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**Proceedings Paper:**

Mackie, Meaghan, Radini, Anita and Speller, Camilla Filomena (2017) The Sustainability of Dental Calculus for Archaeological Research. In: Favreau, Julien and Patalano, Robert, (eds.) *Shallow Pasts, Endless Horiozons: Sustainability & Archaeology: Proceedings of the 48th Annual Chacmool Archaeology Conference.* , pp. 74-81.

<https://doi.org/10.11575/PRISM/10231>

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## The Sustainability of Dental Calculus for Archaeological Research

*Meaghan Mackie, Anita Radini and Camilla Speller*

**Abstract:** Dental calculus is a mineralized plaque biofilm formed by microbiota of the oral microbiome. Until recently, the vast research potential of dental calculus for archaeological study was not fully appreciated and it was often discarded. It is now recognized that dental calculus entombs and preserves valuable microfossils and biomolecules within its matrix. While microscopic and biomolecular analysis of calculus is destructive, judicious sampling of relatively small quantities of material can provide unique information on ancient health and diet. Additionally, dental calculus is not classified as human tissue, but as an ectopic growth, and in some cases may provide an alternative approach to the destructive analysis of human skeletal remains. We present a case study recovering proteins, DNA, and microscopic debris from Roman Age individuals to demonstrate the important insights into diet, health, and disease that can be obtained from even minute quantities of dental calculus.

### Introduction

Our world is becoming increasingly “molecular”. Rapidly developing technologies allow for detailed investigations into cultural and biological heritage through the analysis of ancient remains. This application of destructive methods on unique, irreplaceable artifacts or biological materials creates an inevitable tension in archaeological science. On one hand, archives (museums, repositories, etc.) and researchers alike share the responsibility for preserving invaluable and non-renewable archaeological resources, yet microscopic and molecular technologies can provide unprecedented details about the life history of biological remains. There are at least two interrelated issues at stake in the destructive analysis of ancient materials: first, biological

remains may be degraded or contaminated by age and/or storage conditions, reducing the likelihood of obtaining successful biomolecular results; second, many specimens are so rare that destructive sampling should be avoided when there is limited likelihood of analytical success. In this paper, we present a biomolecular case study of Roman Age materials to highlight the archaeological insights into diet and disease that can be obtained through the destructive analysis of dental calculus (mineralized plaque). We argue that dental calculus represents an extremely robust archive of ancient biomolecules and microscopic debris, with a high probability of analytical success. Moreover, the general ubiquity of dental calculus on archaeological skeletons, combined with the minimal sample sizes required for analysis makes it a sustainable avenue of bioarchaeological research that should be more systematically incorporated into future research.

### Dental Calculus and Bioarchaeology

Dental calculus (tartar, calcified plaque) is a mineralized biofilm created by a wide range of microscopic organisms residing in the oral cavity (Figure 1).



Figure 1. Dental calculus visible on the upper teeth of Skeleton 9 from Oxford Street Cemetery, Leicester.

The human mouth is host to a wide array of bacteria, both commensal and pathogenic, creating what has been called the human oral microbiome. *Streptococcus* and *Actinomyces* species are the most prevalent plaque forming organisms but calculus often includes species from *Veillonella*, *Neisseria*, *Fusobacterium*, and some *Bacteroides*. These organisms adhere to the tooth surface and each other within an extracellular matrix composed of host and microbial derived polymers and extracellular DNA (Hillson 2005; Marsh 2004; Socransky and Haffajee 2002). Studies suggest that the modern oral microbiome comprises of over 700 different bacterial species (Belstrøm et al. 2014) all of which could be incorporated into the calculus matrix. Although the triggers and processes of mineralization are still unresolved, it appears that the mineral content ultimately derives from the calcium phosphate in saliva. Therefore, the areas closest to the salivary glands are the most likely to develop calculus: lingually on the incisors and canines, and buccally on the upper molars in humans (Hillson 1996, 2005). As the plaque biofilm is mineralized, it entraps and preserves the organic content from bacteria as well as human dietary and inhaled microdebris within the matrix. This organic component of calculus represents around 15–20% of the deposit, predominantly representing proteins and lipids (Lieverse 1999:220).

### **Dental Calculus and Sustainability**

Until recently, calculus was treated “unsustainably” and was often scraped off archaeological skeletons and discarded in order to thoroughly characterize dental stress markers (e.g. linear enamel hypoplasia) or dental wear. Within the last decade, dental calculus has emerged as a remarkable archive of both microbial and host biomolecules providing insight into both individual and population analysis of health and diet (Warinner et al. 2015). The mineralized matrix of dental calculus is of high physical hardness and durability, preserving organic microscopic debris and biomolecules in sequentially deposited events throughout the lifetime of an individual, including a range of organic remains not otherwise preserved in the archaeological record. Due to these unique properties, dental calculus has been described as “among the richest biomolecular sources yet

identified in the archaeological record” (Warinner et al. 2014a:343).

Sustainability in bioarchaeology largely revolves around the destruction of non-renewable resources, and this is still applicable to dental calculus research. Beyond recording the presence, abundance, and location within the mouth, all other forms of analysis require destruction of archaeological material. For both microscopic and biomolecular analyses, material must be removed and demineralized; however, dental calculus has some advantages over other skeletal elements. First, due to a lack of modern dental hygiene practices and knowledge about the aetiology of dental disease, dental calculus was more prevalent and abundant in the past – confirmed by the ubiquitous incidence rate in archaeological specimens (White 1997). Second, due to the high concentration of biomolecules or microdebris within the substrate, less than 50 mg of dental calculus is required for analysis. Third, calculus is often found on multiple teeth within the mouth, leaving unsampled material for future analyses. Finally, dental calculus is not considered a human tissue but an ectopic growth, and in some cases may provide an alternative to the destructive analysis of human skeletal remains. Therefore, in spite of its destructive nature, biomolecular analysis of dental calculus may still provide a sustainable and valuable avenue of bioarchaeological research.

### **Case Study: Dental Calculus from British and Italian Roman Age Sites**

In order to demonstrate the sustainable potential of ancient dental calculus, we present data recovered from Roman Age individuals using multiple bioarchaeological techniques to highlight the broad range of information that may be obtained from dental calculus (detailed methods and results may be found in Mackie 2014). Dental calculus was sampled from 22 individuals: 8 individuals from the necropolis of Isola Sacra near Rome dating to 50–200 CE (Prowse et al. 2005); 6 individuals from Driffeld Terrace, York dating broadly to 44–410 CE (Caffell and Holst 2012); and 8 individuals from the Oxford Street Cemetery, Leicester dating to the early 4<sup>th</sup> century A.D. (Keefe and Holst 2013).

## **Biomolecular Methods – Proteomics, DNA, and Microscopy**

Dental calculus samples ranging from 15–60 mg were collected from each individual, and a unified protocol was used to isolate proteins, DNA, and microscopic debris from the same fraction of calculus. First, the samples were demineralized in 0.5 M EDTA (pH 8). Proteins were extracted from the dental calculus and blank controls at *BioArCh*, University of York using a modified filter-aided sample preparation (FASP) method that has been derived specifically for the protein extraction from ancient calculus, dentine, and bone (Cappellini et al. 2012) with detailed methods described in Warinner et al. (2014b). After the extracted proteins were isolated, the retentate within the filter devices was purified through Qiagen MinElute columns to isolate the DNA. Proteins and aDNA were quantified by Qubit® 2.0 Fluorometer, and DNA fragment length was determined using 2100 Bioanalyzer (Agilent Technologies) with a high sensitivity DNA assay. The proteins from the dental calculus were characterized via Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) at the *Proteomics Discovery Institute* at the University of Oxford, and the resulting peptide ions were searched through Mascot Daemon against the *UniProtKB/SwissProt* (<http://www.uniprot.org/>) and *Human Oral Microbiome* (HOMD) (Dewhirst et al. 2010; <http://www.homd.org/>) databases. Finally, aliquots were taken from remaining pellet material and suspended in HCl before centrifuging and rinsing with distilled water. The remains were mounted on slides in 50% glycerol in water, and the slides were then examined under a compound microscope, Olympus IX 71, examined at magnification up to 630x. Observations were complemented by the use of polarized light.

### **Proteomic Analysis of Dental Calculus**

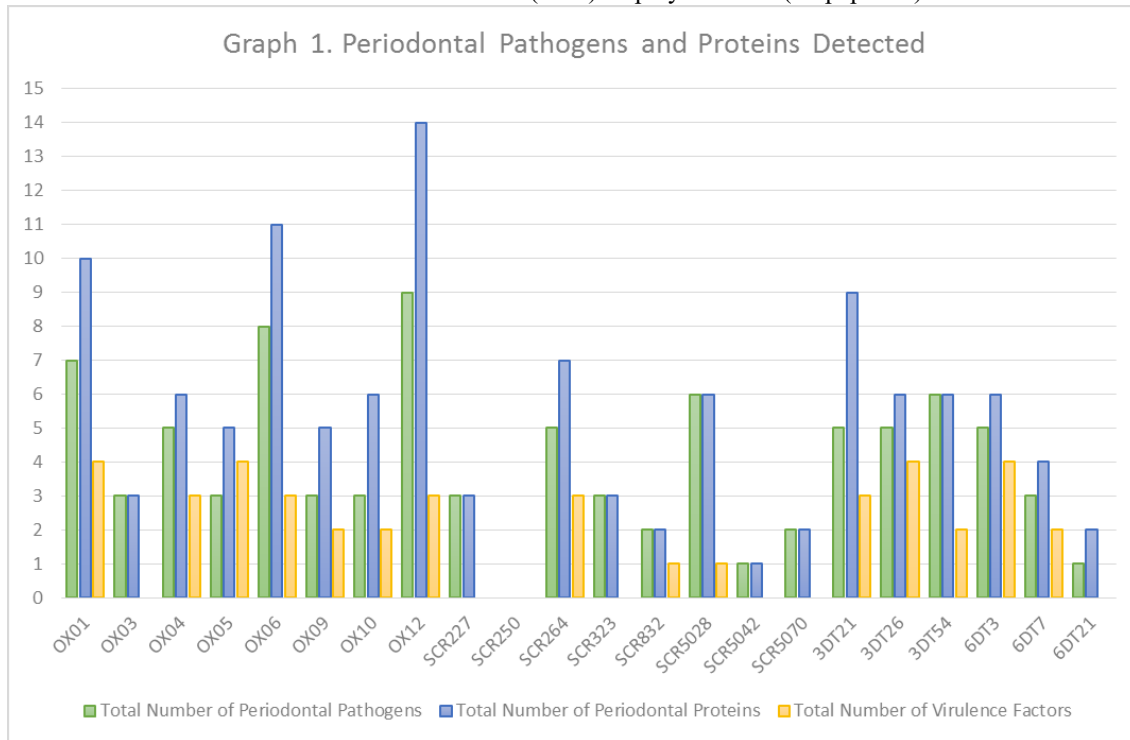
Proteomic analysis of ancient dental calculus is still in its infancy, but already yielding exciting insights into ancient health and diet. Warinner et al.'s (2014a) study was the first to apply “shotgun” proteomic methods to Medieval dental calculus from Germany, identifying proteins from myriad periodontal bacteria, as well as human proteins relating to the innate immune response. Our case study from the Roman period identified the same

rich abundance of proteins, confirming the utility of dental calculus as a promising substrate for analysis.

A total of 143 proteins were confidently identified within the samples, with bacterial proteins making up 62% of the identified proteins from all three sites. The identified bacteria generally fall into three categories: commensal species found within the normal human microbiome, pathogens (disease causing microorganisms), and environmental species. Plaque forming bacteria represent a common subset of the identified commensal species. For example, *Actinomyces* was the most commonly identified commensal bacteria taxon, representing 14.3% of all protein clusters from the three sites. However, typically commensal bacteria (e.g. *Corynebacterium matruchotii*, *Leptotrichia buccalis*) may also act as opportunistic pathogens if an individual is immunocompromised, resulting in periodontal disease (Africa 2012:6) or other systemic diseases such as endocarditis and organ abscesses (Wade 2013:141). Pathogenic bacteria were identified in individuals from all three archaeological sites, including periodontal pathogens, other oral pathogens, and systemic pathogens. The majority of these pathogens were related to dental disease (e.g. *Eikenella corrodens*, *Fusobacterium nucleatum*, *Tannerella forsythia*) especially periodontal disease (Graph 1), which causes inflammation and tissue damage of the gums and bone. In some cases, the virulence factors (or specific protein elements) associated with inflammation and degradation of tissue, were identified (e.g. *E. corrodens* Fimbrial protein EcpC, *Porphyromonas gingivalis* gingipain).

Interestingly, periodontal pathogens and virulence factors were identified in individuals both with and without osteological evidence of periodontitis. Individuals from Oxford Street Cemetery displayed the greatest proportion of periodontal pathogen proteins, and Isola Sacra the least. Periodontal disease is also useful as a general indicator of health, as many studies have shown a moderate causal or associated relationship between periodontal disease and several systemic infections and inflammation, especially cardiovascular and pulmonary diseases (Beck and Offenbacher 2005; Peter et al. 2013).

Graph 1. The number of periodontal pathogens, periodontal proteins and virulence factors identified in the complete dataset. Individuals from Oxford Street Cemetery (OX) demonstrated the greatest evidence for periodontal pathogens (139 peptides overall), while those from Isola Sacra (SCR) display the least (49 peptides).



Systemic pathogens were also identified, albeit at a lower frequency. For example, proteins associated with infectious agent of gonorrhoea (*Neisseria gonorrhoeae*), diphtheria (*Corynebacterium diphtheriae*), and listeria (*Listeria monocytogenes*) were identified with a high degree of confidence. Many of these pathogens can be found as part of the modern human oral microbiome, and may be present without causing disease. While the identification of these pathogens cannot confirm the presence of active infection, they can uniquely provide a record of an individual's exposure to the agents of disease. Moreover, dental calculus offers the potential to link osteological markers of disease (e.g. periodontitis), with biomolecular evidence for periodontal or systemic infections to provide much more insight into individual life histories.

Our proteomic analysis also allowed for insight into the dietary practices of the Roman period. The presence of  $\beta$ -lactoglobulin (BLG) (Figure 2), a milk whey protein present in milk and to a limited extent in soft cheeses, was identified in four of the British individuals (as presented in Warinner et al. 2014b). Due to species-specific variants within the BLG protein, two individuals could be confirmed

as consuming cow's milk, while one was identified as having consumed either sheep or goat milk. BLG has recently been detected in dental calculus dating back to the Bronze Age (Warinner et al. 2014b), providing new means to document the emergence and importance of dairying in archaeological contexts.

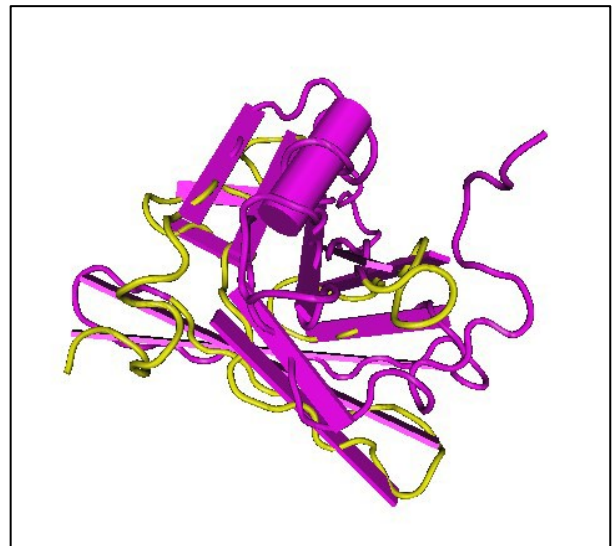


Figure 2. The 3-dimensional structure of the bovine  $\beta$ -lactoglobulin protein, with the lighter highlighted section indicating identified peptides from one Oxford Street Cemetery individual (OX04).

### **Ancient DNA Analysis of Dental Calculus**

Dental calculus has recently been demonstrated as a rich source of ancient microbial DNA. Early biomolecular investigations targeted specific oral pathogens such as *P. gingivalis* and *Streptococcus mutans* through PCR-based approaches (Adler et al. 2013; de la Fuente et al. 2012), while more recent work has applied high-throughput sequencing to reconstruct ancient oral microbial communities (Adler et al. 2013; Warinner et al. 2014a; Zieseimer et al. 2015). These studies have identified ecological shifts in the oral microbiome corresponding with the Neolithic and Industrial Revolution (Adler et al. 2013), as well as reconstructing complete genomes of oral bacteria to investigate evolutionary changes associated with pathogen virulence and antibiotic resistance (Warinner et al. 2014a). These genetic studies have suggested that archaeological dental calculus may preserve orders of magnitude more DNA than bone or dentine (Warinner et al. 2014a). Successful recovery of DNA from dental calculus samples up to 7,000 BP has already been demonstrated (Adler et al. 2013), and likely persists into much deeper periods of prehistory. Importantly, mineralization of the dental calculus seems to create a closed system which prohibits environmental contamination (Warinner et al. 2014a), reducing the possibilities of contamination from exogenous sources.

In our case study, the recovered ancient DNA did not undergo sequencing, but was analyzed principally to ascertain whether DNA and proteins could be simultaneously extracted from the same piece of calculus, and to assess the average length of DNA molecules extracted using this “unified” extraction method. Individuals from the three sites yielded high concentrations of DNA (average of 15–23 ng/mg of calculus), with no detectable DNA recovered from the blank extractions. The modal base pair lengths of the recovered DNA from the British and Italian sites were similar, 70 bp and 62 bp, respectively. Importantly, our study suggests that it is possible to recover relatively well preserved DNA from the same fraction of calculus analyzed for surviving proteins, eliminating the need for additional sampling and increasing the sustainability of dental calculus research.

### **Microscopic Analysis of Dental Calculus**

Previous studies have retrieved a great variety of microdebris from dental calculus such as starch granules, phytoliths, pollen, spores, and even plant fibers (e.g. Hardy et al. 2015; Henry and Piperno 2008; Henry et al. 2012, 2014; Lalueza Fox et al. 1996; Li et al. 2010). Due to the fact that calculus forms in the mouth, food has been seen as the major source of debris recovered, and research conducted so far has focused almost exclusively on dietary remains entrapped within (e.g. Hardy et al. 2009; Henry and Piperno 2008). However, over the past couple years, it has become clear that calculus can also entrap a variety of remains of non-dietary origin, including inhaled environmental microdebris (Buckley et al. 2014; Radini et al. 2016).

In our study, microscopic analysis was performed on the remaining fraction of calculus from one individual to demonstrate the full suite of techniques that could be applied to a single piece of analyzed calculus. Both dietary and environmental information was obtained in the form of starch granules and microcharcoal. Examples of starch include members of the Triticeae tribe (wheat and barley) of the Grass Family (Poaceae); these were very diagnostic due to their bimodal distribution of small and large granules and the characteristic lenticular shape of the large granules; Figure 3 is an example of this starch and the slightly bloated nature of the granule suggests that it may have been cooked prior to consumption. Another example of observed starch resembles the compound granules found in another tribe of grasses, the tribe Aveneae (oats tribe) (Figure 4). However, precise taxonomic identification of the latter is uncertain, since the starch has been crushed or grinded, either through cooking practices or consumption. Additional evidence of cooking and/or smoke inhalation was observed in the form of pieces of microcharcoal and a burnt phytolith (Figure 5A, 5B).

While the microscopic evidence emerging from a single skeleton may not provide particularly comprehensive or representative information, this work showcases a new method for linking specific environmental and dietary practices with both osteological and biomolecular evidence of disease to provide increasingly detailed “osteobiographies” at the individual rather than population level.

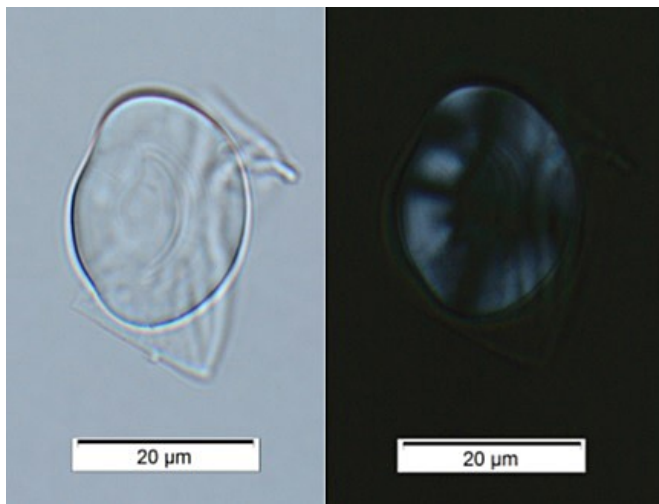


Figure 3. Starch granule of the Triticeae tribe from OX05 (60x, left polarized).

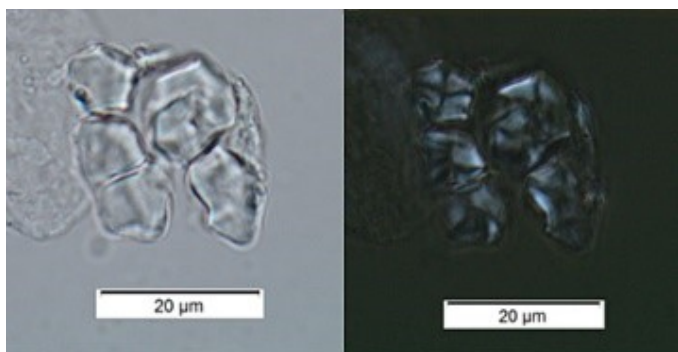


Figure 4. Compound starch granules, potentially from the Aveneae tribe, from OX05 (60x, left polarized).

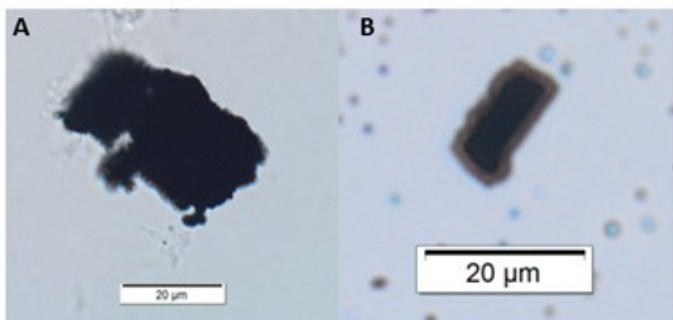


Figure 5. A) Microcharcoal from OX05 (40x); B) Burnt phytolith from OX05 (40x).

## Conclusions

The analysis of ancient dental calculus is not a final solution to the overarching problem of sustainability in bioarchaeology, however, it is an emerging and highly informative avenue of research into the health and diet of ancient peoples. In light of growing sustainability concerns, dental calculus offers the key advantages of being: 1) archaeologically ubiquitous; 2) an exceedingly rich source of preserved biomolecules and microfossils;

and 3) potentially highly resistant to environmental contamination. Single small samples can be analyzed simultaneously to obtain proteomic, genomic, and microscopic data, thus providing insight into multiple facets of the health and diet of the individual and their associated cultural practices. Finally, as an ectopic growth, its analysis is not destructive of human tissue. Bearing in mind the abundance of data that can be obtained from this remarkable archaeological resource, we must still remember to sample judiciously and carefully to sustain the research potential for generations of archaeologists to come.

## Acknowledgements

The authors would like to thank those who provided the samples: *York Archaeological Trust*, the University of Leicester Archaeological Service, and the Italian Ministry of Culture and University of Rome; University of York *Centre for Chronic Diseases and Disorders* for providing funding; and *BioArCh* and the University of York for providing laboratory space and materials. Additionally, we would like to acknowledge and appreciate Malin Holst and Matthew Collins for reviewing the presentation related to this paper.

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**Shallow Pasts, Endless Horizons: Sustainability & Archaeology**

**Proceedings of the 48<sup>th</sup> Annual Chacmool Conference**

**Edited by**

**Julien Favreau and Robert Patalano**

**Cover art by Alexa Lacroix**

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**ISBN 13 978-0-88953-397-4**

**ISBN 10 0-88953-397-0**