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B. CECCANTI (*), A. LANDI (**), F. BARTOLI (**), F. MALLEGNI (**),
G. MASCIANDARO (*), A. CARMIGNANI (*), C. MACCI (*)

STUDY AND CONTROL OF THE GEOCHEMICAL PROCESSES RESPONSIBLE OF DIAGENETIC ALTERATION OF ARCHAEOLOGICAL BONES

Abstract - Many analytical methods have been proposed to study the diagenetic modification of buried soils and bones but not all of them were efficient and conclusive. The objective of this work was to reproduce in the laboratory the conditions to enhance the diagenetic alteration of archaeological bones that might have been induced by soil pH, moisture changes, soil mineral particles (clay-silt-sand) intrusion and reaction into bone pores, by circulating directly soil suspensions and acid buffers through bone porosity. Changes in bone chemistry and mineral soil deposits in the bones during incubation were measured by traditional elemental analysis, SEM-EDX, Pyrolysis Gas-chromatography (Py-GC). The penetration of the soil mineral silt-clay fraction into the bone caused an increase of microbial activity which accelerated collagen protein degradation as demonstrated by the analysis in Py-GC. Combining the analysis of Py-GC, SEM-EDX and AAS, it was possible to asses the preservation status of bone and processes affecting the mineral-organic structure of bone during burial.

Key words - Bone diagenesis, trace elements, phosphorus, experimental approach.

Riassunto - *Studio e controllo dei processi geochimici responsabili dell'alterazione diagenetica delle ossa di interesse archeologico*. Numerosi metodi analitici sono stati proposti per lo studio delle modificazioni diagenetiche di suoli e ossi sepolti, ma non tutti si sono dimostrati efficienti e decisivi. L'obiettivo di questo lavoro è stata la riproduzione in laboratorio di condizioni atte ad aumentare l'alterazione diagenetica che può essere stata indotta nelle ossa di interesse archeologico dal pH del suolo, da variazioni di umidità, intrusione e reazione nei pori di particelle minerali (sabbia-limo-argilla), facendo circolare sospensioni e tamponi acidi direttamente nella porosità dell'osso. Le variazioni della composizione chimica dell'osso e dei depositi di frazioni minerali di suolo entro l'oso durante l'incubazione sono state misurate per mezzo di analisi elementare tradizionale, SEM-EDX/microsonda elettronica, Py-GC/Gas-cromatografia a pirolisi. La penetrazione della frazione limo-argilla del suolo nell'osso ha provocato l'incremento dell'attività microbica accelerando la degradazione del collagene, come dimostrato dall'analisi Py-GC. Combinando tutti questi metodi è stato possibile stabilire le condizioni di conservazione dell'osso e identificare i processi che hanno modificato la struttura organo-minerale dell'osso durante il seppellimento.

Parole chiave - Diagenesi dell'osso, elementi in traccia, fosforo, approccio sperimentale.

INTRODUCTION

Many methods and specific parameters have been proposed to study the diagenetic modification of buried soils and bones from organic burial environments. While organic tissues (animal, plants etc.) may decompose in buried soils and transform from large-sized into unidentifiable small-sized fragments or amorphous organic humus, organo-mineral bones may change in composition as elements in the surrounding matrix equilibrate with the bone. Changes depend on soil physical and microbiological reactions, which are conditioned most by the oxido-reduction potential, pH and moisture level (Stain, 1992). Paleodiet research, bone dating and paleopathology studies, as studied also through the chemical composition and conservation of bone tissue, may be negated by a severe post-depositional alteration. Diagenetic change of buried bones was thought to involve the periosteal surface of the outer layers in a cross-section of the bones; these hypotheses have led some authors to suggest that the outer layer of cortical bone be completely removed if bones were used for isotopic or amino acids analysis (Pfeiffer & Varney, 2000), or that bone had to be corrected for those elements incorporated with time by physical deposition of silt-clay soil particles into bone diagenetic microcavities (Fig. 1a, b) (Ceccanti *et al.*, 1994). This method permits to know the amount of elements in the silt-clay fraction and to subtract it from the total amount of elements found in the diagenised bone. At present, the assessment of bone preservation based on a single criterion is not completely satisfactory and often is elusive; more reliable criteria should be based on the analysis of both organic and inorganic bone structures (Hopkins, 2000).

OBJECTIVES

The objective of this work is i) to assess the diagenetic alteration of ancient human bones excavated from two different burial environments and, ii) verify if they are susceptible of further chemical alteration following incubation with the soil mineral-organic components, under controlled conditions in laboratory experiments and, iii) find a relation between bone structure alteration and losses or increase of the trace elements usually adopted to investigate in-vita paleodiet.

(*) Istituto per lo Studio degli Ecosistemi, sez. Geochimica del Suolo, CNR, via Moruzzi 1, Pisa, 56124, Italy.
(**) Dipartimento di Scienze Archeologiche, Università di Pisa, via S. Maria 53, Pisa, 56100, Italy.

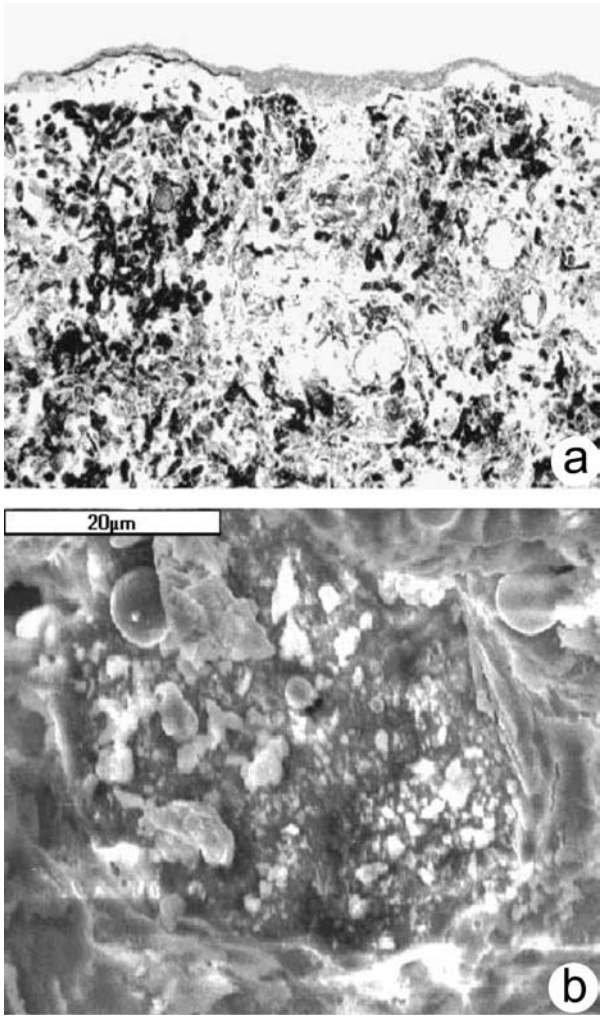

Fig. 1 - a) Light microscopy of a thin section of bone GH 14 showing soil particles partly layered on the external surface and partly permeated into the bone microcavities. Some Havers channels containing anhedral calcite can be observed. PPL, 10x. b) Scanning electron microscopy (SEM-EDX) image showing deposits of soil mineral particles into CD S27 bone cavity.

Materials and methods

Archaeological sites and bone material

Two human femoral bones were used, one (CD S27) collected from a tomb in the Castle of Donoratico (Tuscany region) and subjected to periodic groundwater excursions and soil activity; the other (GH 14) was taken in the Church of S. Francesco in Pisa from a tomb situated in a void space under the Church floor, which was not in direct contact with soil but subject to periodic infiltrations of muddy water during the floods of the Arno river through centuries.

These two different *post-mortem* environments might have differently influenced the integrity and susceptibility of the bone. For this reason the study was aimed to investigate the dynamics of those processes further modifying the collagen and mineral structures of bone, under specific microenvironments artificially recreated in the laboratory.

Fresh bone is not appropriate for studying diagenesis because of the interference caused by the releasing of soluble mineral-organic compounds from the mineralization of easily-degradable bone structures. Experiments with fresh bone would have required 1-2 years mineralization in a re-created laboratory environment before drawing conclusions on the diagenetic pattern of paleodietary elements. In addition, these experiments did not match our scientific goal of investigate and compare mechanisms that might have damaged bone stoichiometry and paleonutritional element diagenesis in their natural environments.

Experimental approach

The bone coming from the Church in Pisa was incubated in laboratory with a sandy soil (pH 5.4) (T.50) taken from the biologically active top-layer (0-30 cm) of an agricultural soil in Tuscany (Leghorn Province). Soil pH and microbial activity are considered the most active soil characteristics affecting diagenetic changes in mineral and organic structural composition, since bone is composed mainly of calcium phosphate (hydroxyapatite) and collagen proteins. Fine sand (250-50 µm), silt (50-2 µm) and clay (< 2 µm) fractions were separated by sedimentation of a water-soil suspension, according to the Stokes law. The soil sample was previously sieved through a 2 mm sieve, then soaked in deionised water overnight to disaggregate soil particles. To separate the clay-silt-fine sand fraction from the > 250 µm coarse sand (which is less important for diagenetic deposition because not capable of entering the bone pores), the freshly stirred sediment suspension was allowed to stand exactly 2 minutes.

Part of the suspension was decanted in a separate beaker for 9 minutes to separate fine sand from the clay-silt fraction. Part of this clay-silt suspension was decanted again for 16 hours to separate clay from the silt fraction. Each gravimetrically prepared fraction was suspended (1% weight to volume) in distilled water and separately filtered through a micro core of bone removed from the femur by a coring tool. The water suspension filtered rapidly through the bone disk (1-2 minutes) leaving part of the mineral deposits on the surface, and part inside the bone microcavities (Fig. 1a, b). The filtration system is schematised in Figure 2a; the bone disk was put in a filter-holder at the bottom of a graduated pipette and assembled on an erlenmayer flask with side arm for vacuum pumping.

This experimental approach allows reproduction of three concomitant effects taking place during burial: a) the passive deposition of elements into the bone with soil mineral-organic components; b) the leaching of bone elements with the flushing water of the soil suspensions and, c) the reaction of soil minerals and microorganisms with bone organo-mineral structure. The filtration was repeated weekly for 5 weeks, from 28th June to 2nd August; the weekly frequency of load-

ing of soil fractions allowed soil particles and bacteria to accumulate into the pores, and to react here with the bone matrix. The change of soil-bone microenvironment from saturated to dry conditions between the filtration intervals, stimulated microbial decomposition of structured collagen. In the experiment with the bone excavated from the soil in the Castle of Donoratico, a closed batch system was used to keep the bone in a biologically active, permanently moist environment (Fig. 2b).

The batch consisted of a sealed plastic cylinder in which a segment of femoral bone of the individual SD S27 was vertically oriented and blocked at the bottom by araldite resin. The void space between the bone and plastic wall (outer chamber) was filled alternatively with soil or buffer solutions. Since the excavated skeleton was supposed to have suffered diagenesis from surrounding soil in the past, a femoral segment was submitted to washing cycles with citrate buffer solutions at pH 6.0 followed by pH 4.8, in order to give quantitative measurements of a selective removal of P by pH.

A second bone segment was left to react with a microbiologically active calcareous soil T.26 (0-30 cm topsoil), which was deposited into the outer chamber of the batch system; thereafter, the soil-bone system was saturated with distilled water and pressurized at 3 bars. The saturated and pressurised environment caused a close contact between bone and soil mineral fractions finely dispersed into the bone porosity, as revealed by scanning electron microscopy SEM-EDX analysis (Fig. 1b). This contact gave rise to a concomitant diffusion of diagenetic elements from or towards surrounding soil. The goal was to enhance – under controlled laboratory experiments – the simulation of diagenetic reactions that might have induced changes in the physical-chemical properties of bones during burial, and to confirm whether soil mineral particles and microbial degradation of the collagen could induce consistent modification in the bone chemistry and, consequently, in its archaeological interpretation.

ANALYSIS

The water solutions flowing out the bone GH 14 micro-disks (dynamic system) or buffer solutions rinsing the bone femoral segments SD S27 were analysed for P content before and after incubation. Changes in the collagen structure were evaluated by means of analytical pyrolysis, which thermically decomposes (750°C) the collagen fibrils into several volatile low-molecular-weight organic compounds whose relative abundance was determined coupling the pyrolyser to a gas-chromatographer (Py-GC). Ion liquid chromatography (DIONEX method) was employed to analyse phosphate ions produced in the mineralization of organic phosphorous compounds, or to analyze inorganic phosphorous released by degradation of mineral hydroxyapatite. Total mineral P was measured spectrophotometrically with molibdate-antimonium tartrate method after acid perchloric-sulphuric digestion (Olsen method). The histological section of bone GH 14 and the surface scanning of bone CD S27 were evaluated through light microscopy observation and SEM-EDX, respectively. Soil analyses were performed according to standard methods (SISS, 2000). The main soil characteristics are reported in Table 1.

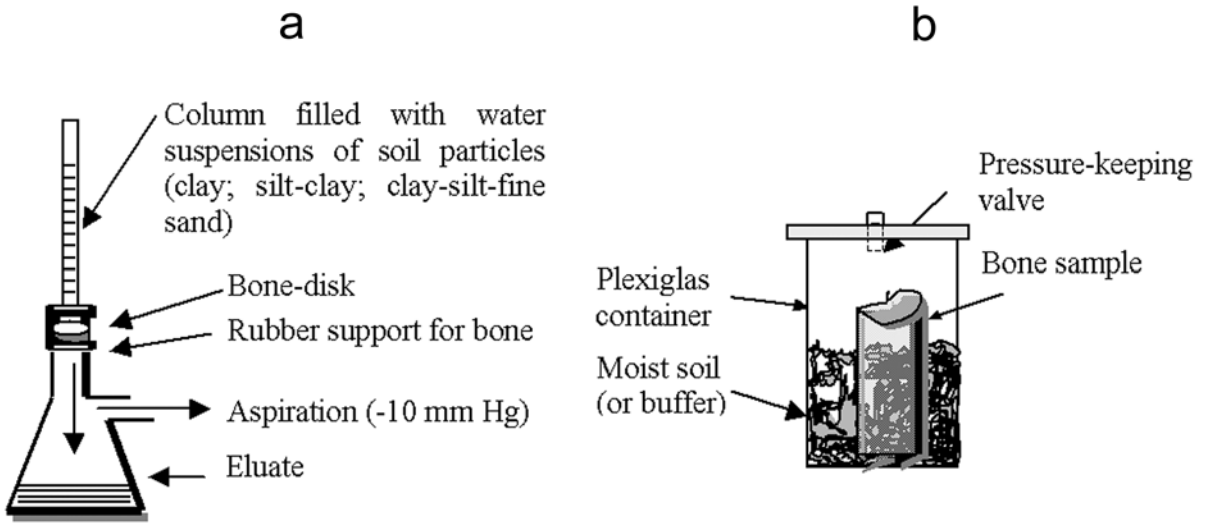

Fig. 2 - Incubation of fragments of femoral bone of two individuals with soil and soil mineral fractions. a) dynamic unsaturated (wet-dry cycles) system to permeate water suspensions of selected soil mineral particles into the bone-disk porosity; b) air pressurized, permanently moist (saturated), soil (or buffer)-bone batch system.

Tab. 1 - Soil characteristics.

	Soils	
Parameters	**Acid T.50**	**Calcareous T.26**
pH (1:10 v/w-H_20)	5.4	7.7
Clay %	10.2	9.8
Silt %	35.8	42.2
Sand %	53.2	48.0
Calcium carbonate %	-	26.0
Microbial activity (activity of the enzyme dehydrogenase, µg INTF*/g x h)	0.1	2.73
Organic Carbon %	0.50	0.16
Total Nitrogen %	0.074	1.44
Total Phosphorus (mg/kg)	214.8	202
Exchangeable Phosphorus (mg/kg)	19.8	6.3
C/N	7.97	8.92
Copper (mg/kg)	49.2	57.0
Manganese (mg/kg)	192	240
Iron (mg/kg)	12,630	23,345

* INTF: iodo-nitrophenyl formazan produced by reduction of the substrate (Iodo-nitrophenyl tetrazolium, INT) for dehydrogenase activity tests.

RESULTS

Preliminary study on the preservation status of the excavated bones

Before beginning the experiments, the femoral bone samples were analysed according Table 2 in order to evaluate the preservation status of bones through the calculation of total nitrogen-carbon percent and their C/N ratios (Stafford *et al.*, 1988; Pfeiffer & Varney, 2000; De Niro, 1985), assuming that changes in these elements demonstrate diagenetic changes in bone chemistry (Tab. 3).

By comparing our values of C/N and total N% (Tab. 1) with those reported in literature (Tab. 2), we assign a «good-modern», classes 1-2 preservation status to our femoral bones, which apparently indicate relatively small and similar diagenetic alteration, although the bones come from two very different burial environments.

Changes in bone chemistry

The changes in the mineral composition of hydroxyapatite in relation to changes of the collagen structure of both bone samples GH 14 and SD S27, were evaluated through the susceptibility of bones to loose inorganic-P (orthophosphate) ion and total-P (orthophosphate plus organic-P) following 5-weeks incubation with aqueous soil fraction suspensions under wet-dry conditions. The cumulative losses of both inorganic-P and total-P from the bone of individual GH 14 were assessed analysing the eluate collected at the bottom of the microcolumns, during the experiments of permeation of water soil fraction suspensions through the bone disk (Fig. 2a). The clayey fraction was the less active in releasing inorganic-P, whereas clay-silt was the most active, and even more active than a silt-clay-fine sand mixture (Tab. 4). Ceccanti *et al.* (1994) reported that the silt-clay fraction was that most contributing to bone contamination by physical deposition, and this caused diagenetic modification of bone chemistry.

The dynamics of P removal through time were reported in Figure 3.

Total and available P in the two soils have been reported in Table 1. The soil P content was in the range 200 µg/g (or 200 mg/kg), while it is known from the literature that the content of P in bone accounts for 10-20% d.w. (or 100-200 mg/g), *i.e.* 1000 times higher than soil P. Although the chemistry of P in the two soils may be supposed different under diverse incubation systems, soil-P certainly contributed very few or nothing to the overall P-chemistry (bone + soil mineral fractions).

The results proved that actually the continuous removal of the organic P through time was responsible for the release of significant amounts of inorganic P in silt-clay treatment but not in the other treatments; these, on the contrary, showed a peak of organic P removal only during the first week. As a consequence, total cumulative P increased linearly with time in the silt-clay experiment, while tended to a plateau in the other treatments. Although the dynamics of inorganic-P released with time resulted in different outcomes for each treatment, the results of P losses suggested that mineral hydroxyapatite was chemical destructurated as a consequence of organic collagen mineralization. This suggests that the organic collagen matrix was actually involved in P (and probably other elements) release from diagenetically

Tab. 2 - Analysis of total organic carbon and nitrogen and C/N ratios in the femoral samples of the two individuals before carrying out laboratory experiments.

Human bone	C %	N %	C/N
GH 14	3.57	1.51	2.36
CD S27	4.89	2.34	2.09

altered hydroxyapatite. The analyses of those elements which are usually considered important in paleonutritional studies are reported in Figure 4 (Fornaciari *et al*., 1988; Ceccanti *et al*., 1984).
The element contents were expressed as g/g bone-ash at 600°C, while Ca and P were expressed as mg/g for graphic comparison; these elements showed a decrease related to the type of soil mineral fraction, and in the order: silt-clay > clay, control > silt-clay-fine sand. Silt-clay-fine sand fractions showed the lowest values, with some exceptions probably due to the occlusion of pores by sand grain deposits which impeded further migration of smaller-sized soil silty and clayey fractions into bone microcavities. These results have demonstrated unequivocally the significant contribute of silt-clay fraction to bone contamination and diagenetic removal of elements as found by Ceccanti *et al*. (1994) studying a pool of 60 bone samples and their buried soils. However, since the Ca/P ratios calculated from data of Figure 4 varied from 2.00 to 2.25, *i.e*. in a range not far from the biological standard value of 2.15 (White & Hannus, 1983), bone GH 14 was apparently not severely diagenised.

Elemental analysis
The elemental analysis of the treated bone CD S27 coming from the soil burial excavation (Donoratico Castle) showed a decrease of element concentrations with respect to untreated bone sample (Fig. 5). This demonstrated that there was a migration of diagenetic elements from the bone towards the surrounding soil, as frequently reported in bibliography (Lambert *et al*., 1984, 1985). The «reverse» migration of elements back to the soil might be due to the exceptionally high concentration of soluble forms of organic and inorganic P measured on the external surface of bone, as reported in Figure 6 (more evident at pH 4.8), that created a gradient diffusion when soil was added. These P forms probably were responsible of the secondary depositions and accumulation of soil elements into bone matrix during post-mortem burial period. The acid washing of diagenetic material from bone samples is recommended before proceeding to the analysis for archaeological interpretation (Price *et al*., 1992). The Ca/P ratios before and after citrate buffer treatments were 1.67 and 2.19 respectively, meaning that the removal of diagenetic material was possible and this allowed regeneration of the near-original Ca/P ratio of 2.
The diagenetic alteration as shown on the basis of the low Ca/P ratio 1.67 was not evidenced by the analysis of collagenic organic C and N percentage and their ratios (Tab. 2). Collagen and probably collagen-associated humic-proteic compounds (also defined as «tanned protein») may better reflect the diagenetic status of the organic matrix of an archaeological bone and its evolution under laboratory incubation (Hopkins, 2000).

Pyrolysis
The Py-GC technique (Ceccanti *et al*., 1986), gives the relative abundance of volatile pyrolitic nitrogenated compounds pyrrole and acetonitril (Fig. 7). Pyrrole (O) is very likely produced from the pyrolitic decomposition of complex nitrogenated organic-materials (bacterial cells, structured collagen fibrils, some humic acids etc.), while acetonitril (E1) is prevalently formed during the decomposition of destructurated, partially hydrolysed collagen proteins, peptides and simple amino acids. There was, however, indication from the literature of the existence of not-collagenic structures (humic-protein, lipids, fats and carbohydrates) (Hopkins, 2000), so that other volatile pyrolitic fragments

Tab. 3 - Indicators of the preservation conditions of excavated archaeological bones (from Petchey: *et al*., 1988; http://www.c14dating.com/bone.html).

	Preservation classes (status)				
	1 (modern bone)	**2 (good)**	**3 (fairly good)**	**4 (bad)**	**5 (very bad)**
Total-N %	4.5-3.5	3.5-0.6	0.9-0.4	0.5-0.1	0.1 < 0.001
C/N ratio	2.9-3.6	3.5-4.0	4.0-4.5	4.5-6.0	> 6.0

Tab. 4 - SH 14 femoral bone. Five-week cumulative values of P losses (µg/g, dry bone ash at 600°C) from bone samples treated with soil particle suspensions.

Treatments	Inorganic P	Total P	Organic P (total-inorganic)
Clay	78	754	676
Clay-silt	878	6498	5620
Clay-silt-fine sand	304	626	322

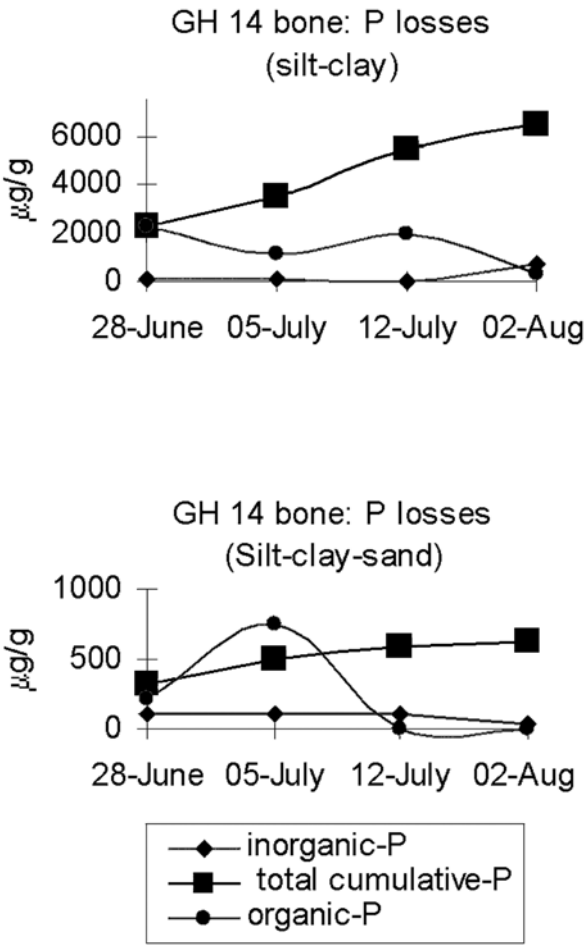

Fig. 3 - Phosphorous losses versus time, following incubation of bone GH 14 with water suspensions of different sized soil fractions.

were characterised; these were: acetic acid (K), Benzene (B), Toluene (E3), furfural (N) and phenol (Y), which prevalently originated from humic-protein compounds (B, E3, Y), fats and lipids (K), carbohydrates (N). As an example of the applicability of the Py-GC technique to bone collagen characterization, bone samples of experiments CDS27-T.26 incubated in batch system and GH14-T.50 (silt-clay fraction treatments) were considered, passed to pyrolysis and compared with their untreated control bones. The relative abundance of pyrrole + acetonitril (O + E1) in all pyrolised samples accounted for more than 50%, meaning that the collagen was still considerably structured and apparently well preserved in the two burial environments, thus confirming suppositions made on the basis of C and N elemental analysis. Due to their high relative abundance even after long time of burial (centuries), and considering the small variation after laboratory treatments, E1 and O pyrolitic fragments were considered not sensitive of collagen degradation. However, the relative abundance of the other specific pyrolitic fragments changed, giving evidence that those related to stable humic-proteic (B, E3, Y) and to lipidic and fatty (K) compounds were very sensitive, since they increased or decreased with respect to the control bone in function of the bone sample considered. Fragments related to humic-protein compounds benzene (B) and toluene (E3) increased with respect to the control in GH 14 but decreased in CD S27, while phenol (Y) decreased in both samples. These results suggested that diagenesis induced in the laboratory should have affected the mineralisable part of humified collagen represented by phenol Y and, but to a less extent, by acetonitril E1. In fact, the ratios B/E3 and O/Y – traditionally accepted as indices of the humification and mineralisation status of the organic matter (Ceccanti *et al.*, 1984) respectively – correlated very well (r: 0.996, $p < 0.5\%$) with the mineralisation index of collagen E1/Y and (E1 + O)/Y. These indices increased considerably after the treatments, meaning that the accelerated diagenesis had caused a further mineralization of collagenic structures giving rise to acidic semi-humified material which was capable of solubilising great amounts of organo-mineral phosphorous in GH 14 and mineral elements in CD S27. The mineral fractions of the acidic soil T.50, which were permeated in a dynamic system into GH 14 sample, and the calcareous soil T.26 incubated in a static aerated system with CD S27 bone, might have accelerated physical cracking, thus permitting a rapid colonization of microorganisms into bone microcavities where collagenase enzyme activity was previously triggered by collagen cleavage. Later, the proteolitic activity hydrolysed collagen-free proteins (Hopkins, 2000). The biological activity and the physical destructuration of bone gave rise to a considerable losses of mineral elements.

CONCLUSIONS

From the methodological point of view, the reconstructed bone-soil incubation experiments, along with the observation of several other parameters, and the combination of different techniques, have given new criteria for assessing archaeological bone diagenesis and mechanisms affecting bone structure during burial. The mineral structure of bone was weakened by the chemical and biological attack of organic (collagen and collagen-associated humic-proteic compounds) and mineral matrices, as demonstrated by the analysis in Py-GC and P-losses from hydroxyapatite. The dynamics of P losses suggested that mineral hydroxyapatite was chemically destructured as a consequence of organic collagen mineralization. This suggests that the organic collagen matrix was actually involved in P (and probably other elements) releasing from diagenetically altered hydroxyapatite. The clay-silt fraction was the most active in releasing mineral-P from diagenised bone (Tab. 4), even more active than the silt-clay-fine sand and clay fractions. This fraction is also responsible of the bone contamination by physical deposition that caused diagenetic modification of bone

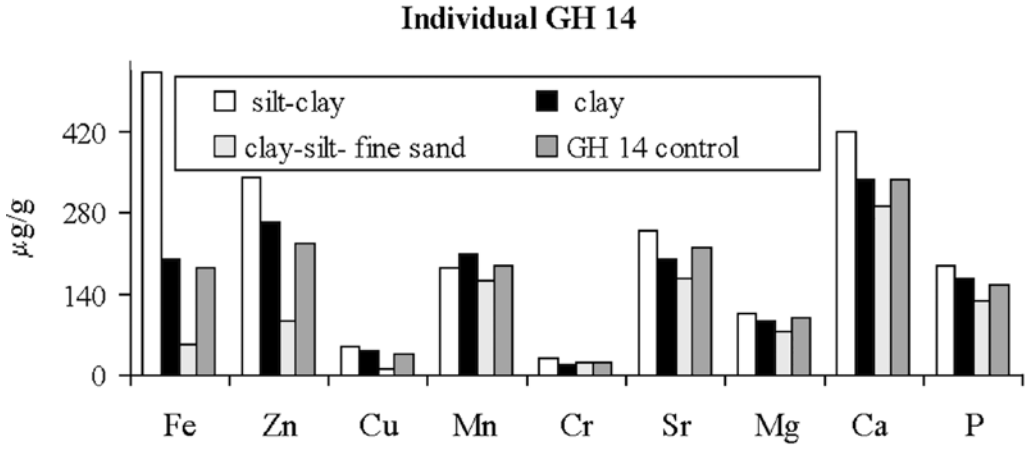

Fig. 4 - Distribution of minor (µg/g) and major elements Ca and P (expressed in mg/g, same axis) in the bone sample of individual GH 14 excavated in the Church of S. Francesco in Pisa, in function of the soil mineral particles permeated.

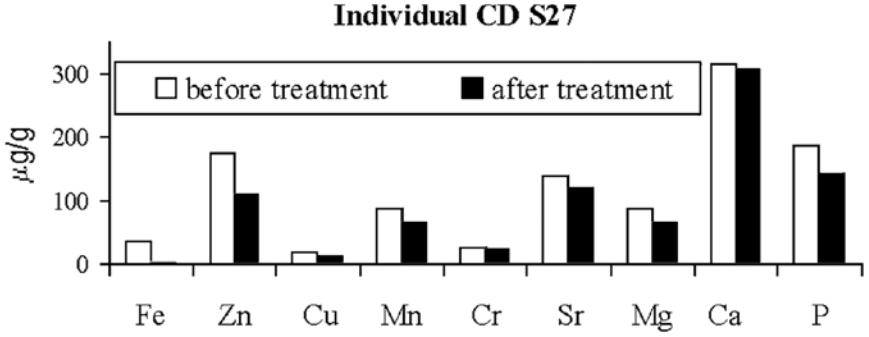

Fig. 5 - Dstribution of minor (µg/g) and major elements Ca and P expressed in mg/g, same axis values) in the bone sample of individual CD S27 excavated in the Donoratico Castle (Leghorn Province), before and after treatment with soil T.26 in a static batch system, under a permanently wet-saturated environment.

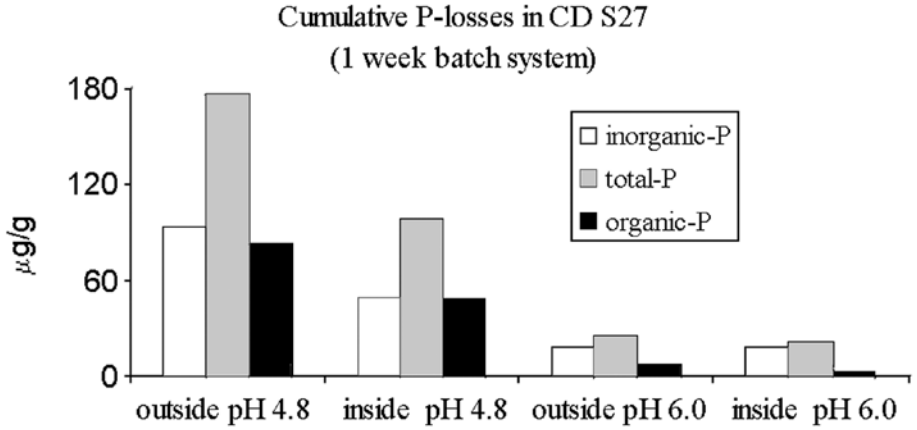

Fig. 6 - Phosphorous removal from outside and inside bone surfaces of CD-S27 sample by citrate buffer at different pH during 1 week incubation (cumulative values).

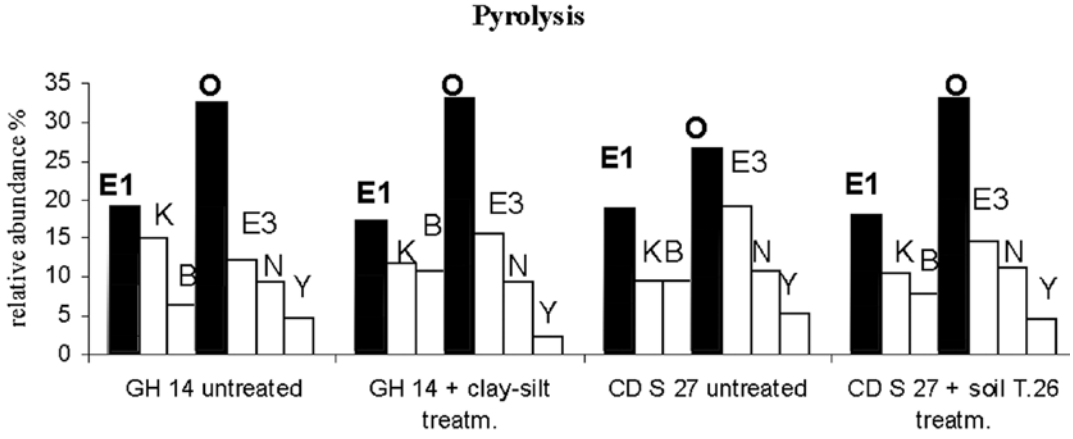

Fig. 7 - Relative abundance (%) of pyrolitic products before and after treatments of bone with soil T.26 and mineral fractions of soil T.50.

chemistry (Fig. 4). Physical deposition of soil particles may occur at the bone surface and inside (Fig. 1a, b). In both cases, the altered bone stoichiometry can be correct adopting the method suggested by Ceccanti *et al.* (1994) when the deposition took place inside, or making a pre-washing of bone with weak acid buffer when the deposition took place on the outer layer. The acid washing of diagenetic material has been recommended before proceeding to analyses for archaeological interpretation (Price *et al.*, 1992). In fact, our results showed that the Ca/P ratios before and after acid citrate buffer treatments (citrate buffer pH 4.8 or 6.0) were 1.67 and 2.19 respectively, meaning that the removal of diagenetic material was possible, and this allowed regeneration of the near-original Ca/P ratio of 2.
This study provides a valid analytical tool to assess the diagenetic status of bones in two different burial environments and to know, by combining conventional analysis with advanced Py-GC and SEM-EDX analysis, the dynamics of diagenetic processes taking place when soil mineral particles infiltrate into bone cavities.

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