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BIOSTRATIGRAPHIC EVIDENCE RELATING TO THE AGE-OLD QUESTION OF HANNIBAL'S INVASION OF ITALY, II: CHEMICAL BIOMARKERS AND MICROBIAL SIGNATURES*

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As discussed in Part I, a large accumulation of mammalian faeces at the mire site in the upper Guil Valley near Mt. Viso, dated to 2168 cal ^{14}C yr., provides the first evidence of the passage of substantial but indeterminate numbers of mammals within the time frame of the Punic invasion of Italia. Specialized organic biomarkers bound up in a highly convoluted and bioturbated bed constitute an unusual anomaly in a histosol comprised of fibric and hemist horizons that are usually expected to display horizontal bedding. The presence of deoxycholic acid and ethylcoprostanol derived from faecal matter, coupled with high relative numbers of Clostridia 16S rRNA genes, suggests a substantial accumulation of mammalian faeces at the site over 2000 years ago. The results reported here constitute the first chemical and biological evidence of the passage of large numbers of mammals, possibly indicating the route of the Hannibalic army at this time. Combined with the geological analysis reported in Part I, these data provide a background supporting the need for further historical archaeological exploration in this area.

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INTRODUCTION

Because no physical artefacts have been located on any potential Hannibalic Alpine route, a recent find of organic remains in a massive 'churned-up' bed could offer critical scientific evidence that may be extended to further historical archaeological exploration regarding Hannibal's crossing of the Alps. Moving beyond traditional historical arguments to answer the Hannibalic route question and the analysis of physical and/or environmental parameters (Mahaney 2008; Mahaney *et al.* 2016—hereinafter, 'Part I′), we investigate sediment geochemistry and microbiology found at a mire site that may have been used for forage along the southern route first identified by Sir Gavin de Beer (1974). This deposit, described in Part I, is clearly bioturbated to an extreme compared with normal transhumance sites, and has been linked to radiocarbon dates precisely at 2168 cal yr. BP (for standard deviations on this age, see Fig. 4 of Part I).

Organic compounds, sourced from people and animals, lend themselves to relatively easy detection in the bioturbated sediments, with such materials persisting for millennia (e.g., Bull *et al.* 2003; Baeten *et al.* 2012), which offers a contrast to transhumance control sediment recovered in younger beds described in Part I. We can hypothesize that Hannibal's army may have produced the unusual non-laminated, massively churned-up sediment during passage over the Alps. To investigate this possibility, we carried out an intensive study of the mire site at 2580 m asl, located along the southern route in the Alps, which could have been used as a foraging/watering area for a large group of people and animals.

Based on our study of faecal compounds and recalcitrant microbial populations described below, we identify a distinct increase in usage of the area by mammals, such activity occurring during the period in which the churned-up layer was formed. Based on this evidence, we hypothesize that Hannibal's army may have used the mire site, producing the unusual non-laminated, massively churned-up sediment during passage over the Alps. We encourage further archaeological study of the area.

MATERIALS AND METHODS: THE BIOLOGICAL RECORD

Total organic carbon (TOC)

Elemental analysis was performed in triplicate, using a Fisons NCS 1500 NA elemental analyser, and average values are reported here. Samples were treated with 2 N HCl prior to analysis to remove carbonates. Samples were then weighed (~5 mg) into tin capsules and combusted in the presence of pure O_2. The CO_2 evolved from this process was then measured and the percentage of organic carbon (OC) was calculated by comparison with the certified reference standard acetanilide, which was analysed in conjunction with samples.

Lipid biomarker analysis

All samples for organic geochemical analysis were freeze-dried and homogenized using a mortar and pestle, and sieved (850 μm pore size). All glassware was heated in a furnace for 8 h at 480°C prior to use. All Teflon equipment was sonicated in chloroform for 30 min. Lipids were extracted using ultrasonically assisted extraction, following the method of Otto *et al.*

(2005). Samples (1–3 g) were sonicated for 15 min with 10 mL of methanol and centrifuged for 15 min at 6000 rpm. Supernatants were then filtered through Whatman GF/A glass-fibre filters. This procedure was then repeated with solvents of decreasing polarity (10 mL of 1:1 methanol: dichloromethane, followed by 10 mL of dichloromethane). Filtrates were combined, concentrated and transferred to a 2 mL glass vial.

Faecal stanols and bile acids were isolated and analysed following the method of Birk *et al.* (2012). The dried total lipid extract (TLE) was saponified overnight with 0.7 M methanolic KOH, at room temperature. Following the addition of chloroform-extracted deionized H_2O, the TLE was extracted (×3) with chloroform to provide a neutral fraction including stanols. The remaining aqueous fraction was acidified to pH <2 with 6 N HCl and extracted (×3) with chloroform to provide an acid fraction including bile acids.

The neutral fraction transferred to a 5% deactivated silica SPE cartridge (Isobe *et al.* 2002), which was preconditioned with 5 mL of hexane. Less polar compounds were removed with 5 mL of hexane. Steroidal compounds were eluted with 3 mL of dichloromethane followed by 2 mL of 2:1 dichloromethane:acetone, dried under anhydrous N_2 and transferred to a 2 mL glass vial.

The acid fraction was methylated by addition of 1 mL of 1.25 M methanolic HCl and heating at 80°C for 2 h. Then, 1 mL of organic-free DI H_2O was added and methyl esters were extracted (×3) with 1 mL of hexane. The combined deionized extracts were transferred to an activated silica SPE cartridge (Elhmmali *et al.* 2000), which was preconditioned with 5 mL of 2:1 dichloromethane:hexane. Less polar substances were removed with 5 mL of 2:1 dichloromethane:hexane. Bile acids were eluted with 5 mL of 2:1 dichloro-methane:methanol, dried under anhydrous N_2 and transferred to a 2 mL glass vial.

Steroidal compounds were silylated with a 100 μL mixture of hexamethyldisilazane (HMDS), trimethylchlorosilane (TMCS) and pyridine (3:1:9) at 70°C for 1 h. Excess silylating agent was evaporated and the extract resuspended in 100 μL of 100 ppm 5α-cholestane (in dry toluene) as an internal standard. Bile acids were silylated with 50 μL of dry toluene, 98 μL of BSTFA and 2 μL of 1-(trimethylsilyl)imidazole (TSIM) at 80°C for 1 h. Then, 50 μL of 400 ppm 5α-cholestane (in dry toluene, providing 100 ppm concentration) was added as an internal standard. All fractions were analysed by gas chromatography mass spectrometry according to Birk *et al.* (2012). Individual compounds were identified by the use of mass spectral library databases (NIST) and comparison of mass spectrometric patterns with published mass spectra and authentic standards (see Fig. 1).

Microbial population analysis

Sediments for DNA extraction were taken with sterilized implements from carefully cleaned exposed surfaces in the G5a profile. The samples were kept cold (~3°C) immediately in the field and stored at −20°C immediately upon return to the laboratory prior to DNA extraction (Griffiths *et al.* 2000). We then performed 16S rRNA gene pyrosequencing on all DNA extracts using established primers and methodology (Berry *et al.* 2011). The 16S rRNA gene sequencing was performed at the Department of Biochemistry, University of Cambridge, UK, on a 454 Junior Sequencer. The bacterial composition of samples collected between depths of 15 and 55 cm was estimated by 16S rRNA gene pyrosequencing, followed by analysis using the QIIME pipeline (Caporaso *et al.* 2010). Sequences assigned to phylogenetic groups are based upon a limit of 97% sequence identity to representative genes.

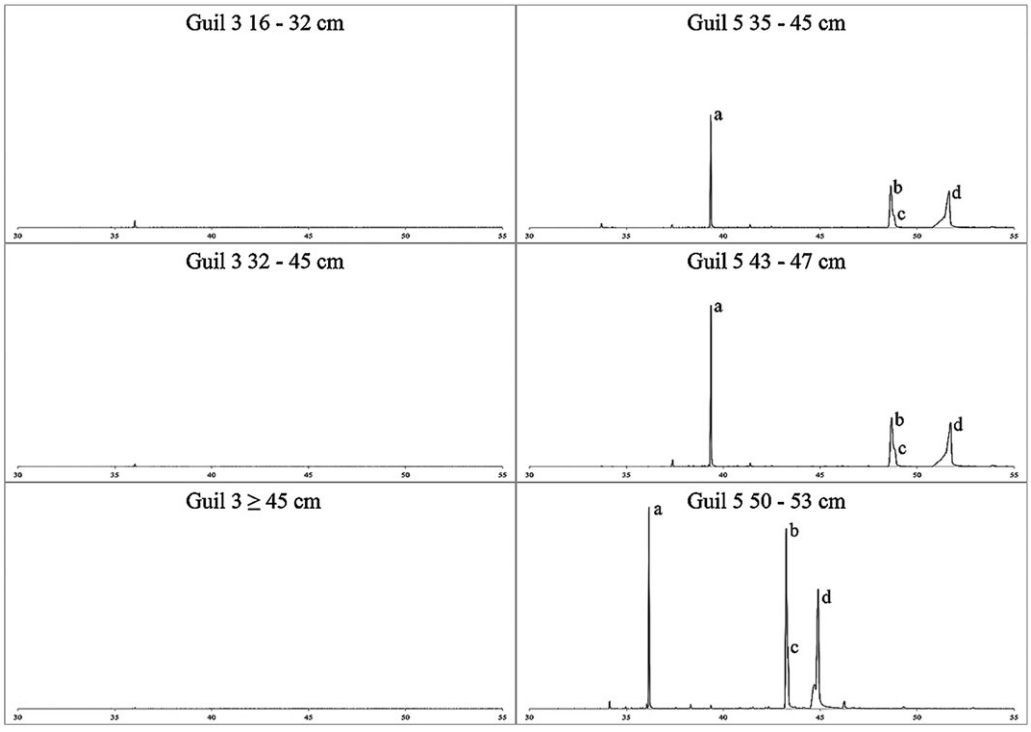

Figure 1 *A comparison of extracted ion chromatograms (EIC m/z 398) for three similar layers from control sample Guil 3 and mire site sample Guil 5. Identified compounds in Guil 5 chromatograms are labelled: a, 1-tricosanol; b, 24-ethyl-5β-cholestan-3β-ol; c, 24-ethyl-5β-cholestan-3α-ol; d, 24-ethyl-5α-cholestan-3β-ol. Guil 5 35–38 cm and 43–47 cm were run on a different instrument to the 50–53 cm sample; hence the difference in retention times.*

RESULTS AND DISCUSSION

Organic matter (OM)

TOC (%) values for each individual depth at the Guil 5a site are listed inTable 1. The results show a minimum value of 6.24% at 15–20 cm and a maximum value of 19.92% at 30–35 cm. The value obtained for the churned-up layer at 40–45 cm was 16.6%, with somewhat lower concentrations in the G5a section than values obtained from the combined cores, as discussed in Part I of this paper.

As expected in any soil or sediment profile, the results from analysis of %TOC show a decreasing trend with depth overall. However, there is a sharp peak at ~20–35 cm, suggesting a period of higher OM input. The Bayesian statistical analysis indicates that the dates for this depth match that of the Medieval Warm Period (MWP) at *c.*1000–1200 yr. BP (Mangini *et al.* 2005; Mann *et al.* 2009; Cronin *et al.* 2010; Goosse *et al.* 2012) and therefore a higher OM input would be expected during these warmer years. The level of TOC then decreases sharply, followed by another peak at ~40–50 cm. This depth corresponds to the bioturbated layer, indicating a significant influx of organic matter during this period. The decreasing OM trend continues below these depths. Despite differences in organic matter content between the section (G5a) and the core (G5b), these trends for G5a concur with the OM% results (obtained by loss on ignition (LOI) observed in Part I of this study).

Table 1 *An overview of geochemical and microbiological results for the 2013-G5a samples: the geochemistry section displays organic carbon (%), concentrations of faecal compounds [μg $(gOC)^{-1}$] and values of the stanol ratio, while the microbiology section provides a summary of alpha diversity measurements of pyrosequencing data*

Depth (cm)	*Geochemistry*				*Microbiology*			
	OC (%)	*5β-Stigmastanol* [μg $(gOC)^{-1}$]	*Deoxycholic acid* [μg $(gOC)^{-1}$]	*Stanol ratio*	*Sequences per sample*	*Chao1 average from first 602 sequences analysed*	*Observed species average value*	*Shannon diversity average*
15–20	6.24	26.11	40.44	0.78	1876	236.57	166.8	6.481
20–25	18.96	30.59	28.05	0.78	3549	268.189	171.4	6.362
25–30	18.72	10.45	11.92	0.68	4244	254.947	168.8	6.464
30–35	19.92	10.62	6.47	0.73	3625	312.87	197.2	6.812
35–40	10.85	22.66	31.55	0.84	2721	246.775	156.6	6.099
40–45	16.6	16.96	15.83	0.83	2543	247.434	155.2	6.308
45–50	18.35	13.12	1.95	0.76	4357	216.855	129	5.74
50–55	16	4.62	4.23	0.65	2244	158.939	106.4	5.096
55–60	14.23	4.29	3.62	0.89	3535	153.328	79.7	4.187

Faecal biomarkers

Lipid biomarker analysis was carried out on samples taken from the 2011 Guil Valley sites ($n = 9$). This led to the identification of 24-ethyl-5β-cholestan-3β-ol (5β-stigmastanol) at site 2011-G5, located within the mire. No faecal biomarkers were found at any of the other sampling sites in the region (e.g., Mahaney *et al.* 2013, fig. 1). The discovery of this biomarker compound prompted further organic geochemical investigations of this mire site.

Analysis of the 2013-G5a samples confirmed the occurrence of 5β-stigmastanol as well as the secondary bile acid, deoxycholic acid (DCA). Both of these compounds were present at all depths and displayed similar trends, with increased input observed between depths of 20–25 cm and ~35–45 cm (Fig. 2 and Table 1). Faecal contamination is supported by the use of the previously reported stanol ratio (Grimalt *et al.* 1990; Bull *et al.* 1999), which corrects for non-faecal sources of 5β-stanols and also any microbial or diagenetic alterations that may affect the relative concentrations of these compounds:

$$\frac{(\text{24-ethyl-5β-cholestan-3β-ol} + \text{24-ethyl-5β-cholestan-3α-ol})}{(\text{24-ethyl-5β-cholestan-3β-ol.} + \text{24-ethyl-5β-cholestan-3α-ol} + \text{24-ethyl-5α-cholestan-3β-ol})}.$$

The resulting values from all but two depths (25–30 and 50–55 cm, 0.68 and 0.65, respectively) were above the proposed lower limit for faecal contamination of 0.7 (Fig. 2 and Table 1) (Grimalt *et al.* 1990).

5β-stigmastanol is a 5β-stanol commonly used as a biomarker for faecal material produced by ruminant mammals (Evershed *et al.* 1997; Bull *et al.* 2002). It was also the most abundant stanol observed in horse faeces by Leeming *et al.* (1996). DCA is a major secondary bile acid in the faeces of humans and some higher animals (Bull *et al.* 1999; Elhmmali *et al.* 2000; Bull *et al.* 2002). The presence of DCA in the absence of lithocholic acid (LCA) indicates a ruminant animal source for the faecal material (Bull *et al.* 2002).

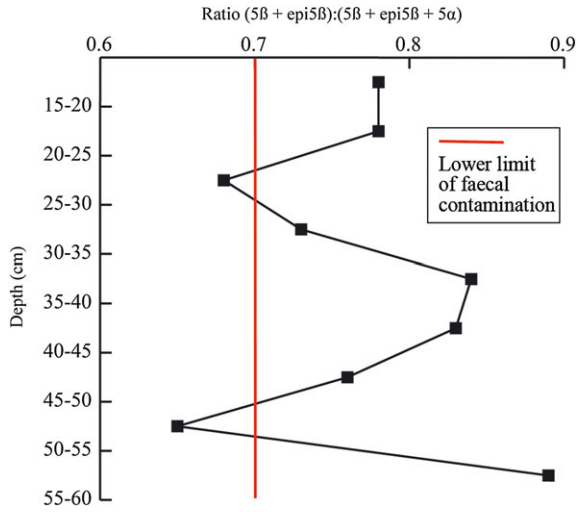

Figure 2 *A plot of the stanol ratio for use in archaeological studies (Grimalt 1990; Bull 1997). The vertical marker line indicates the lower limit of faecal contamination, of 0.7.*

Concentrations of faecal biomarkers 5β-stigmastanol and DCA show almost identical profiles throughout the mire section (see Fig. 3). The maximum concentrations of both compounds across the 20–30 cm depths could be due to increased animal faecal matter input coinciding with the MWP. Increased temperatures could possibly have led to reduced snow cover at these altitudes, making them more accessible for transhumance and providing longer grazing periods, as well as slightly drier conditions providing more space and plant cover for animals to graze. Where increased TOC values are observed at 35–40 cm, the churned-up layer correlating to the Hannibalic event, both of these faecal biomarkers observe a surge in concentration, moving away from the decreasing values at higher depths.

According to values obtained from the faecal stanol ratio, a higher proportion of the 5β-stigmastanol in the MAD bed (Mass Animal Deposition bed, defined in Part I) is derived from faecal contamination than in the 20–30 cm depths. The highest value for faecal contamination was observed in the 55–60 cm depth: this would suggest that these compounds in this layer originate primarily from faecal material. However, the concentrations of both biomarker compounds are relatively low and signify a minor input, possibly due to leaching of material from soil layers above. Taking this evidence into account, it would appear that the MAD bed is the layer most significantly contaminated with faecal material in the G5a soil profile.

The 35–45 cm depth lies within the MAD bed. As this is a waterlogged mire site, there is potential for anaerobic microbial hydrogenation of higher plant sterols (i.e., β-sitosterol: see, e.g., Elhmmali *et al.* 2000), which could lead to the false assignment of a faecal source for the organic matter (Leeming *et al.* 1996). Micro-organisms in the intestinal tract convert primary bile acids, formed in the liver, to secondary bile acids such as DCA (Elhmmali *et al.* 2000; Bull *et al.* 2002; Tyagi *et al.* 2008). To the authors' knowledge, bile acids are not known to be produced outside of the digestive system, which suggests a faecal input to this site. Also, the high input of each individual compound, coupled with the additional evidence of faecal contamination provided by the stanol ratio, is further indicative of elevated mammalian faecal deposition in the MAD bed.

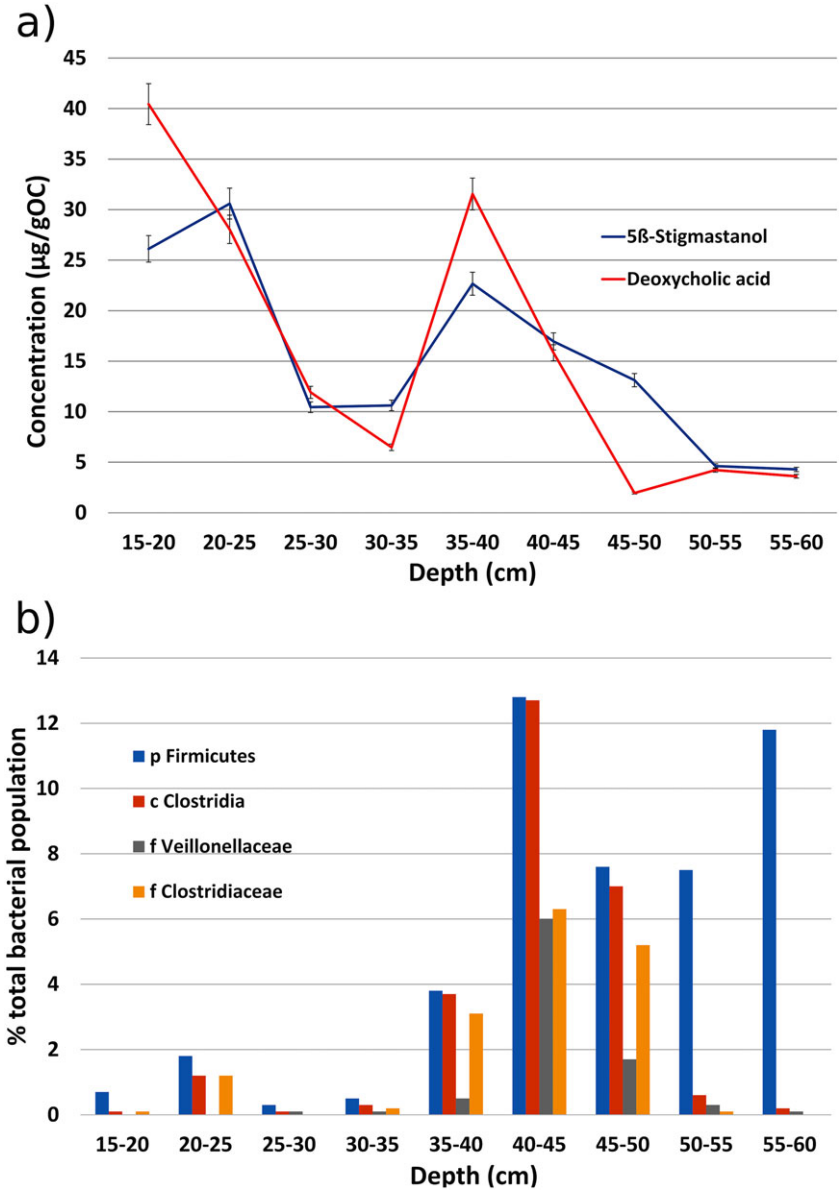

Figure 3 *(a) Relative concentrations, in µg g^{-1}, of the faecal biomarker compounds 5β-stigmastanol and deoxycholic acid throughout the 2013-G5a Histosol profile: one abundance peak for both occurs within the bed from 40 to 50 cm. (b) Selected metagenomic analysis of 16S rRNA gene sequences from the MAD bed: closer analysis of the Firmicutes reveals that the Clostridia (class), Clostridiaceae (family) and Veillonellaceae (family) are maximally associated with the sediment sample dated to the time of the Punic invasion (40–45 cm).*

The presence of both 5β-stigmastanol and DCA at all depths within the soil section could be due to one of two factors. Baeten *et al.* (2012) observed migration of both stanols and bile acids in archaeological soils. They suggested that due to the solubility of bile acids, they are prone to

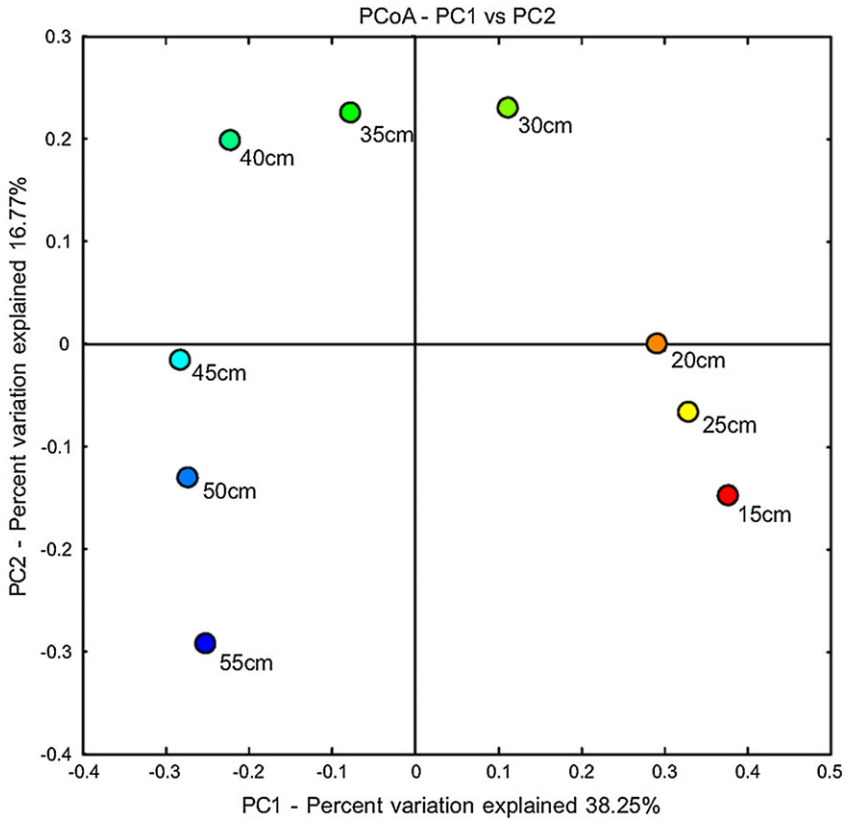

Figure 4 *A comparison of beta diversity in the 16S rRNA gene populations studied, using principal coordinates analysis.*

leaching. Migration of *n*-alkanoic acids, organic molecules of similar hydrophilicity, has also been suggested to have been the result of leaching in soils (Bull *et al.* 2000). Stanols are hydrophobic compounds, thus making leaching less likely; however, they have been associated with particulate matter in sewage and water columns, and therefore may be subject to physical migration within the mire (Isobe *et al.* 2002; Baeten *et al.* 2012). However, as the elemental ratios (Part I) show no evidence of leaching, this scenario is unlikely.

It is possible that local fauna could be responsible for the low levels of faecal contamination that can be seen outside of the spikes in concentration at the 20–25 cm depth (attributed to the MWP) and the MAD bed. The *Capra ibex*, *Rupicapra rupicapra* and *Marmota marmota* that are native to the region are ruminants, and therefore would contribute similar faecal signatures to those identified.

The most important factors in the argument proposed in this research are that no faecal biomarker compounds were observed in the control samples taken from the surrounding areas, and that the concentrations of the identified faecal compounds within the mire site show an increase at the depth associated with Hannibal's crossing. This suggests that at some point *c.*218 BC there must have been an increase in animal activity at this grassy mire site and not in the other surrounding locations investigated thus far. It is likely that Hannibal's army would have

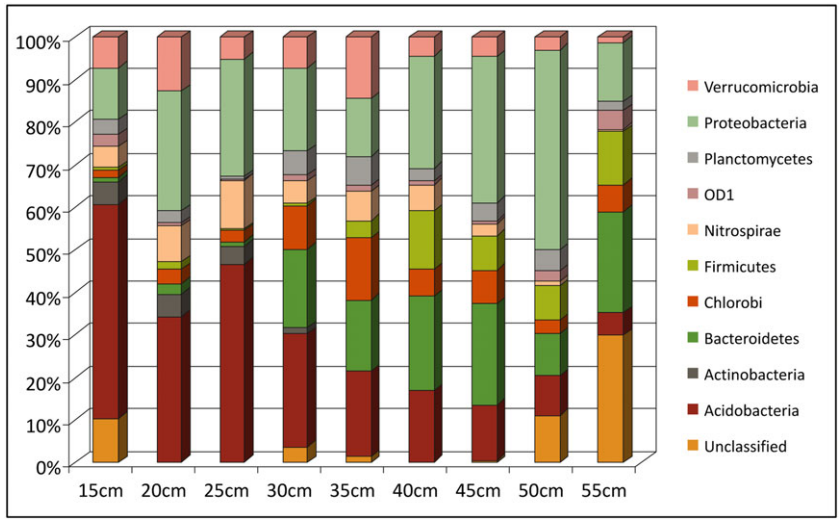

Figure 5 *A comparison of major phyla detected in the nine samples analysed.*

taken the opportunity to rest at this site, where both fresh water and ample grazing were available for the cavalry, valuable resources that can be scarce in this mountainous landscape.

Microbiology

Alpha diversity analysis through Chao1 indices, average species diversity and Shannon diversity analysis (see Table 1) of nine samples collected at equidistant 5-cm intervals in the G5a profile of the mire suggested that our sequencing results are representative of the total eubacterial population within the depth range—with typically 2000+ gene sequences retrieved per sample analysed.

Comparison of beta diversity between samples suggests very clearly that there are major differences between eubacteria detected in the 40–45 cm depth range when compared to the other samples, and that bacterial populations are more closely related in adjacent as opposed to more distant samples throughout the total depth sequence (Fig. 4). Samples were compared in depth order from the 15 cm sample to the 60 cm sample depth. These data indicate that there is a clear sample depth-related trend, suggesting that the 16S rRNA gene populations in adjacent samples are more closely related to each other than they are to the other gene populations analysed in this set. This is consistent with the conclusions that (a) the samples are taken from a single core/section at regular depth intervals, and (b) that there is no cross-contamination between the samples. One sample pair that may not follow this sample depth trend is the 20–25 cm and 25–30 cm samples. However, both samples are clearly relatively closely related.

Of particular note is a maximal abundance of 16S rRNA genes from the Clostridia class in the 40–45 cm sample of the MAD bed, representing the period dated to Hannibal's transit. While Clostridia are well-established soil bacteria, it is surprising to find representatives of this group comprising more than 12% of the total bacterial flora in this specific sample. Previous studies suggest that Clostridia are rarely found in soil above 2–3% of the total eubacterial population (Jansen 2006). It is also important to note that fractions analysed at sediment depths above and below the 40–50 cm range had a much lower relative distribution of Clostridia sequences, mostly

less than 2% of sequenced 16S rRNA genes in each sample. Furthermore, the abundance of Bacilli is notably lower than Clostridia in the 40–50 cm fractions—which suggests that these Firmicutes arose from mammalian faecal deposition (Harmsen *et al.* 2002; Ley *et al.* 2006; Steelman *et al.* 2012). Typically, horse gut bacterial flora is comprised of +70% Clostridia (Costa and Weese 2012). The Veillonellaceae family also constitutes 6% of all 16S rRNA gene sequences in this sample, which is consistent with the hypothesis that gut-derived bacteria have survived in these sediments (Ley *et al.* 2006). While we cannot presently be certain that the Clostridia detected are associated with endospore-forming gut bacteria, the detection of increased levels of bile acids and faecal lipid biomarkers in the same layer provides compelling evidence in favour of this hypothesis. The relatively low numbers of other known faecal bacteria observed in the 40–45 cm layer may actually provide further evidence to strengthen this hypothesis, as their absence could be a result of the lower stability of non-endospore-forming gut bacteria over the ~2000-year time frame since deposition. If the changes observed in the MAD bed were the result of a recent faecal deposition event, we would expect to see corresponding increase in Bacilli and other gram-negative bacteria associated with mammalian gut, which is not observed (see Fig. 5).

CONCLUSIONS

The bioturbation event coupled with inordinate faecal deposition, still registered today by a residual organic carbon increase and the presence of atypical levels of faecal biomarkers and abundant Clostridia 16S rRNA gene sequences in metagenomic analysis specifically linked to sediments dated to the time of Hannibal's invasion, provides some of the first direct evidence of the route followed by the Punic Army. Given that frost churning of soils in the area is not known to produce convoluted horizons (Mahaney *et al.* 2013) and, further, that organic materials are good insulators to avoid freezing if snow covered, only progressive animal disruption (including humans) of organic sediment is likely to have produced the bioturbated bed described here. This analysis of the MAD bed at G5a and G5b provides the most direct evidence yet uncovered to date for the passage of Hannibal's army.

It appears that the fibrist/hemist soil boundary falls near the end of the MWP and is marked by the highest concentration of biomarker compounds, but relatively low values of the faecal stanol ratio and levels of Clostridia. High organic matter input (without bioturbated layers) and these biomarkers (Fig. 3 (a)) may register long-lived peak transhumance occurring in the high alpine environment during and near the end of the MWP, in contrast to the heavy environmental impact in *c.*2168 cal yr. BP.

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