calculus by using the same method long used by palynologists. So we added one *Lycopodium* tablet to each micro centrifuge tube containing a dental calculus sample.

We added up to 1.5 ml 10% hydrochloric acid to each tube and the tubes were observed until it was apparent that the cal-

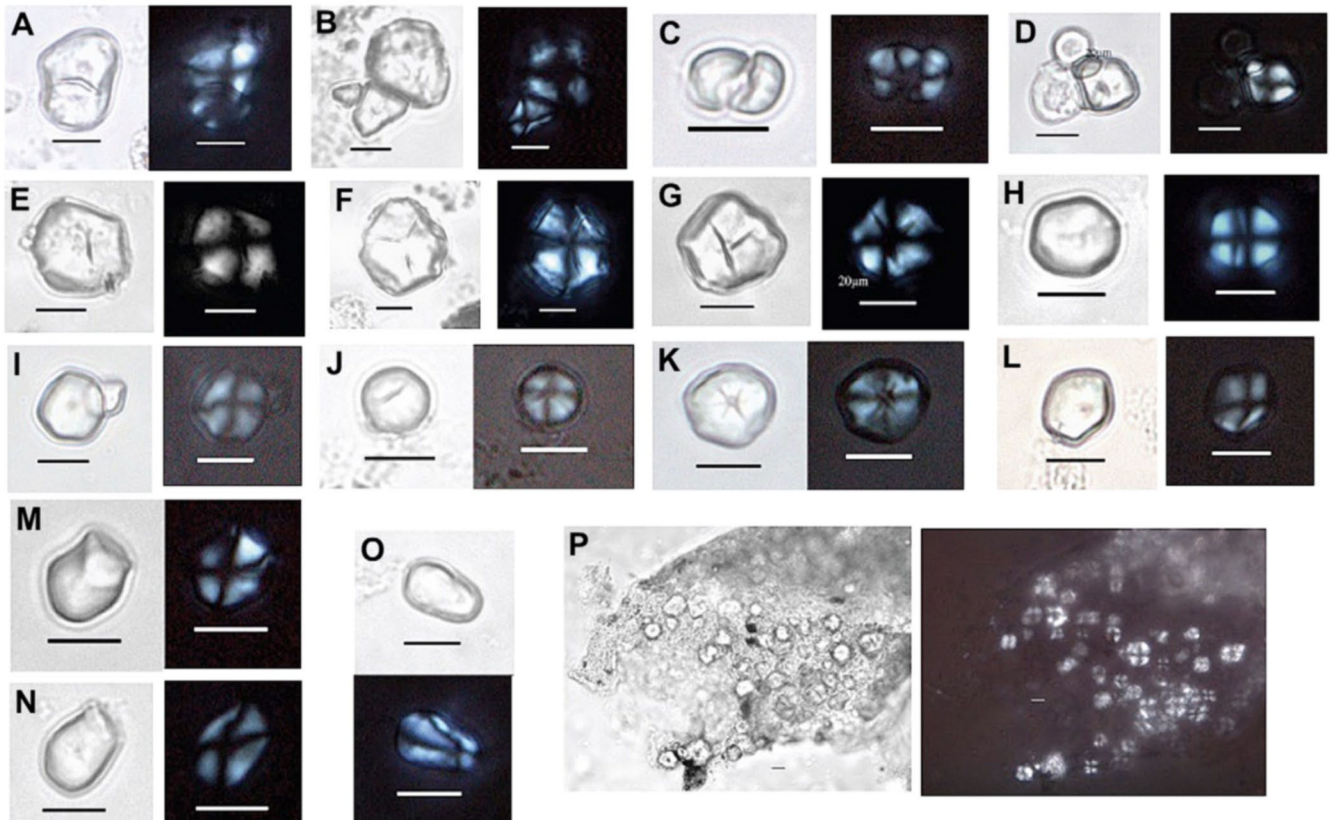

Figure 4. Starch grains recovered from Morro do Ouro area "A". A–D illustrate aggregates of altered starch that can not be identified to taxon. In this view, 4D may not appear as an aggregate, but its aggregate nature is clear when rotated in other planes. E–G represent a type of faceted starch with central hila consistent with *Ipomoea batatas* (sweet potato). H–L represent a type of globular, faceted starch grains with hila that resemble dark points. These are consistent with Araceae (arum family). M–N are represent an elongate, faceted starch grains that can not yet be identified to a specific taxon. O is consistent with the genus *Dioscorea* (yam). P is consistent with *Ipomoea batatas* (sweet potato) that are retained in plant tissue. The bar scales equal 20 µm. Areas R and A were stratigraphically distinct but contemporaneous.

culus was completely dissolved. When it was no longer possible to see individual fragments of calculus, and when gas no longer appeared in the tubes, we judged that the calculus had dissolved satisfactorily. The time necessary to dissolve each sample was noted. After the calculus was dissolved, the tubes were centrifuged in a Dynac II centrifuge at 1000 RPM for 5 min and the supernatant was pipetted off of the solid plug. The samples were then washed in water as follows. Up to 1.5 ml of distilled water was added to each tube until each tube was full of fluid. The plug was homogenized with the water with a Genie vortex mixer and the samples were centrifuged as above. This process of water wash was repeated twice.

After the final water wash, 1.5 ml of 100% ethanol was added to each tube to dehydrate the samples. The samples were homogenized and centrifuged as above with the exception that the centrifugation lasted 8 min. The dehydration process was repeated twice. After this process, 100% ethanol was added to each sample until the total liquid volume inside each tube reach 0.5 ml.

To assess loss of microfossils in the process of decanting, all acid, water, and alcohol from the processing of each sample was saved in a glass, conical base, 12 ml centrifuge tube. These were centrifuged at 1000 RPM for 10 min. After centrifugation, the tubes were inspected for any evidence of solid deposits in the base of the tubes. Then the fluid was examined with a Jenaval compound microscope at 250 and 400 power magnifications looking for microfossils. The fluid from the 14 control samples was examined completely for microfossils and ambient microstructures.

The microscopic examination of the calculus samples was done first in the Microfossil Processing Laboratory, University of Nebraska – Lincoln and then at the Laboratório de Anatomia Vegetal, Instituto de Biociências, Universidade de São Paulo. Both laboratories are equipped with research-grade Jenaval and Leica microscopes. The preliminary examination was done in Nebraska and final examination in São Paulo. For preliminary examination, one microscope slide was prepared from each dehydrated calculus sample. The materials used for mounting the calculus samples included glycerine (refraction index 1.475), heat activated Gargille Meltmount™ (refraction index 1.539), Gold seal microscope slides, and Gold Seal cover slips, 24 × 32 µm. The microscope slides and cover slips were pre-cleaned and sterilized. For pipetting, an automatic pipette set for 10 µl with low-retention disposable tips was used.

For each sample, a microscope slide was placed on a hot plate at approximately 50 °C and a thin, open rectangle of Meltmount was drawn around the center of the slide within the dimensions of a cover slip. The slide was removed from the hot plate until the Meltmount was solid. The calculus sample was homogenized and 10 µl of calculus sample was placed in the area within the Meltmount rectangle. When the ethanol was almost completely evaporated, a drop of glycerin was stirred into the sample and mixed with the microfossils. The slide was returned to the hot plate and the Meltmount became viscous instantly. Using a plastic spatulette, a cover slip was placed on one corner of the Meltmount rectangle. Gentle pressure was applied to the cover slip gradually from one corner to the entire slip to flatten the Meltmount seal, and glycerin–microfossil mixture. The slide was removed from the hot plate and the Meltmount hardened.

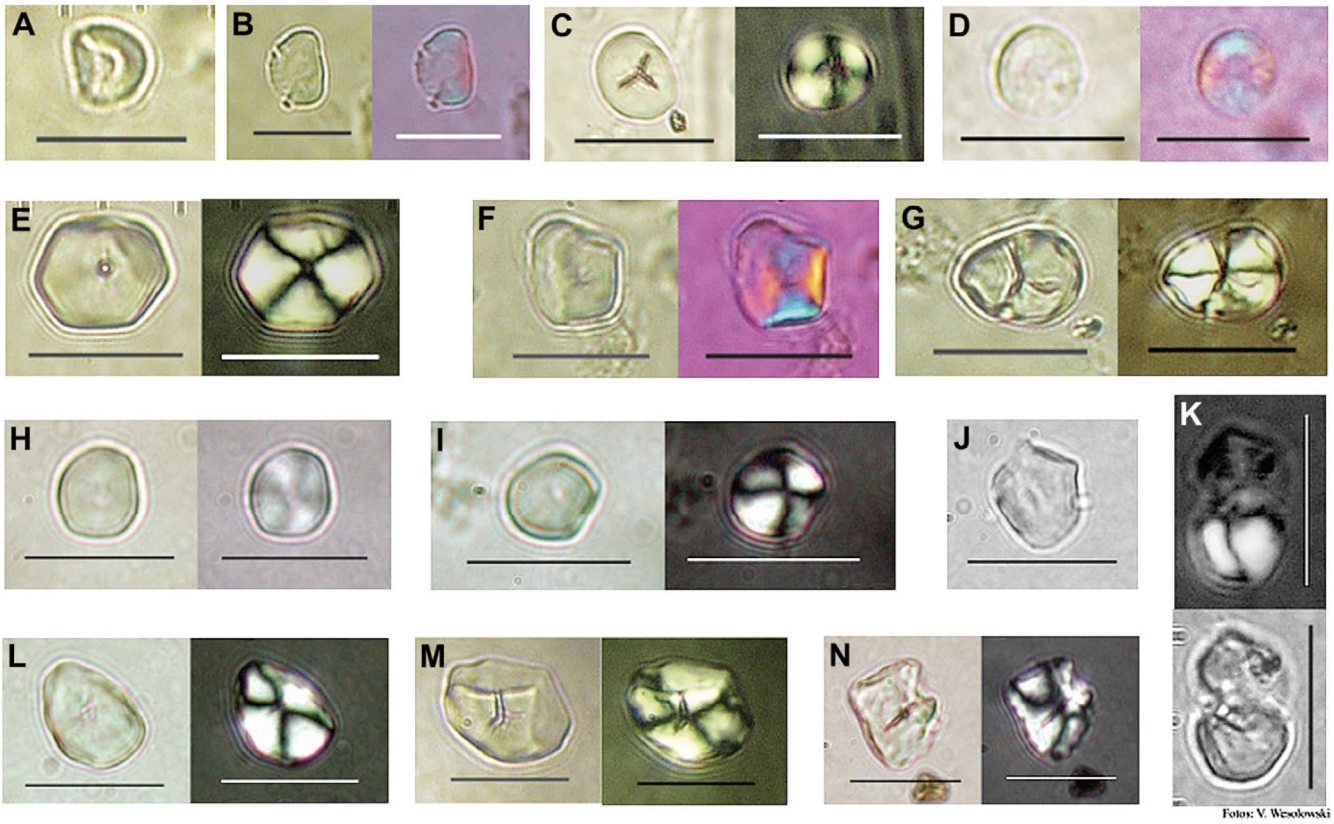

Figure 5. Microfossils from Enseada I. B, D, and G are altered starch that cannot be identified. E is well preserved but cannot be identified. A and F are consistent with *Ipomoea batatas* (sweet potato). C is altered *Zea mays* (maize). H, I, and K are similar to the Araceae genus, *Alocasia*. J, M, and N are suggestive of *Araucaria angustifolia* (Paraná pine). L is very well preserved but does compare well with reference samples. The bar scales equal 25 µm.

Even though the time of exposure of micro residues to heat was very brief, less than 15 s, we were concerned that the heat might alter the starch. Therefore, we tested our method of mounting microfossils with modern starch using the same methods. Starch was examined and photographed before and after mounting. No degeneration of the starch was observed.

All slides were examined with optical compound microscopes with polarized light at 400× magnification using lambda interference filters. To examine the three dimensional morphology of the microfossils, gentle pressure was applied to the cover slip to turn the microfossils in the glycerin. Examination of the microscope slide commenced at the upper left corner of the cover slip. The slide was scanned to the right upper corner. Then the slide was moved 1.5 fields below and scanned to the left. This was repeated until the lower right corner of the slide was reached. All encountered microfossils and *Lycopodium* spores were counted and recorded. Microfossils included phytoliths, starch granules, charcoal, and mineral fragments. The condition of each microfossil was recorded. For starch, relevant observations were made regarding hylum shape, granule shape, partial loss of three dimensional aspects, and decreased birefringent properties. To calculate the numbers of microfossils, the method of Maher (1981) was adapted using this formula.

$$\text{Concentration} = ((m/l) \times a)/w$$

m = microfossils counted
l = marker *Lycopodium* sp. spores counted
a = marker *Lycopodium* sp. spores added
w = weight of calculus

In those cases where the calculus weight was less than 0.001 g, we arbitrarily used the weight 0.0009 in calculating concentration values. Although we know that concentration for these samples could be underestimated this was the only way to make the calculations possible.

A comparative collection of starch and phytoliths was used to identify some of the microfossils. The collection was derived from modern plant samples and from archaeological plant samples.

2. Results

There was a wide variation in the time needed for the samples to dissolve, ranging from 1 h and 15 min to 60 h (Figure 2). This time variation was not related to weight.

We were concerned that increased time in acid affected the morphology of the starch granules. Control studies with modern starch were undertaken to assess possible damage to starch with increased time in acid. Manioc (*Manihot esculenta*), sweet potato (*I. batatas*), yam (*Dioscorea* species) and araucaria (*A. angustifolia*) were selected for analysis. Starch samples were scraped from the source plant and put directly in 10% HCl for 48 h at room temperature. There was no clear change in form of sweet potato (*I. batatas*), yam (*Dioscorea species*) and araucaria (*A. angustifolia*). There was a slight change in manioc but these changes would not hinder identification of starch. We also cooked araucaria pine seed for 1 h 30 min at 85 °C. The cooked araucaria starch exhibited minor change in birefringence and morphology, but it was still identifiable.

At the same time we found that the time of processing dental calculus samples was not correlated to the numbers of starch grain alterations. According to the Spearman correlation coefficient there is no statistically significant relation between time in acid and number of degraded starch granules.

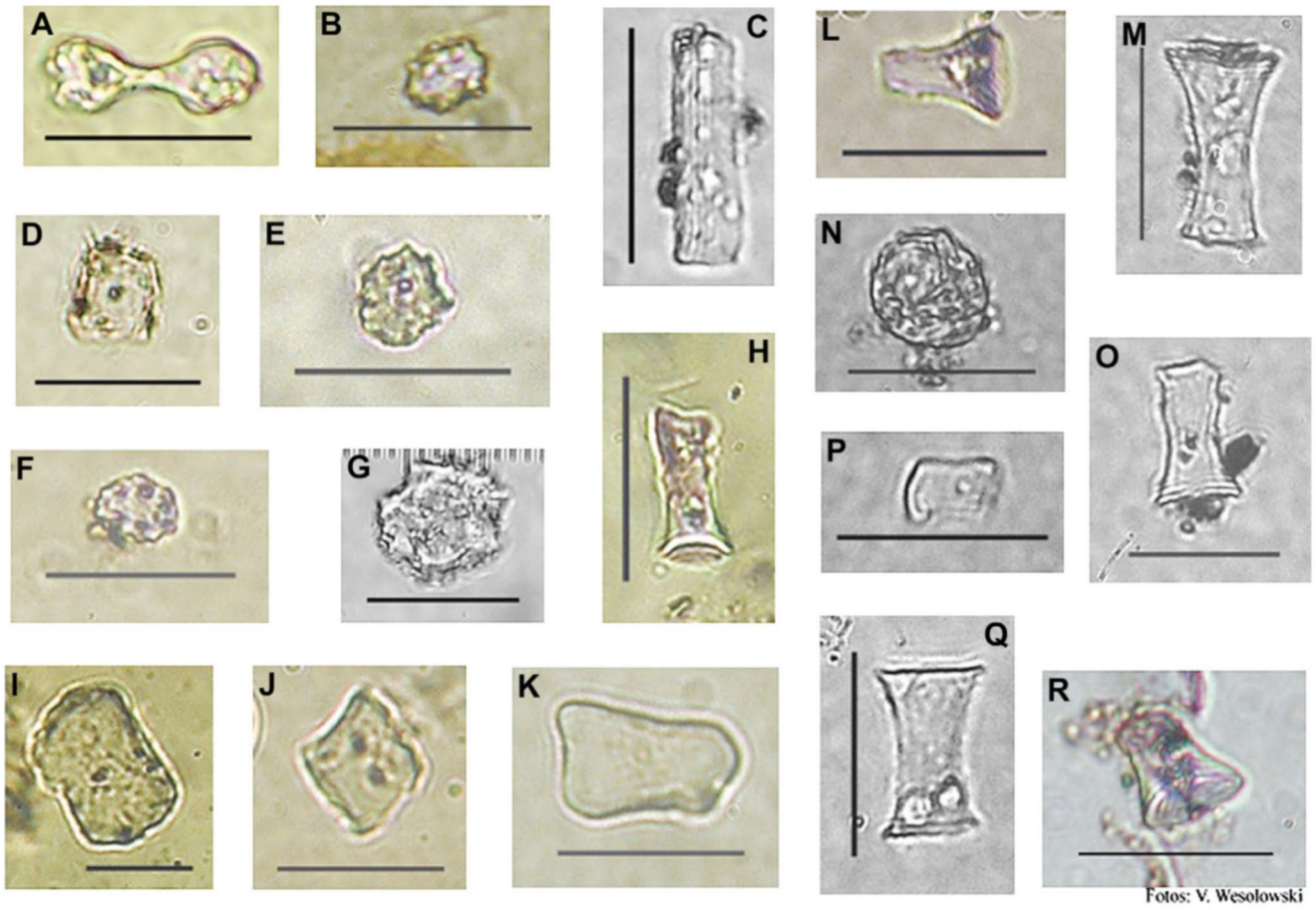

Figure 6. Phytoliths from Enseada I. A is a bilobed structure consistent with Panicoudeae and Bambusoidea. C–D and I–K are unidentified. B is consistent with Bromeliaceae. F is similar to species in the genus *Geonoma*. H, L–M, and O–R are Poaceae. G is consistent with Marantaceae. N is similar to two taxa, the Boraginaceae genus Heliotropium and also *Araucaria angustifolia* (Paraná pine). E is consistent with *Araucaria angustifolia*. The bar scales equal 25 μm.

No evidence of contamination during the processing of calculi was found in the 14 control samples or in the decanted supernatants. Microscopic particles specific to the dental calculus samples included starch granules and phytoliths, but mineral particles and dark opaque structures that are probably charcoal fragments were also found. These four types of particles were found in all sites, but not necessarily in every calculus sample.

Starch granules were present in all 53 calculi (Figure 3; Figure 4; Figure 5; Figure 6; Figure 7; Figure 8) and 39 samples contained phytoliths. Fragmented plant epidermis was seldom observed. Table 3 presents the direct counts of microfossils found in the dental calculi, Table 4 presents the concentration values derived from the direct counts.

The majority of the starch granules exhibited morphological alterations. These alterations included breakage of the hylum, deformation of shape, partial loss of three dimensional shape, and decreased birefringent properties. These observations are consistent with partial gelatinization of the starch (Torrence and Barton, 2006). The phytoliths are consistent with types in the Poaceae, (grass family) and *A. angustifolia* (Paraná pine). Some phytoliths are fragmented, others are intact. *A. angustifolia* phytoliths and starch were sometimes found in the same samples.

The numbers of samples positive for each type of microfossil are presented in Table 5. The Forte Marechal Luz sample size was very small and is excluded from comparison to the other, larger samples. Starch was found in all individuals from all sites. The taxa discovered are listed in Table 6. The starch was represented by *Dioscorea* (yam), *A. angustifolia* (Paraná pine), and probably Araceae (arum family), *I. batatas* (sweet potato), and *Z. mays* (maize).

Interesting variation was found in altered starch abundance (Table 7). Starch alteration could be due to cultural practices such as cooking. It is less likely that diagenesis affected the starch (Hardy et al., 2009). Morro do Ouro had the highest frequency of altered starch followed by Itacoara and Enseada 1. The reverse is true regarding phytolith abundance. As graphed in Figure 9, there is an inverse relationship between the abundance of phytoliths and altered starch, when the total sample of each site is considered.

Our analysis of different teeth from the same individuals suggests microfossil variation between different dental calculi in the same mouth can occur. The plant microfossil concentration data from different teeth is presented in Table 8. The results, according to the Mann–Whitney Test, show no significant difference in the number of micro residues, when two or more calculi of the same individual are compared. However, examination of the variance of plant microfossils shows apparent differences. Future work must focus on this problem by analysis of larger samples.

In the sites as a whole, there was a linear relation between concentrations of microfossils and the weight of the calculus samples (Figure 10), pointing to an inverse relation between weight and starch and phytolith concentration values. This difference was statistically significant as demonstrated by the Pearson and Spearman correlation statistics. For the other two variables of probably charcoal and mineral fragments, only the Pearson

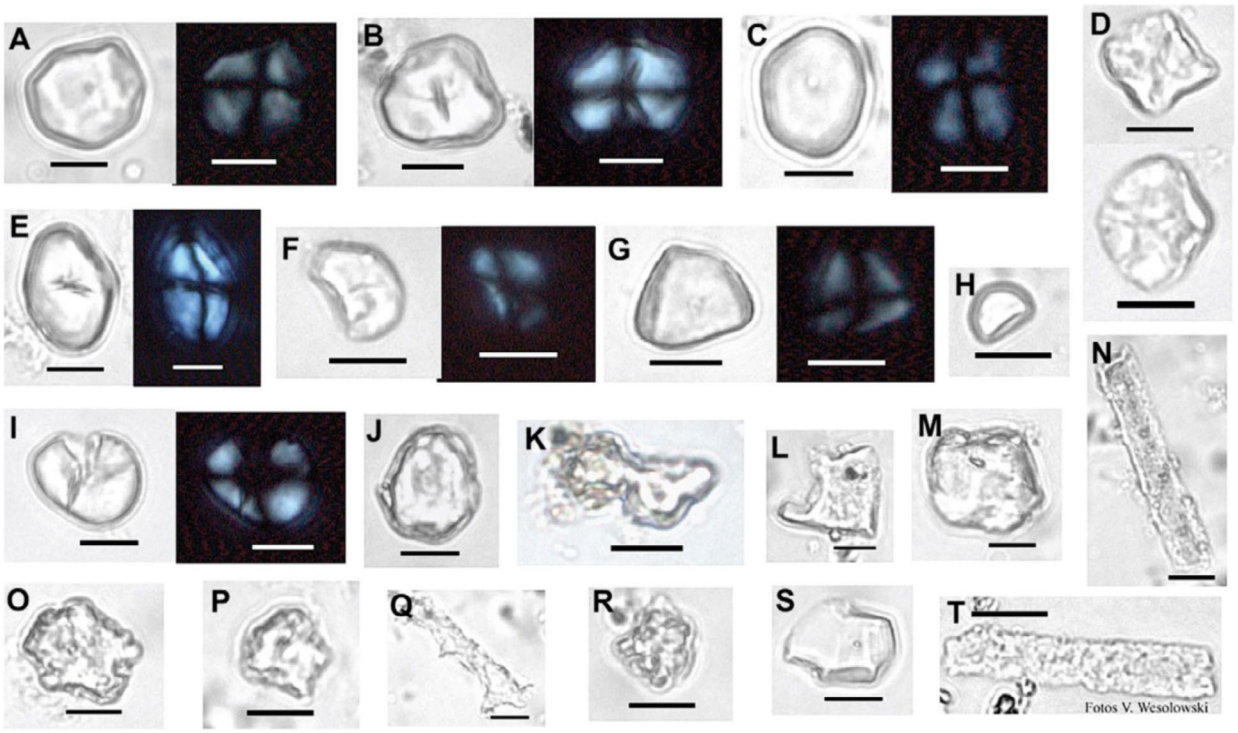

Figure 7. Microfossils from Forte Marechal Luz. A–I are starch grains and K–T are phytoliths. A is consistent with Araceae (arum family). B is consistent with *Ipomoea batatas* (sweet potato). C is a rounded, globular starch with a central hilum that is possibly in the Araceae, genus *Alocasia*. D and F are altered starches consistent with *Araucaria angustifolia* (Paraná pine). E and G are well preserved but do not match our reference material. H–J are altered starches that cannot be identified. K is a cruciform quadrilobate phytolith consistent with Panicoideae. L–T illustrate a variety of phytolith forms that we could not identify. The bar scales equal 20 µm.

index demonstrated statistical significance. In summary, larger calculus samples had lower microfossil concentrations.

3. Discussion

Some bioarchaeologists assume that calculus forms relatively uniformly in all people who did not have modern dental hygiene practices. However, dental calculus formation is actually quite variable and complicated. Various systemic factors, including disease, affect the amount of deposition as well as the velocity of deposition of dental calculus (Beiswanger et al., 1989, Lieverse, 1999, Poff et al., 1997, Epstein et al., 1980, Mandel, 1995, Scheie, 1994, Shasha et al., 1983, Eigner et al., 1986 and Fujimaki et al., 1998). Levels and location of calculi are population-specific and are influenced by ora1l hygiene habits, diet, age, ethnicity, and use of teeth as tools (White, 1997). In this regard, some prehistoric habits that might have limited calculus formation include quid-chewing, abrasive diets, and preparing fibers for cultural uses using the teeth.

The speed of calculus deposition is also affected by diet. Protein-rich diets increase the urea in saliva and accelerate the mineralization of bacterial plaque (Kleinberg et al., 1981 and Sissons et al., 1988), at the same time the putrefaction of meat residues attached to the teeth can also accelerate dental calculus formation by increasing urea in the mouth (Jin and Yip, 2002). Thus, even for one individual, the rate of dental calculus may change over a year period based on seasonal access to resources.

Our find that the size of dental calculus varied between sites, and that the largest dental calculi had the lowest concentrations of microfossils suggests that some individuals had relatively rapid precipitation of dental calculus. Apparently in these individuals the relative amount of microfossils was dwarfed by the increased accumulation of saliva minerals. This is in accord to clinical studies that relate higher meat diets to rapid precipitation of calculus. This could potentially complicate comparison across sites because diet and oral physiology seems to affect not just the rate of dental calculus formation but also the concentration of microfossils in calculi.

Variation in dental calculus starch content may be related to the genetic inheritance of the numbers of genes coding for the salivary amylase gene (AMY1). Perry et al. (2007) found that individuals from populations with high-starch diets have more AMY1 copies than those with traditionally low-starch diets. They assert that starch consumption is a main part of diet in agricultural societies and arid environment hunter-gatherers. They suggest that diet is a selective pressure for increase in inherited numbers of the AMY1 gene. Future work should explore the relation of sambaqui diet over time to investigate whether or not starch consumption increased from early sites to later sites and whether more efficient digestion of oral starch relates to microfossil content in calculi.

Although the differences found in dental calculi weight are not statistically significant, we believe that the large Forte Marechal Luz dental calculi make them incomparable to the other three sites with similar calculi sizes. Indeed, Forte Marechal Luz exhibits the lowest concentrations of starch and phytoliths relative to the other sites. The larger calculi at Forte Marechal Luz suggest that the diet in this sambaqui had a higher meat intake compared to the others. According to Bryan and Gruhn (1993) the zooarchaeological records point to considerable amounts of faunal remains, with emphasis in shark meat which is rich in urea.

Both Forte Marechal Luz and Enseada 1 could be used seasonally, being partially abandoned at winter. This is suggested by zooarchaeological analysis (Bandeira, 1992). Only at these sites did we found starch grains from A. angustifolia (Brazilian Pine). The Brazilian pine forests are located some kilometers from seashore, in the highlands of southern states. This is a natural resource available at the end of fall and during in the winter.

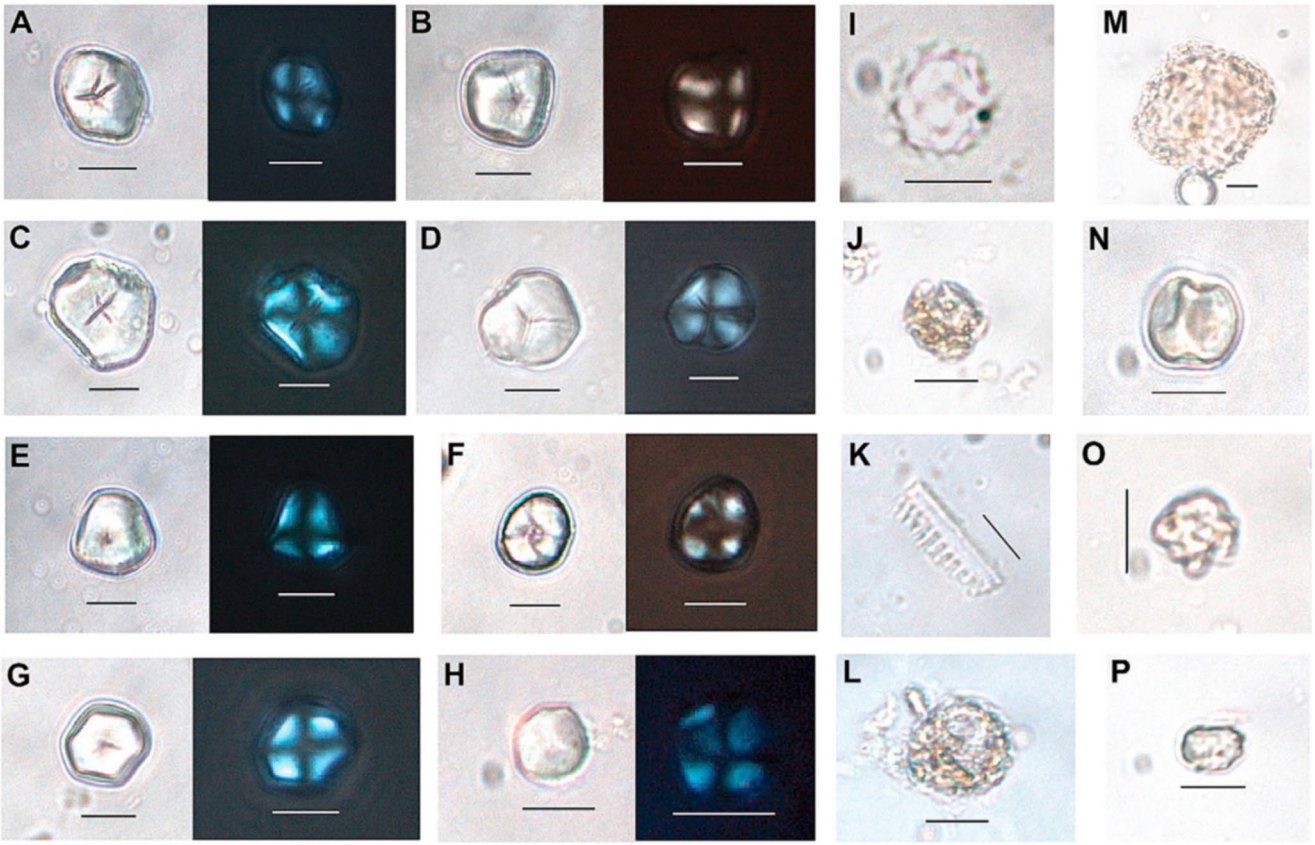

Figure 8. Microfossils from Itacoara. A–H are starch grains and I–P are phytoliths. A is consistent with *Araucaria angustifolia* (Paraná pine). B is consistent with *Ipomoea batatas* (sweet potato). C–E are well preserved grains with unspecific morphology. F is an altered, unidentifiable grain. G is consistent with *Zea mays* (maize). H is consistent with Araceae (arum family). The phytolith in image I is consistent with the Arecaceae (palm family). J, L, N, and P were not identified. K and O are similar to two taxa, the Boraginaceae genus *Heliotropium* and also *Araucaria angustifolia* (Paraná pine). M is a long cell Poaceae phytolith. The bar scales equal 20 μm.

There is a question of how many months or years of diet are represented in a given dental calculus deposit. On this question, Henry and Pipperno (2008, P. 1944) write, "Few empirical data are available with regard to the rate of calculus formation on teeth and how much of an individual's lifespan evident calculus represents. Nevertheless, because calculus does accumulate over an individual's life if not removed, we may surmise that at least several years diet is represented in every individual studied. Additional research is needed on this point." Qualitatively, we observed broken and worn areas in the biggest dental calculi as well as the spontaneous loosening of some of calculi in the sambaqui human remains. This suggests that, as in present people, relatively stable dental calculus may be modified by breakage. Parts are lost and new precipitation occurs.

These observations are supported by the dental literature. The plaque may be formed and removed, depending on the daily diet. Subsequent crystal formation in the calculi follows sequential mineral phases (hidroxiapatite, brushite, whitlockite, octacalcium phosphate). Calculi only become stable and resistant one year after its initial formation when the original plaque was deposited. According to Abraham et al. (2005), the mineralization process and the subsequent crystal-growing process are affected by chemical and thermodynamic factors. This makes the application of anti-calculus agents in clinical treatment more effective prior to the sixth month of maturation before the more stable phase of hydroxyapatite is reached. The implication of this is that there is fluctuation in calculus formation and also in its fixation as a permanent oral deposit.

Therefore, dental calculi may express variation between teeth in the same mouth, as suggested by our analysis of multiple teeth from the same individuals. As can be seen in the tabulated data, none of the six individuals studied for multiple teeth exhibit consistency in the concentrations of starch and phytoliths. We suggest that this variation reflects different periods of microfossil accumulation before death or the active reworking of dental calculi as fragments erode or break off and newer precipitation occurs. We echo Henry and Piperno's (2008) call for more research on the mechanisms of dental calculus accumulation.

Although we acknowledge that phytoliths are damaged and even dissolved in very high pH aqueous solutions (Fraysse et al., 2006 and Fraysse et al., 2009), it is unlikely that the relationship shown in Figure 9 is due to environmental conditions such as pH. Phytoliths are embedded in dental calculus as shown by ESEM studies presented by Reinhard et al. (2001). Because of this, phytoliths in dental calculus are insulated from water that might percolate through sambaquis. Villagrán (2008) addresses the pH and microfossil preservation potential of terra preta (black earth) levels in the Jabuticabeira II shell-mound. She finds that the maximum pH for these levels are 7.5. She conducted experiments to define the pH threshold at which opaline silica dissolves in aqueous solutions. She found that opaline silica dissolves between pH levels of 9 and 13. Therefore, it is not likely that the alkalinity of sambaquis would damage phytoliths within dental calculus.

This study shows that it is possible to recover dental calculus from museum specimens without contamination. In our experience in Brazilian laboratories, the main sources of contamina-

Table 3. Direct counts of microfossils observed per calculus sample.

Burial	Weight	*Lycopodium*	Starch	Starch altered	Phytolith	Sand	Charcoal
MO-REC 8	0.0009	1650	7	2	0	2	1
MO-REC	0.010	1042	7	6	1	26	19
MO-REC 80	0.0009	1023	6	0	2	21	50
MO-REC 60A	0.0009	1281	6	1	1	0	1
MO-REC 60B	0.001	515	1	0	0	0	1
MO-REC 10	0.070	890	10	2	4	2	2
MO-ANT 44B	0.008	945	6	4	0	0	3
MO-ANT 29	0.009	1786	15	9	0	0	0
MO-ANT 50	0.028	1743	127	6	0	3	5
MO-ANT 51	0.013	1367	13	3	3	0	9
MO-ANT 23	0.002	573	7	5	1	1	1
MO-ANT 28	0.001	768	13	8	0	6	9
MO-ANT 25	0.0009	1316	20	6	1	0	4
MO-ANT 22	0.009	983	7	4	0	4	8
Enseada 1 8529	0.022	1076	5	0	10	24	0
Enseada 1 8530	0.014	1040	4	0	2	0	0
Enseada 1 8532	0.010	1689	14	0	7	21	123
Enseada 1 8636	0.007	875	4	0	0	0	0
Enseada 1 8693	0.003	1575	5	2	1	0	0
Enseada 1 8693	0.003	1582	10	2	23	18	0
Enseada 1 8703	0.015	714	4	0	2	8	0
Enseada 1 8704	0.005	620	7	0	1	1	0
Enseada 1 8533	0.007	712	10	1	3	0	0
Enseada 1 8534	0.052	1293	13	3	0	0	0
Enseada 1 8623	0.014	804	8	0	2	0	0
Enseada 1 8634	0.012	1492	1	0	8	4	0
Enseada 1 8634	0.020	592	4	0	0	0	0
Enseada 1 8647	0.004	1895	7	0	10	0	0
Enseada 1 8647	0.017	1011	2	0	10	2	0
Enseada 1 8681	0.010	1431	11	4	2	0	0
Enseada 1 8681	0.020	1969	10	1	1	2	0
Enseada 1 8638	0.007	2319	21	10	3	0	0
Enseada 1 Sn2	0.0009	1403	12	5	4	3	0
Enseada 1 8680	0.001	1221	6	1	3	1	0
Enseada 1 8638	0.005	894	10	4	3	0	0
FML-SCR 6	0.004	999	3	1	4	27	24
FML-SCR 10	0.029	1085	12	4	6	12	23
FML-SCR 10	0.075	1208	6	3	18	156	251
FML-SCR 22	0.009	1089	8	3	4	4	10
FML-SCR 23	0.014	1610	8	4	0	5	55
FML-SCR 23	0.004	1305	5	4	0	5	17
FML-SCR 23	0.012	846	3	2	2	8	15
Itacoara 11	0.0009	1305	11	2	1	7	10
Itacoara 21	0.003	868	3	1	0	0	2
Itacoara 27	0.010	1490	11	2	1	5	35
Itacoara 13	0.005	1536	12	5	18	11	9
Itacoara 34	0.010	1662	6	2	14	24	8
Itacoara 25A	0.024	1013	6	0	13	7	8
Itacoara 12	0.004	1372	11	6	0	7	3
Itacoara 29	0.010	1570	3	0	1	2	0
Itacoara 26	0.0009	865	4	3	4	1	10
Itacoara 25B	0.003	1332	21	2	1	9	10
Itacoara 31	0.028	1529	9	0	3	6	40

tion are fibers from paper towels and airborne pollen. Contamination can be prevented by establishing simple protocols of enclosing the analysis area in plastic sheeting, cleaning tables' surfaces, brushing tooth surfaces, and sealing lab windows and doors. The use of laboratory control samples provides a method to demonstrate successful control of contamination. For dental calculus analysis, we recommend that laboratory workers maintain an obsessive attitude towards cleanliness.

Dental calculus does not always dissolve rapidly in hydrochloric acid. We demonstrate that starch granules in slowly dissolving samples do not exhibit more alteration than granules in rapidly dissolving samples. We recommend that if calculus is dissolving slowly it is simply sufficient to leave the calculus in the acid for a longer period. It is not necessary, and perhaps inadvisable, to increase the concentration of acid. We note that in this study, we encountered silica phytoliths. In areas where calcium oxalate phytoliths are common in prehistoric foods, the analyst must be very careful in controlling the time and concentration of acid treatment.

It is implicit in the work of Reinhard et al. (2001) that large calculi samples, 0.1 g or larger, are best for analysis. Our work reduces the potential sample size to less than 0.001 g. It is noteworthy that we found that large calculi samples have the lowest concentrations of microfossils. Therefore, we advocate that the analysts simply use smaller centrifuge tubes such as the 1.5 ml conical tubes with attached caps used in this study.

In contrast to Reinhard et al. (2001) and Fox et al. (1996), we find that starch granules are the most common microfossil type encountered in dental calculus. We recommend that the dental calculus analysts be prepared for starch analysis by preparing starch reference collections and by using compound microscopes with polarized light capabilities to view interference crosses in starch granules.

Table 4. Calculated concentration values for each calculus sample.

Burial	% Starch Altered	Starch altered	Starch	Phytolith	Sand	Charcoal
MO-REC 8	28.57	16820	58871	0	16820	8410
MO-REC 2	85.71	7191	8390	8390	31163	22773
MO-REC 80	0.00	0	81388	27129	284858	678234
MO-REC 60A	16.67	10833	64996	10833	0	10833
MO-REC 60B	0.00	0	24250	0	0	24250
MO-REC 10	20.00	2005	401	802	401	401
MO-ANT 44B	66.67	6608	9912	0	0	4956
MO-ANT 29	60.00	6993	11655	0	0	0
MO-ANT 50	4.72	1535	32499	0	768	1280
MO-ANT 51	23.08	2108	9136	2108	0	6325
MO-ANT 23	71.43	54490	76285	10898	10898	10898
MO-ANT 28	61.54	130094	211402	0	97570	146355
MO-ANT 25	30.00	63267	210892	10545	0	42178
MO-ANT 22	57.14	5647	9882	0	5647	11293
Enseada 1 8529	0	0	2110	5276	12662	0
Enseada 1 8530	0	0	3431	1716	0	0
Enseada 1 8532	0	0	10352	5176	15528	90950
Enseada 1 8636	0	0	8156	0	0	0
Enseada 1 8693	40.00	5286	13216	2643	0	0
Enseada 1 8693	20.00	5263	26315	60524	47367	0
Enseada 1 8703	0	0	4664	2332	9329	0
Enseada 1 8704	0	0	28201	4029	4029	0
Enseada 1 8533	10.00	2506	25058	7517	0	0
Enseada 1 8534	23.08	557	2415	0	0	0
Enseada 1 8623	0	0	8876	2219	0	0
Enseada 1 8634	0	0	698	5580	2790	0
Enseada 1 8634	0	0	4219	0	0	0
Enseada 1 8647	0	0	11533	16476	0	0
Enseada 1 8647	0	0	1453	7267	1453	0
Enseada 1 8681	36.36	3491	9600	1745	0	0
Enseada 1 8681	10.00	317	3171	317	634	0
Enseada 1 8638	47.62	7694	16157	2308	0	0
Enseada 1 Sn2	41.67	49454	118689	39563	29672	0
Enseada 1 8680	16.67	10229	61371	30686	10229	0
Enseada 1 8638	40.00	11176	27940	8382	0	0
FML-SCR 6	33.33	3125	9376	12502	84385	75009
FML-SCR 10	33.33	1588	4763	2382	4763	9129
FML-SCR 10	50.00	414	827	2481	21504	34600
FML-SCR 22	37.50	3823	10194	5097	5097	12743
FML-SCR 23	50.00	2216	4433	0	2770	30474
FML-SCR 23	80.00	9570	11963	0	11963	40673
FML-SCR 23	66.67	2460	3691	2460	9842	18453
Itacoara 11	18.18	21267	116968	10633	74434	106335
Itacoara 21	33.33	4796	14388	0	0	9592
Itacoara 27	18.18	1676	9220	838	4191	29337
Itacoara 13	41.67	8131	19514	29271	17888	14636
Itacoara 34	33.33	1503	4509	10520	18035	6012
Itacoara 25A	0	0	3082	6678	3596	4110
Itacoara 12	54.55	13654	25033	0	15930	6827
Itacoara 29	0	0	2386	795	1591	0
Itacoara 26	75.00	46514	62019	62019	15505	155047
Itacoara 25B	9.52	6251	65633	3125	28128	31254
Itacoara 31	0.00	0	2625	875	1750	11669

Charcoal was quantified because our initial thought was that this class of residue related to cooking. However, we also considered that the charcoal could be contamination from the sambaqui sediment matrix. The three sites that had the greatest charcoal concentrations in calculi also had hearths associated with the burials. Many charcoal layers were encountered in the sites of Itacoara (Bandeira, 2004Tiburtius et al., 1951), Morro do Ouro (Beck et al., 1969, Tiburtius, 1996 and Goulart, 1980) and Forte Marechal Luz (Bryan and Gruhn, 1993). The only site where hearths were not associated with burials was Enseada I (Beck, 1974, Bandeira, 1992 and Tiburtius, 1996), and calculi of that site also had minimal charcoal micro residues. Thus, it is possible that contaminant charcoal adhered to the porous surface of the calculi. We found that it is very difficult to eliminate contaminant ash by washing and brushing tooth surfaces. We suggest the future research includes efforts to develop more effective cleaning methods.

Table 5. % of burials positive for microfossils. "*n*" refers to numbers of skeletons analyzed.

Site	Starch	Altered Starch	Phytoliths	Sand	Charcoal
MO *n* = 14	100	86	50	57	93
Itacoara *n* = 11	100	73	82	91	91
Enseada I *n* = 17	100	47	94	59	6
FML *n* = 4	100	100	75	100	100

Table 6. Plant starch and phytoliths identified per site.

Site	*Dioscorea*	Araceae	*Ipomoea batatas*	*Araucaria angustifolia*	*Zea mays*
MO	Yes	Yes	Yes	No	No
Itacoara	No	Yes	No	Yes	Yes
Enseada	No	Yes	Yes	Yes	Yes
FML	No	Yes	No	Yes	No

Table 7. Summary of altered starch.

Sites	% Starch Altered	Starch altered	Starch total
Enseada	14	4570	18458
Itacoara	26	9436	29580
MO	38	21971	57854
FML-SCR	50	3314	6464

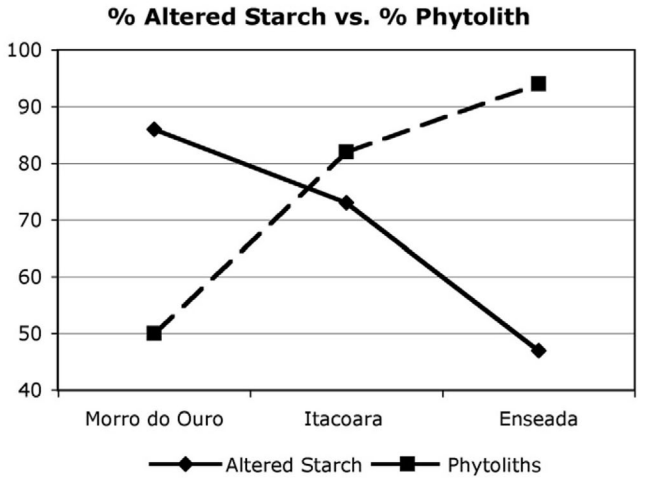

Figure 9. Phytoliths plotted against altered starch. There is an inverse relation between abundance of altered starch and phytoliths.

Table 8. Variation was observed between dental calculus samples from separate teeth from the same individual. These are the concentration values for multiple samples from the same individual. See Table 2 for explanation of abbreviations.

Burial number	Tooth Sampled	% Starch Altered	Starch altered	Total Starch	Phyt.
Enseada 1 8693	LRCI	40	5286	13216	2643
Enseada 1 8693	1ULM	20	5263	26315	60524
Enseada 1 8634	3LRM	0	0	698	5580
Enseada 1 8634	LRLI	0	0	4219	0
Enseada 1 8647	1LLM	0	0	11533	16476
Enseada 1 8647	2URM	0	0	1453	7267
Enseada 1 8681	2ULM	36	3491	9600	1745
Enseada 1 8681	1LRPM	10	317	3171	317
FML-SCR 10	1ULM	33	1588	4763	2382
FML-SCR 10	3LRM	50	414	827	2481
FML-SCR 23	LRC	50	2216	4433	0
FML-SCR 23	2LLM	80	9570	11963	0
FML-SCR 23	3LLM	66	2460	3691	2460

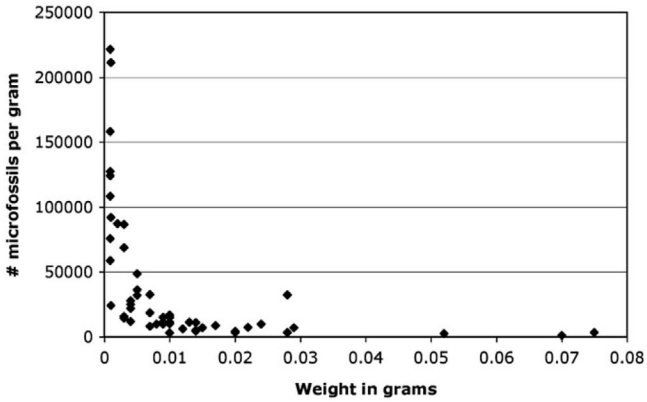

Figure 10. Microfossil calculated concentration plotted against sample weight. This shows that larger samples have lower concentrations of starch.

Initially, we quantified calculus mineral particles as potential evidence of grinding stones. We now suspect that mineral particles can be contaminants. Mineral fragments from sand, silt, clay and grit are abundant in the archaeological sambaqui soils. Although their presence in dental calculi could be also associated to food processing (grinding, cooking inside pits, roasting on the fireplaces, etc.), the soil in direct contact with the teeth could add mineral particles that would cement to the calculi surface. As discussed by Middleton (1992), minor amounts of mineral fragments trapped in the exposed surface of the calculi may introduce impressive mineral content in the samples. The methods presented in this paper would clean away most minerals from the surface of calculi, but smaller, adherent particles might not have been removed. Because of this, any inference based on minerals must be dependent on control samples taken from sambaqui sediment in the oral cavity, especially when small size calculi are processed.

We are continuing LEM and ESEM studies of dental calculus to assess whether or not carbon and grit are contaminants. We are preparing thin slices of dental calculus for examination to determine if the carbon and grit are adherent to the calculus surfaces that were in contact with the site sediment following the methods of Reinhard et al. (2001).

In summary, the methods presented here are the best devised to date for recovery of microfossils from small calculus deposits from fragile skeletal remains. When these methods are widely applied to dental calculus samples, we are certain that calculus analysis will open new doors to explore the relation of humans to plant use and domestication and also elucidate the relation between dental disease to diet and food preparation.

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