Chapter 11
Bone Diagenesis at Azokh Caves

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Abstract Bone diagenesis is a set of processes by which the organic and mineral phases and the structure of bone are transformed during fossilization. To understand how these processes have affected skeletal material recovered from Azokh Caves (particularly the organic preservation), we measured 'diagenetic parameters' of skeletal material from Holocene, Late Pleistocene and Middle Pleistocene deposits from Azokh Caves. Additionally, we used this study to further test the application of both nitrogen adsorption isotherm analysis and mercury intrusion porosimetry for measuring the porosity of fossil bone. The skeletal material from the Pleistocene layers of Azokh Caves can be characterized as generally poorly preserved (especially collagen preservation). Porosity values of the bones are lower than might be expected as many bones show evidence of extensive infilling of the pores with secondary minerals. The pore infilling in the Middle Pleistocene layers is most extensive and this type of preservation has not previously been described in archaeological material.

Резюме Диагенез костей – это совокупность процессов, в результате которых органические и минеральные составляющие структуры кости трансформируются благодаря распаду и фоссилизации. Чтобы понять, как эти процессы воздействовали на скелетный материал, обнаруженный в Азохской пещере (и, в частности, оценить степень сохранности органических веществ в костях), были измерены определенные "диагенетические параметры" скелетного материала. Тридцать три кости из трех главных участков Азохской пещеры были исследованы для выяснения степени сохранности в зависимости от места находки и возраста образца. Голоценовый материал из *Азох 2* был сопоставлен с костями из *Азох 1* (подразделения *II–III* – поздний плейстоцен и средние горизонты подразделения *V* – средний плейстоцен).

Мы оценили количество коллагена, оставшегося в костях после деминерализации, и степень сохранности минералов с использованием метода FTIR (инфракрасная спектроскопия на основе преобразования Фурье). Изменения на поверхности костей и гистологическая структура поперечного сечения были исследованы с помощью обычного светового и сканирующего электронного микроскопов с электронной информационной системой (EDS). Степень гистологической сохранности была оценена с использованием шкалы *Oxford Histological Index*. Изменения в пористости костей были измерены с помощью изотермального анализа поглощения азота (NAIA) и ртутной интрузионной порометрии (HgIP), а результаты этих двух методов в дальнейшем были сопоставлены.

Согласно величинам "диагенетических параметров", материал из *Азох 2* представлял собой смесь из хорошо сохранившегося материала и костей, которые лишились коллагена химическим путем, а также некоторых костей, потерявших коллаген из-за микробного воздействия. Мы объясняем этот конгломерат различных типов сохранности как возможный результат смешения современного и ископаемого материала на поверхностных слоях *Азох 2*. Скелетный материал из плейстоценовых слоев *Азох 1* в целом плохо сохранился. Содержание коллагена бедное, с большими изменениями в кристалличности структуры. Результаты гистологического исследования и анализа на пористость показывают, что во многих случаях кости лишились коллагена по причине химической деградации, хотя

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потеря коллагена, вызванная микробами, также может быть значительной, особенно в подразделениях *II–III*. Степень пористости костей оказалась ниже, чем ожидалось, учитывая показатели потери коллагена и микробного воздействия. Многие кости имеют обширную заполненность пор вторичными минералами. Содержание пор в среднеплейстоценовых горизонтах наиболее экстенсивное, и данный тип сохранности ранее не был описан в археологическом материале.

Обнаруженные уровни коллагена как показателя сохранности органического материала свидетельствуют о низком содержании древней ДНК (*aDNA*) в пещере; более того, сильно измененные минералы костей также оставляют мало надежд на сохранность *aDNA*.

Данное исследование представляет собой интересный пример сравнения двух методов измерения пористости. Оно показало, что поры диаметром ниже порога чувствительности метода HgIP, но исследованные с помощью NAIA (с диаметром пор меньше 0,1мкм), возникли по причине потери коллагена; они заполняются таким же образом, как и поры диаметром 0,01–0,1мкм.

Keywords Diagenetic parameters • Mercury intrusion porosimetry • Nitrogen adsorption isotherm analysis • Collagen • Histology • Fossilization

Introduction

Diagenesis is the process of physical, chemical, and biological changes of sediments after their deposition. The term can also be applied to bones as part of a soil component deposited at an archaeological or paleontological site and the term 'Bone diagenesis' can be used to describe specifically the changes that bones undergo during fossilization. Bone is a composite biological material with a complex structure and is composed principally of bone mineral (bio-apatite) and the tough fibrous protein collagen (about 25% by weight in fresh bone). Typical diagenetic changes include the degradation and loss of organic matter such as collagen and DNA, changes in the bone mineral, and often microbial destruction of the morphological structure (which also alters the organic and mineral components) (Collins et al. 2002). Increases in the bone porosity are also common as a result of these diagenetic changes (Hedges et al. 1995).

It is important to understand how and why diagenetic changes take place, as they control the formation of the archaeological and fossil record as a whole. Understanding the reasons why bones do or do not survive in particular sites helps to improve site prediction and detection, and can help develop *in situ* heritage site protection strategies (Kars and Kars 2002). Moreover, archaeological bones are used for laboratory analyses such as radiocarbon dating, stable isotope analysis and ancient DNA studies, and it is imperative to understand how diagenetic changes affect the quality of this data.

There are many factors that influence the types and rates of diagenetic changes to bone (Hedges 2002). The intrinsic factors (the properties of the bone itself) need to be considered; for example different skeletal elements have different structural properties (and these vary with species, sex and age) and will have different proportions of collagen and mineral at a micro-scale. The soil environment in which the bone is deposited will also have a major influence on the diagenetic processes. Sediment conditions, such as, soil chemistry, pH, redox potential of the soil, and temperature as well as water interaction with the bone especially site hydrology (Hedges and Millard 1995), are major factors. The results of bone degradation vary from complete destruction, to fossilization where the organic material is degraded and the mineral heavily altered. Between these two extremes is a spectrum of preservation types that depends on the factors mentioned above, history of deposition and age of the material.

The number of factors that influence diagenetic processes and the length of time that they take means that they cannot be easily replicated in laboratory conditions or field experiments, so often the process of studying bone diagenesis relies on the examination of the properties of the bones excavated from sites and relating these to the properties of the sediments and history of the site.

A popular mode of investigation has been to measure 'diagenetic parameters' of bones in order to characterize the physical and chemical characteristics of the material, i.e.; mineral alteration, collagen loss, micromorphological preservation and pore structure changes of bone (e.g., Hedges et al. 1995; Colson et al. 1997; Gutierrez 2001; Trueman et al. 2004; Smith et al. 2007). These parameters can be compared with each other in order to observe how the different aspects of bone degradation are related. Furthermore, the characteristics of bones from a site can be compared with each other, and with bones from other sites, and these can also be related to the specific depositional contexts and histories of the bones in order to build models of diagenetic trajectories and processes (Hedges 2002).

Building on the diagenetic parameter approach pioneered by Hedges et al. (1995), Smith et al. (2007) described four major types of bone preservation in European Holocene deposits, based on their diagenetic parameter values (see also Nielsen-Marsh et al. 2007). Figure 11.1 displays example pore structures (a plot of pore volume against pore diameter, determined by mercury intrusion porosimetry) as well as typical diagenetic parameter values of the main diagenetic types (after Smith et al. 2007). In brief the 'Well Preserved Bone' category has diagenetic parameter values similar to those of modern bones. A second category of bones are those

that have undergone 'Accelerated Collagen Hydrolysis' (ACH), where the bones have only small amounts of collagen remaining and often extreme mineralogical changes, but no evidence of histological damage caused by microbes. Notably these bones have a significant increase in their pore volume in the smallest pore range (~0.01–0.1 μm diameter). Bones that have undergone 'Microbial Attack' have porosity increases in the >0.1 <10 μm diameter pore range and damage to the histological structure of the bone caused by microbes and fungi (semi quantified in a histological index, from 5-unaltered to 0-heavily damaged). Collagen yields of the microbially damaged bone vary ranging from 0 to 20% by weight and there are some mineralogical changes. It should be noted that the ACH type and microbial attack appear to be mutually exclusive pathways of diagenesis.

A fourth type of preservation described is bone that is undergoing 'Catastrophic Mineral Dissolution'. These bones tend to be poorly preserved in most aspects with large pore structures, low collagen yields and high levels of mineral alteration, but with variable levels of histological damage. This research has indicated that some bone degradation processes such as microbial attack (Jans et al. 2004) or accelerated collagen loss (Smith et al. 2002) can occur rapidly post-mortem and that these processes can lead to extensive changes in the diagenetic state of the bone in a short period of time. In contrast, under other circumstances very little change can occur over hundreds or even thousands of years and the bone remains in the 'Well Preserved' state. It is also important to be aware that these early stages of bone diagenesis can affect subsequent longer-term changes that occur in bone fossilization (Trueman and Martill 2002; Smith et al. 2007; Marin-Monfort et al. 2016). Besides helping us to understand the processes of fossilization and the formation of the archaeological record (at a site level and more generally), understanding diagenetic changes to the mineral and organic fraction of bone helps us to understand how these changes can affect the biogenic signals that they contain (i.e. isotopic and DNA data) and inform us as to where and for how long such information might be preserved in bone.

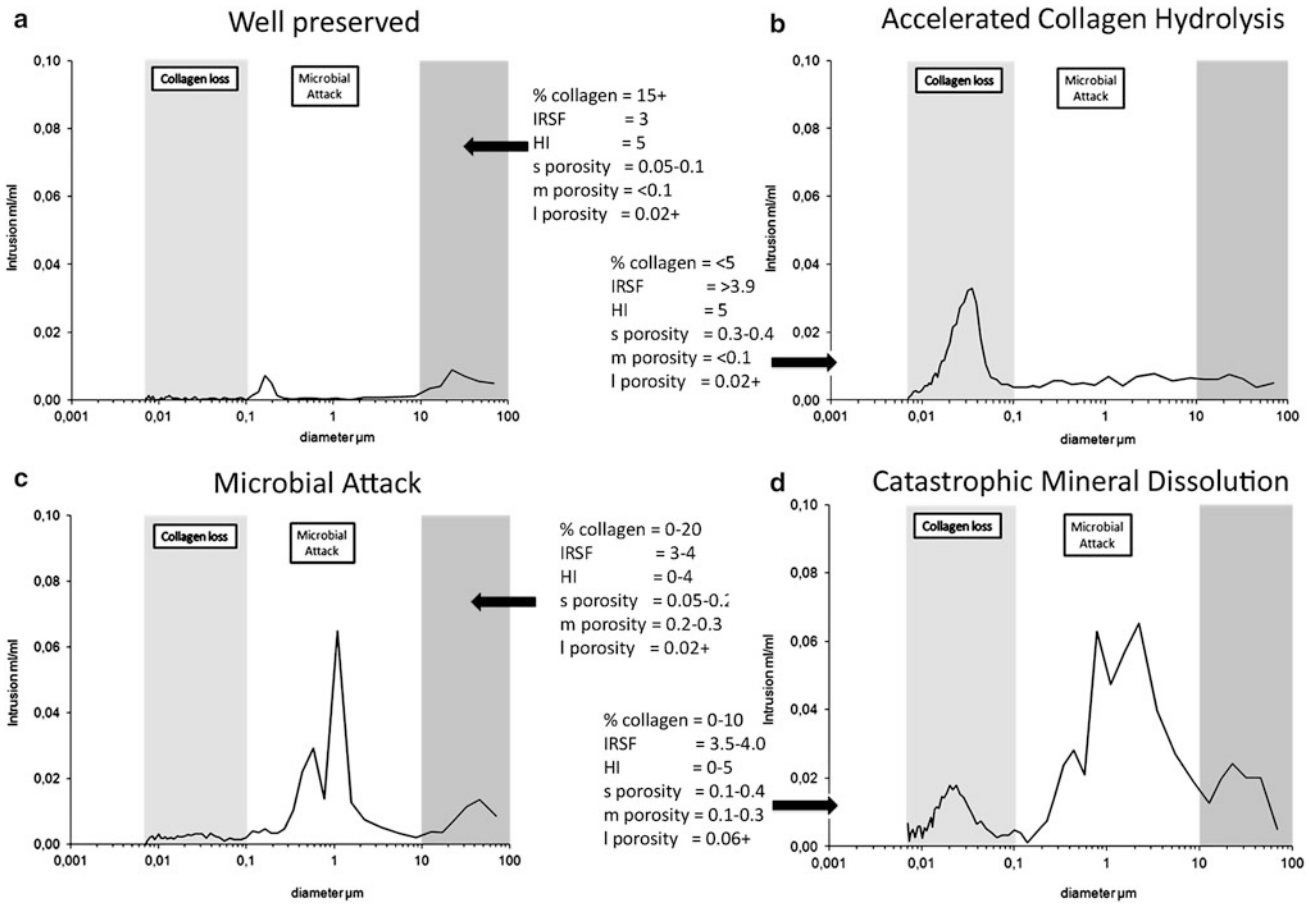

Fig. 11.1 Examples of typical pore size distributions (measured by mercury intrusion porosimetry) of four types of archaeological bone. **a** "Well preserved bone". **b** Accelerated collagen hydrolysis, **c** Microbially Attacked bone and **d** Catastrophic Mineral Dissolution (After Smith et al. 2007 and Nielsen-Marsh et al. 2007). Typical diagenetic parameter values are also given

Porosity as a Diagenetic Indicator

Measuring the porosity using mercury intrusion porosimetry (HgIP) has become a valuable tool in determining diagenetic changes, as plotting the pore size distribution provides a clear visual way of comparing bones and reveals the signature pore structures of the preservational types (see Fig. 11.1). HgIP does, however, have some disadvantages; firstly the minimum pore diameters that HgIP can measure are limited to around 0.005–0.01 μm and bone has a significant amount of porosity in pores of smaller diameter (Robinson et al. 2003), secondly, HgIP fills the bone sample with toxic mercury and is thus, in effect, destructive. A complementary method of porosity analysis that has been applied to archaeological bone is that of Nitrogen Adsorption Isotherm Analysis (NAIA) (Robinson et al. 2003; Smith et al. 2008; Bosch et al. 2011). This method is capable of measuring the volume in pores with sub nanometer diameters and leaves the sample of bone intact so that it can be used for subsequent analysis (e.g., HgIP, histological examination or sub sampling for other diagenetic parameters). NAIA is not useful for measuring the larger pore diameters associated with microbial attack, however it has been used to measure pores between approximately 0.0005–0.1 μm in a limited archaeological bone data set and shown that it also records changes occurring in ACH bone in the 0.01–0.1 μm range (Smith et al. 2008). NAIA has yet to be applied extensively to archaeological and paleontological bone but holds great potential in investigating changes in sub nanometer pore sizes that have yet to be explored in detail.

Bone Diagenesis at Azokh Caves

Azokh Caves site is located in the Lesser Caucasus (Fernández-Jalvo et al. 2010a). Azokh 1 yielded a Middle Pleistocene human mandible discovered in the 1960s (Kasimova 2001; King et al. 2016), and it was accompanied by an abundant contemporaneous fauna and human made tools. Detailed sedimentology and stratigraphy has been described by Murray et al. (2010, 2016). In 2002 excavation at the site was resumed by an international team which discovered two new entrances (Azokh 2 and Azokh 5), and which has extended the research into this interesting western Eurasian area from Holocene to middle Pleistocene (Fernández-Jalvo et al. 2016; see also Appendix of this volume).

Bone diagenesis at Azokh Caves was investigated in order to understand the general level of bone preservation at the site and to help to establish how this can contribute to the discussion about the poor aDNA preservation at Azokh (see Bennett et al. 2016). In addition it presented an opportunity to measure material from a Pleistocene cave site using the same parameters used by Smith et al. (2007). Smith et al. (2007) tested mainly Holocene open air European sites, so the characterization of diagenesis at Azokh is a useful addition to compare preservation at an older and contextually different site. Moreover, material was analyzed from Holocene, late Pleistocene and Middle Pleistocene layers from the site giving an overview of diagenesis over a period of approximately 300 kyr. It also enabled further testing and evaluation of a new method of investigating pore size distribution in archaeological bone with the application of combined nitrogen adsorption isotherm analysis with subsequent mercury intrusion porosimetry on the same sub-sample of bone. As mentioned above, this approach was first implemented by Smith et al. (2008) but has yet to be fully employed in diagenetic investigations.

Materials Analyzed

The skeletal material analyzed here was excavated from Azokh Caves during the 2003 field season (Fernández-Jalvo et al. 2010b, 2016). The material available for analysis was comprised of mainly unrecognizable fragments of bone (i.e. unknown species or element), so as not to destroy useful material that could be identified to species level using morphological characteristics. In addition some more complete bone pieces were also analyzed for diagenetic parameters as they were also analyzed for ancient DNA. There was no obvious macroscopic difference between fragmentary or more complete bones in terms of preservation (Marin-Monfort et al. 2016), and so we believe that the bones represent a faithful sample of the overall assemblage. Fossil bones were collected from three main parts of the site. In Azokh 1, Units II and III represent Late Pleistocene layers which date from around 100 ka to less than 200 ka (see Appendix, ESR). Bone was also excavated from Unit Vm from Azokh 1, which is a Middle Pleistocene layer and probably dates to approximately 300 ka. Bone from Unit Vm appears to be heavily fossilized. Bone was also sampled from the initial excavation of the surface layers at Azokh 2 (another entrance to the Azokh Cave system). Bone found on the surface of Azokh 2, or in the first 30–50 cm of test pit excavations, was also taken for analysis. Whilst anticipating that the majority of the material from Azokh 2 (from the 2003 season) is of recent modern origin, it was noted that some appeared to be heavily fossilized and it is believed that the top layers of the site are a mixture of recent and fossil material, where fossil material may have become mixed as the result of geomorphological cave collapses, producing a sediment mixture of different strata (Fernández-Jalvo et al. 2010b; Domínguez-Alonso et al. 2016; Murray et al. 2016). One sample was taken from the section between Unit III and Unit Vm (i.e. Unit Vu) from Azokh 1. Further descriptions of the material are given in Table 11.1.

Methods

Diagenetic Parameters

The material was analyzed using a suite of diagenetic parameters to measure collagen preservation (% 'collagen'), mineral alteration (IRSF and carbonate phosphate ratio), histological preservation (Oxford Histological Index), (Hedges et al. 1995; Smith et al. 2007 and references therein).

% 'Collagen'

Bone shards of known weight (<60 mg) were demineralized in 2 mls of 0.6 M HCl overnight in Eppendorf tubes. The tubes were centrifuged (at 6000 rpm for 5 min), the acid decanted, and the remaining acid insoluble residue was washed three times in 2 mls of distilled water under centrifugation. The acid insoluble fraction was then oven dried overnight at 65 °C, and weighed. Elemental analysis was carried out in duplicate to obtain the % carbon and nitrogen values to calculate the C:N ratio (molar ratio) to assess if the insoluble fraction is collagen (DeNiro 1985) with values between 2.9 and 3.6 being acceptable collagen values.

Crystallinity Index and Carbonate Phosphate Ratio

The crystallinity index and carbonate phosphate ratio of the mineral fraction was measured using infrared spectroscopy of hand ground bone powder crushed into a potassium bromide (KBr) pellet. The crystallinity index or Infrared Splitting Factor (IRSF) was calculated using the splitting ratio of the phosphate $\nu 4$ doublet at 567 and 605 cm^{-1} in the infrared spectrum following Weiner and Bar-Yosef (1990). The carbonate:phosphate ratio was calculated using the peaks at 1415 cm^{-1} (CO_3^{2-}), and 1035 cm^{-1} (PO_4^{3-}). It should be noted however that this measurement is only semi-quantitative as it can be interfered with by collagen that also absorbs in the 1415 cm^{-1} region of the spectrum.

Surface Modifications and Histological Analysis

Surface modifications were recorded with the naked eye and by examination using a binocular light microscope (10× to 80× magnification), and with an environmental scanning electron microscope (ESEM) QUANTA 200 housed at the Museo Nacional de Ciencias Naturales. Observations were made in backscattered electron mode, combined with secondary electron emission mode, at 20–30 kV, 0.6–0.33 Torr (Fernández-Jalvo et al. 2010a). Histological sections were prepared in the manner described by Fernández-Jalvo et al. (2010a) to produce polished sections of bone (fragile samples were embedded in resin while harder samples were polished without the need for resin support). The sections were examined using ESEM in backscatter mode to determine the extent of damage to the original bone histology caused by microscopic focal destructions and assigned a histological index score (Hedges et al. 1995; Millard 2001; Jans et al. 2004). Other observations were also noted (Table 11.2) and some areas were analyzed using energy dispersive x-ray spectroscopy (EDS) to determine the composition of inclusions or other notable features. Using the elemental compositions from the EDS analysis, possible secondary minerals were suggested in Table 11.2.

Pore Size Analysis Using Nitrogen Adsorption Isotherm Analysis and Mercury Intrusion Porosimetry

Samples of bone (approximately 1 g chunks) were cut from the main sample using an electrically powered circular hand saw at its slowest speed. Porosity analysis was carried out by nitrogen adsorption isotherm analysis (NAIA), which is non-destructive, and then by mercury intrusion porosimetry on the same piece of bone. The following pre-treatment was carried out so that the sample was dry prior to analysis. The samples were frozen at −20 °C for 18–24 h and then lyophilized (for at least 18 h), no more than 48 h prior to the analysis. After lyophilization the samples were stored in an airtight container until required. Immediately before analysis samples were degassed in a Micromeritics VacPrep 061 system for 20 h.

Nitrogen adsorption isotherm analysis was carried out at 80 K in a Micromeritics Tristar 3000 automatic system dosing nitrogen following a custom made pressures table. Equilibrium time and other parameters were optimized to assure the best assay reproducibility. Nitrogen adsorption isotherm analysis works by applying nitrogen to a sample, which adsorbs to the pore walls in a theoretical monolayer. Adsorbed nitrogen does not contribute to the pressure in the system and thus adsorption results in a pressure change. Changes in the partial pressure of nitrogen can be monitored and related to the surface area covered by the nitrogen. Larger pores are filled by increasing the partial pressure of nitrogen and thus at each pressure increment the volume of pores at a certain diameter can be calculated. Following B.J.H. theory (Barrett et al. 1951), the

pore size distribution and specific surface area can be calculated by knowing the volume of nitrogen adsorbed and its relative pressure. Using this technique we were able to measure the BET Surface Area m^2/g and the pore volume (cm^3/g) contained in pores of 0.001–0.1 μm diameters. Further descriptions of the technique and its application to bone porosity measurements can be found in Robinson et al. (2003) and Smith et al. (2008).

Following the non-destructive nitrogen adsorption isotherm analysis, mercury intrusion porosity analysis was carried out. No additional pre-treatment was required other than maintaining dry storage of the samples. A Micromeritics 9320 Poresizer was used for mercury intrusion porosimetry analysis, the volume of mercury intruded was measured following a customized pressure table from 0 to 30 000 Psi (0–2000 MPa). Mercury intrusion porosimetry has been used extensively to investigate bone diagenetic changes and details of the method can be found in Nielsen-Marsh and Hedges (1999), Smith et al. (2002, 2008), among others. Calculations were made using a mercury-apatite contact angle of 163.1° after Joscheck et al. (2000), and a mercury surface tension of 485 dyn/cm. The analysis produces bulk density values (density including pore space) and apparent (skeletal) density (density of the structure excluding pore space). The pore size distribution can be calculated indicating the volume of pore space within certain pore diameters. Table 11.1 gives the values for certain pore diameter ranges relevant to bone diagenesis after Smith et al. (2007).

Results and Discussion

The diagenetic parameter results can be seen in Tables 11.1 and 11.2. Diagenetic changes to bone can be compared with the typical values of modern bone in the tables.

Table 11.1 Surface modifications, collagen and mineral diagenetic parameter values of fossil bones from Azokh Cave

	Sample code	Site	Skeletal element	% Collagen (mean)	% Collagen (s.d.)	C:N ratio of 'collagen'	Crystallinity index (IRSF)	Carbonate: phosphate ratio (by IRSF)
Typical modern values								
	Typical modern bone	N/A	N/A	20–25%	2.0	3.2	2.8	0.40
Azokh 1 Units II–III	AZUM D46 181	Azokh 1 Units II–III	Calcaneous *Ursus spelaeus* (no apparent damage on surface)	0.0	N/A		3.4	0.30
	AZUM D46 2	Azokh 1 Units II–III	Long bone fragment	31.8	10.8	6.3	3.4	0.19
	AZUM D46 3	Azokh 1 Units II–III	Long bone fragment	0.1	0.4		4.3	0.03
	AZUM D45 25	Azokh 1 Units II–III	Radius *Ursus spelaeus*	0.8	0.3		3.6	0.25
	AZUM D45 4	Azokh 1 Units II–III	Long bone fragment	0.4	0.1		3.9	0.20
	AZUM D45 42	Azokh 1 Units II–III	Radius *Ursus spelaeus* (Mn deposit on fractures and bone/sediment surface)	0.2	0.0		2.9	0.47
	AZUM D45 9	Azokh 1 Units II–III	Calcaneous *Ursus spelaeus*	2.1	0.4	7.0	3.3	0.51
	AZUM-D46G	Azokh 1 Units II–III	Several fragments of various bones [Mn staining/carbonatic crust]	0.6	0.4		3.0	0.38
	AZUM-D46G 27 A	Azokh 1 Units II–III	Several fragments of various bones [Mn staining/carbonatic crust]	2.4	0.1	6.3	3.0	0.33
	AZUM-D46G 27 B	Azokh 1 Units II–III	Fragments	1.4	0.0	5.9	3.5	0.25
	AZUM-D46G 19-A	Azokh 1 Units II–III	Several fragments of various bones many 3–5 cm long [Mn staining and trampling]	1.4	0.9	8.7	3.0	0.33
	AZUM-D46G 19-B	Azokh 1 Units II–III	Fragments [trampling marks]	0.1	0.1		3.4	0.28
	AZUM-D46G 19-C	Azokh 1 Units II–III	Fragments	0.0	0.0		3.3	0.24
	AZUM-D46G 19-D	Azokh 1 Units II–III	Fragments [trampling, rounding, Mn staining]	2.6	1.4	7.0	3.5	0.24

(continued)

Table 11.1 (continued)

	Sample code	Site	Skeletal element	% Collagen (mean)	% Collagen (s.d.)	C:N ratio of 'collagen'	Crystallinity index (IRSF)	Carbonate: phosphate ratio (by IRSF)
Azokh 1 Unit Vu	AZU-Section	Azokh 1 Unit Vu	Long bone fragment [black staining, mainly trabecular bone]	0.0	0.3		3.5	0.26
Azokh 1 Unit Vm	AZM E39 1	Azokh 1 Unit Vm	Long bone fragment	0.9	0.5	6.1	3.4	0.20
	AZM E41 4	Azokh 1 Unit Vm	Long bone fragment	0.5	0.1		3.6	0.20
	AZM-E40G	Azokh 1 Unit Vm	Fragments [fibrous texture]	0.7	0.2	6.1	3.3	0.23
	AZM-E41G	Azokh 1 Unit Vm	Fragment of Mandible(?) [splitting and exfoliation on surface]					
	AZM-F42 9	Azokh 1 Unit Vm	Long bone (fragment) [heavily mineralized, fibrous surface]	0.6	0.3		3.9	0.17
	AZM-G41 5	Azokh 1 Unit Vm	Fragments	0.5	0.2		3.9	0.18
Azokh 2	AZN P11	Azokh 2		16.9	1.3	3.3	3.8	0.25
	AZN-Q10	Azokh 2	Long bone [modern root marking, shallow trampling marks]	7.5	0.8	3.3	2.9	0.43
	AZN-SL-HDU	Azokh 2	Metapodial [some skin still present, cut marks, one side weathered, the other not]	23.1	0.2	3.2	3.7	0.36
	AZN-SL-HWU	Azokh 2	Metapodial [surface corroded]	18.7	0.0	3.2	3.3	0.32
	AZN-SL-HDW	Azokh 2	Metapodial [moderately weathered, cracked surface]	21.7	0.1	3.2	3.4	0.31
	AZN-SL-HWW	Azokh 2	Metapodial	21.4	0.4	3.2	3.4	0.28
	AZN-SL-A	Azokh 2	Fragments [root marks]	0.9	0.1		3.4	0.34
	AZN-SL-B	Azokh 2	Epifysis [spots of gypsum and Mn stains]	2.6	0.1	7.1	3.5	0.32
	AZN-SL-C	Azokh 2		0.3	0.1		3.7	0.27
	AZN-SL-D	Azokh 2	Tibia/Fibia proximal end (?) epifysis [heavily mineralized corroded surface]	0.1	0.1		3.4	0.30
	AZN-SL-F	Azokh 2	Fragments of long bone [heavily mineralized, root marks]	0.8	0.1		3.4	0.31
	AZN-SL-G	Azokh 2	Fragments of long bone	0.3	0.1		3.4	0.28

Azokh 1 Units II–III

Units II–III at Azokh 1 are represented by heavily degraded bone, with low levels of organic preservation (none of which displays a collagen like C:N ratio (see DeNiro 1985)) and with the exception of a few samples can be characterized as having highly altered mineral (IRSF values are typically 3.4 or above and C:P values typically less than 0.3). It should be noted that a critical error appears to have occurred in the collagen extraction from sample AZUM-D46-2 that had two disparate values from the duplicate analysis, so this value should be ignored, as it is unreliable. The histological preservation varies in these deposits with some bones showing signs of extensive microbial attack (Histological

Table 11.2 Histological and Porosity values of fossil bones from Azokh Caves

Sample code	Site	Oxford Histological Index and Histology notes	BET Surface Area m^2/g (by N_2 Porosimetry)	Volume (cm^3/g) between 0.001 and 0.1 μm (N_2 porosity)	Volume (ml/ml) in pores <0.1 μm (Hg porosity)	Volume (ml/ml) in pores <10 >0.1 μm (Hg porosity)	Volume (ml/ml) in pores >10 μm (Hg porosity)	Total Porosity (ml/ml) (Hg porosity)	Bulk Density g/ml (Hg porosity)	Skeletal Density g/ml (Hg porosity)
Typical modern values										
Typical Modern Bone	N/A	OHI 5	0.2–1.4	0.002–0.008	0.05	0.02	0.0200	0.0900	1.90	2.10
Azokh 1 Units II–III										
AZUM D46 181	Azokh 1 Units II–III	OHI 5 Microfissures, most infilled by Mn. Sediment illite	101.4	0.2123	0.1791	0.0052	0.1472	0.3314	1.61	2.41
AZUM D46 2	Azokh 1 Units II–III		23.0	0.0993	0.1185	0.2710	0.0652	0.4547	1.01	1.85
AZUM D46 3	Azokh 1 Units II–III	OHI 5 Intense cracking. Abundant secondary osteons (pathology?) Canals filled by sediment	89.3	0.2880	0.3633	0.0417	0.0382	0.4432	1.28	2.29
AZUM D45 25	Azokh 1 Units II–III	OHI 5 Enlarged canaliculi, possibly mineralised by Mn	77.5	0.1964	0.2251	0.0225	0.0481	0.2957	1.63	2.31
AZUM D45 4	Azokh 1 Units II–III	OHI 0 Areas of organised and areas of chaotic bacterial attack all over. Radial and peripheral tubes on bacterial corroded areas (fungi?)	35.6	0.1133	0.1445	0.2518	0.0601	0.4564	1.39	2.56
AZUM D45 42	Azokh 1 Units II–III	OHI 4 Initial bacterial attack. Canals infilled by illite rich in carbonates, and also sulphur detected? gypsum? $Ca(SO_4) \cdot 2(H_2O)$	87.5	0.1958	0.2222	0.0072	0.0225	0.2519	1.74	2.33
AZUM D45 9	Azokh 1 Units II–III	OHI 5 Some osteones are infilled carbonatic minerals (calcite) and minerals enriched in phosphorous and sulphur related to bat guano breakdown. Corrosion of the cortical area	65.4	0.1290	0.1153	0.0524	0.1025	0.2702	1.73	2.37
AZUM-D46G	Azokh 1 Units II–III	OHI 5 Canals partially infilled clayish sediment rich in phosphorous	84.7	0.1715	0.1945	0.0075	0.0274	0.2294	1.92	2.49

(continued)

Table 11.2 (continued)

Sample code	Site	Oxford Histological Index and Histology notes	BET Surface Area m^2/g (by N_2 Porosimetry)	Volume (cm^3/g) between 0.001 and 0.1 μm (N_2 porosity)	Volume (ml/ml) in pores <0.1 μm (Hg porosity)	Volume (ml/ml) in pores <10 >0.1 μm (Hg porosity)	Volume (ml/ml) in pores >10 μm (Hg porosity)	Total Porosity (ml/ml) (Hg porosity)	Bulk Density g/ml (Hg porosity)	Skeletal Density g/ml (Hg porosity)
AZUM-D46G 27 A	Azokh 1 Units II–III	OHI 5 Porous bone with good histology. Mn infilling osteocites, also Ni has been detected with Mn(EDS)	80.6	0.1752						
AZUM-D46G 27 B	Azokh 1 Units II–III	OHI 5 Porous bone, osteocytes and small cracks infilled with Mn (EDS)	76.9	0.1529						
AZUM-D46G 19-A	Azokh 1 Units II–III	OHI 5 Porous bone though good histology. EDS sediment Illite	54.8	0.0705	0.0324	0.0042	0.0475	0.0841	2.22	2.42
AZUM-D46G 19-B	Azokh 1 Units II–III	OHI 0 Bacterial MFD surround Haversian canals at the outer cortical layer chaotic distribution. Radial and peripheral microtunnelling	33.7	0.0897	0.1235	0.0125	0.0288	0.1648	2.01	2.41
AZUM-D46G 19-C	Azokh 1 Units II–III	OHI 0 Similar to B, microtunnelling damaging bacterial attack (fungi?), peripherally dispersed	36.6	0.0933	0.0883	0.0034	0.0171	0.1088	2.14	2.40
AZUM-D46G 19-D	Azokh 1 Units II–III	OHI 2 Histological traits partially disappear, radial distribution of small tubes, fungi? from Haversian canals. EDS Barium sulfate (Barite), and illite (sediment). Enlarged canaliculi, mineralised	48.6	0.0647	0.0416	0.0127	0.0552	0.1096	2.31	2.60
Azokh 1 Unit Vu										
AZU-Section	Azokh 1 Unit Vu	OHI 5 Some canals infilled with clayish sediment rich in phosphorous	80.7	0.2268	0.2674	0.0124	0.0435	0.3233	1.44	2.13
AZM E 39 1	Azokh 1 Unit Vm	OHI 5 Mineralized osteons. Sediment (illite), manganese, Sn and Ti are also present. Mineral "acicular" shape with illites, Mn and unknown minerals with Zn, Ni. (EDS)	31.6	0.0378						

(continued)

Table 11.2 (continued)

Sample code	Site	Oxford Histological Index and Histology notes	BET Surface Area m^2/g (by N_2 Porosimetry)	Volume (cm^3/g) between 0.001 and 0.1 μm (N_2 porosity)	Volume (ml/ml) in pores <0.1 μm (Hg porosity)	Volume (ml/ml) in pores <10 >0.1 μm (Hg porosity)	Volume (ml/ml) in pores >10 μm (Hg porosity)	Total Porosity (ml/ml) (Hg porosity)	Bulk Density g/ml (Hg porosity)	Skeletal Density g/ml (Hg porosity)
AZM E41 4	Azokh 1 Unit Vm	OHI 5 Strong cracking on the outer and inner cortical area. Enlarged canaliculi	33.3	0.0437	0.0104	0.0005	0.0153	0.0262	2.47	2.53
AZM-E40G	Azokh 1 Unit Vm	OHI 4–5 Good histology except in a lateral side that has localized corrosion and enlarged canaliculi	55.8	0.0687	0.0254	0.0089	0.0186	0.0529	2.33	2.46
AZM-E41G	Azokh 1 Unit Vm	OHI 5 ESEM surface of rounded holes, of unknown cause. Nodules of Si (EDS). Heavily cracked	30.7	0.0376	0.0000	0.0000	0.0694	0.0694	2.11	2.27
AZM-F42 9	Azokh 1 Unit Vm	OHI 5 Haversian canals are infilled with secondary minerals of apatite. Enlarged canaliculi	4.2	0.0074	0.0000	0.0000	0.0515	0.0515	3.08	3.25
AZM-G41 5	Azokh 1 Unit Vm	OHI 4–5 Some strong and localized cracking, mainly at the outer cortical area, unknown origin, maybe mineral loss, Sediment illites and Mn Enlarged canaliculi on cortical	36.7	0.0461	0.0244	0.0378	0.0683	0.1305	2.44	2.80
Azokh 2										
AZN P11	Azokh 2	OHI 0 Chaotic distribution of bacterail attack. Slightly enlarged canaliculi	22.1	0.0678	0.0927	0.1515	0.1159	0.3602	1.30	2.05
AZN-Q10	Azokh 2		18.6	0.0486	0.0714	0.0108	0.0173	0.0994	1.76	1.96
AZN-SL-HDU	Azokh 2	Surface analysis, bacterial attack apparent	0.6	0.0026	0.0155	0.0239	0.0000	0.0393	1.91	1.99
AZN-SL-HWU	Azokh 2	OHI 4 Bacterial attack on the outer cortical, very incipient. Deep cracks < 2 mm deep. Enlarged canaliculi	0.1	0.0012	0.0000	0.0000	0.0503	0.0503	2.13	2.24

(continued)

Table 11.2 (continued)

Sample code	Site	Oxford Histological Index and Histology notes	BET Surface Area m^2/g (by N_2 Porosimetry)	Volume (cm^3/g) between 0.001 and 0.1 μm (N_2 porosity)	Volume (ml/ml) in pores <0.1 μm (Hg porosity)	Volume (ml/ml) in pores <10 >0.1 μm (Hg porosity)	Volume (ml/ml) in pores >10 μm (Hg porosity)	Total Porosity (ml/ml) (Hg porosity)	Bulk Density g/ml (Hg porosity)	Skeletal Density g/ml (Hg porosity)
AZN-SL-HDW	Azokh 2	OHI 5 Deep cracking >2 mm deep of weathering. Wedl tunneling dispersed. Most lacunae infilled	1.9	0.0097	0.0303	0.0230	0.0746	0.1279	1.95	2.24
AZN-SL-HWW	Azokh 2		4.6	0.0134	0.0396	0.0341	0.0000	0.0737	1.95	2.10
AZN-SL-A	Azokh 2	OHI 5 Surface affected by root marks, histology is not etched. EDS sediment rich in Fe and Mn	109.4	0.2324	0.2659	0.0226	0.0228	0.3113	1.52	2.21
AZN-SL-B	Azokh 2	The white dots contain gypsum (EDS)	87.9	0.2038	0.2313	0.0442	0.1175	0.3930	1.37	2.26
AZN-SL-C	Azokh 2	OHI 0 Completely invaded by bacteriae. EDS Barium sulfate (Barite) crystals	48.3	0.1153	0.1035	0.2624	0.0109	0.3768	1.47	2.37
AZN-SL-D	Azokh 2	OHI 4 Pitted periosteal cortical layer, possibly fungi								
AZN-SL-F	Azokh 2	OHI 5 ESEM surface analysis, root marks. Good histology, no evidence of root etching in section	102.0	0.1961	0.1603	0.0231	0.0123	0.1957	1.34	1.66
AZN-SL-G	Azokh 2	OHI 5 Root marks on surface. Section: Good histology, no evidence of root etching. Barite deposits	105.5	0.2238	0.2581	0.0303	0.0521	0.3404	1.48	2.25

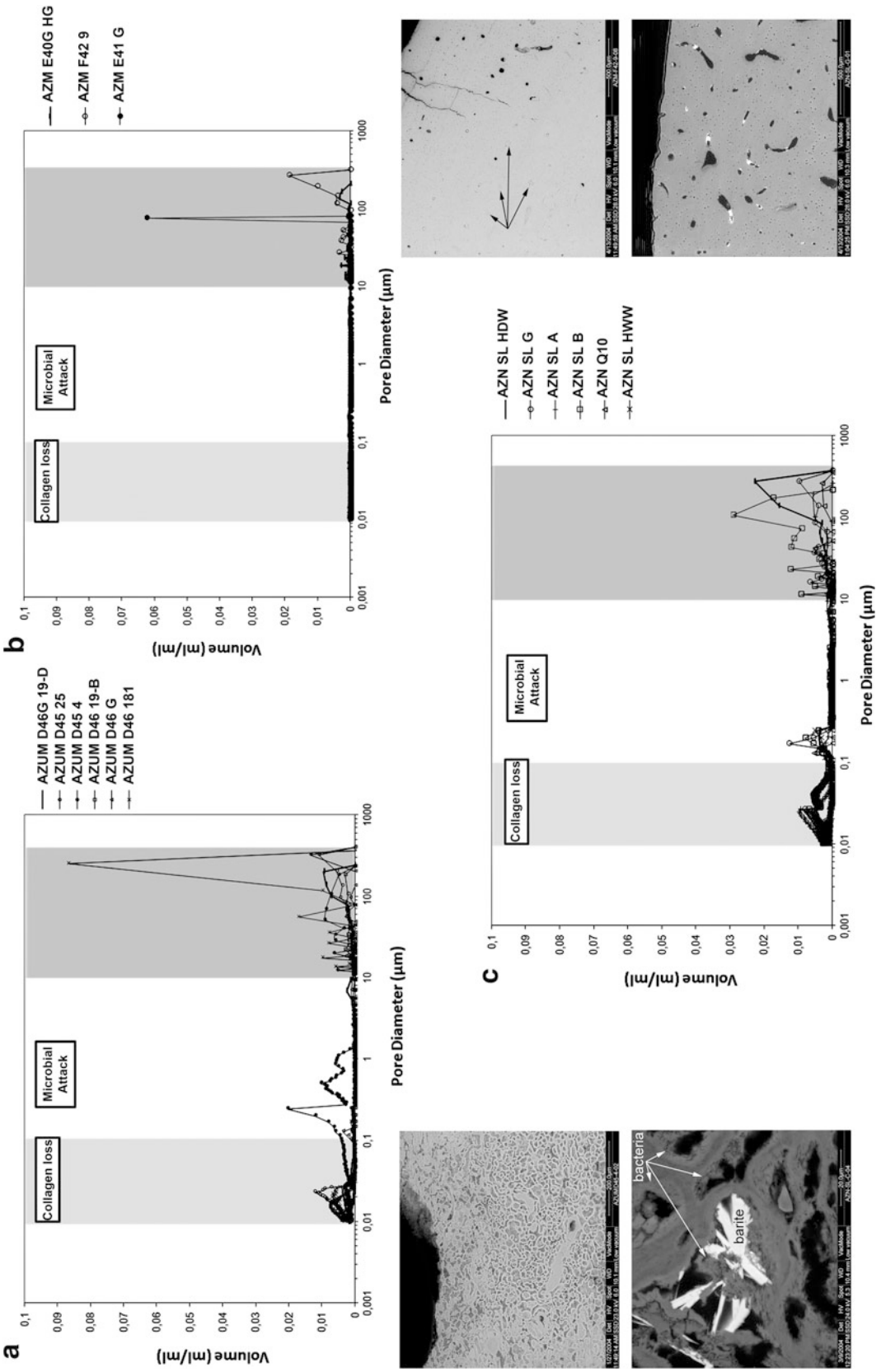

Fig. 11.2 Pore size distributions of Azokh fossil bone samples measured by mercury intrusion porosimetry. **a** Units II–III (inset middle left, AZUM D45 4 SEM microphotograph showing intense bacterial attack), **b** Unit Vm (inset middle right: AZM F42 9 SEM microphotograph showing Canals of Havers filled by calcareous secondary minerals, black arrows), **c** Azokh 2 (inset bottom left: AZN SL C SEM microphotograph showing bacterial attack and barite ($BaSO_4$ identified by EDS) damaging bacteria colonies [microscopic focal destruction, MFD], so bacteria predated secondary growth of minerals; inset bottom right: SEM microphotograph of AZN SL G with histological cavities filled by secondary minerals, see Marin-Monfort et al. 2016)

Index 0) and others none (Histological Index 5). The pore structure of bone from these units (Fig. 11.2a) is somewhat unusual, but it is most similar to that of bone recovered from Etton Causewayed Enclosure (Brock et al. 2010). Such material is similar to those having undergone accelerated collagen hydrolysis (ACH) (Smith et al. 2002, 2007), i.e. it has a significant increase in the porosity in pores of less than 0.1 μm, however there is less volume in this pore space. This pore space is interpreted as the pore space that remains after collagen loss but is only apparent following non-microbially mediated loss of collagen, i.e. it occurs when the collagen is chemically removed. This collagen loss can occur rapidly and has been observed in bones as young as 700 years (Smith et al. 2002), however, the bones from Units II–III in Azokh 1 are likely to be around 100–200 ka (see Appendix, ESR). This Azokh material and that from Etton Causewayed Enclosure (Brock et al. 2010) differs from that of ACH bone as the pore volume is smaller and the pore space is distributed in smaller pores within this range. The smaller pore volume and smaller diameter pore range in the Azokh and Etton Causewayed Enclosure material, compared to that of previously published material from European deposits and boiled bone (Smith et al. 2002, 2007; Roberts et al. 2002; Turner-Walker et al. 2002), is probably the result of some pore infilling during deposition. This observation is supported in the Azokh material by observations under ESEM of bone sections where secondary mineralization can be observed (Table 11.1), suggesting exogenous mineral sources related to cave environments and decay (calcite, tinsleyite, barite, brushite), are contributing to the infilling (Marin-Monfort et al. 2016; Murray et al. 2016).

There are two probable scenarios as to how these bones have been preserved in this state. They either underwent a rapid phase of degradation, like ACH bone during early diagenesis, remaining stable for the following millennia with some pore infilling. Or the observed changes occurred slowly over the whole taphonomic history of the fossils, so that bones with characteristics similar to those of ACH bone can be formed by an alternative slower process.

AZUM D45 4 16/8/3 is a sample that shows extensive histological damage and displays the characteristic increase in porosity (Fig. 11.2a) in pores of diameter 0.1–10 μm (Jans et al. 2004). Samples AZUM-D46G 19- B, C and D also have a low histological index, but do not show this increase in porosity. Indeed they display very low porosity considering that they have no collagen and evidence of microbial attack. This again must be attributed to the pores being in-filled during deposition.

Azokh 1 Unit Vm

The material from Unit Vm, the oldest part of the Azokh 1 sequence excavated so far, is heavily fossilized. The samples analyzed had no collagen preserved (and have yielded no DNA, Geigl 2012 personal communication). They have highly altered mineral (IRSF ranges from 3.3 to 3.9 and C:P ratio 0.26–0.17) and good histological preservation (Histological Index 4 or 5). They have little porosity in the detectable range of mercury porosimetry (on average ~6%) and high density values (both bulk and skeletal). As stated earlier, when collagen is lost from the bone, the porosity of the bone increases (in pores less than 0.1 μm diameter) and there is a concomitant decrease in bulk density and an increase in apparent skeletal density. In the fossil bone from Unit Vm there is a small pore volume in the <0.1 μm diameter pore range (Fig. 11.2b), but it is much smaller than that observed in ACH bone (see Smith et al. 2002) and that observed in bones from Units II–III of Azokh 1 and Etton Causewayed Enclosure (Brock et al. 2010). Even though the fossil bone from Unit Vm of Azokh 1 has lost its collagen, its density is greater than that of fresh modern bone (e.g., Nielsen-Marsh and Hedges 1999), suggesting that the pore space has been in filled with material denser than collagen.

This type of preservation is not prevalent in European Holocene bone (Smith et al. 2007), but the pore structure and lack of collagen is similar to that of dinosaur fossils measured by Trueman and Tuross (2002, in particular Fig. 1 therein). We can speculate about the processes that have formed this material from Unit Vm as being similar to those that may have occurred to the bones in Units II–III. Possible initial ACH type bone may have been formed with subsequent infilling of the pore space, or a different process, where the collagen is slowly degraded and replaced with mineral.

Azokh 2

Interpretation of the samples from Azokh 2 is difficult as the bones are probably a mixture of both modern and fossil material. Based on appearance and diagenetic parameter values the modern bones are represented by AZN P11, AZN Q10 and four samples from the same metapodial AZN H- DU, DW, WW, WU. Four samples were taken from this one metapodial as the bone exhibited an obviously weathered side and an unweathered side. Furthermore, the effect of rudimentary cleaning of the

bone (dry brushing and wet brushing) was also tested on this one specimen, giving four parameters: DU, dry/unweathered, DW, dry/weathered, WW, wet/weathered and WU, wet/unweathered. In general the modern bones show high levels of collagen remaining with the exception of AZN Q10, which has only a moderate amount of collagen. AZN P11 has lost some collagen and has evidence of microbial attack (0 histological index and increased porosity in the 0.1–10 μm pore diameter range). Although the AZN H metapodial is differentially weathered and has been cleaned differently there is little difference in the diagenetic parameters of the four samples. The bone is "well preserved" in terms of collagen preservation although the mineral component of the bone is heavily altered (IRSF 3.3 or above and C:P ratio ranging from 0.36 to 0.28). Interestingly, the surfaces of the un-weathered side show signs of some microbial attack, which is absent in samples taken from the weathered (exposed) side. In general the porosity of the modern samples from Azokh 2 is low (as would be expected), with the exception of sample AZN P11, mentioned above.

The other samples recovered from Azokh 2, probably represent either; rapidly degraded modern samples or, more likely, semi-fossil material that has been transported from inside the cave and deposited in the top layers at the cave entrance during the sedimentation of the cave (Fernández-Jalvo et al. 2010b; Murray et al. 2016). They are typically ACH type bone, with low levels of collagen, and high levels of mineral alteration and porosity in the <0.1 μm diameter pores (Fig. 11.2c), although it should be noted that AZN SL C has been heavily microbially attacked (evidenced by increased porosity in the 0.1–10 μm pore diameter range). They are from a diagenetic perspective similar to the material from Azokh 1 Units II–III.

Assessment of Nitrogen Adsorption Isotherm Analysis and Mercury Intrusion Porosimetry

The investigation of pore structure using both nitrogen adsorption isotherm analysis (NAIA) and mercury intrusion porosimetery (HgIP) worked well in this sample set. The majority of the samples retained little collagen so that they were easy to dry and outgas and amenable to analysis. HgIP

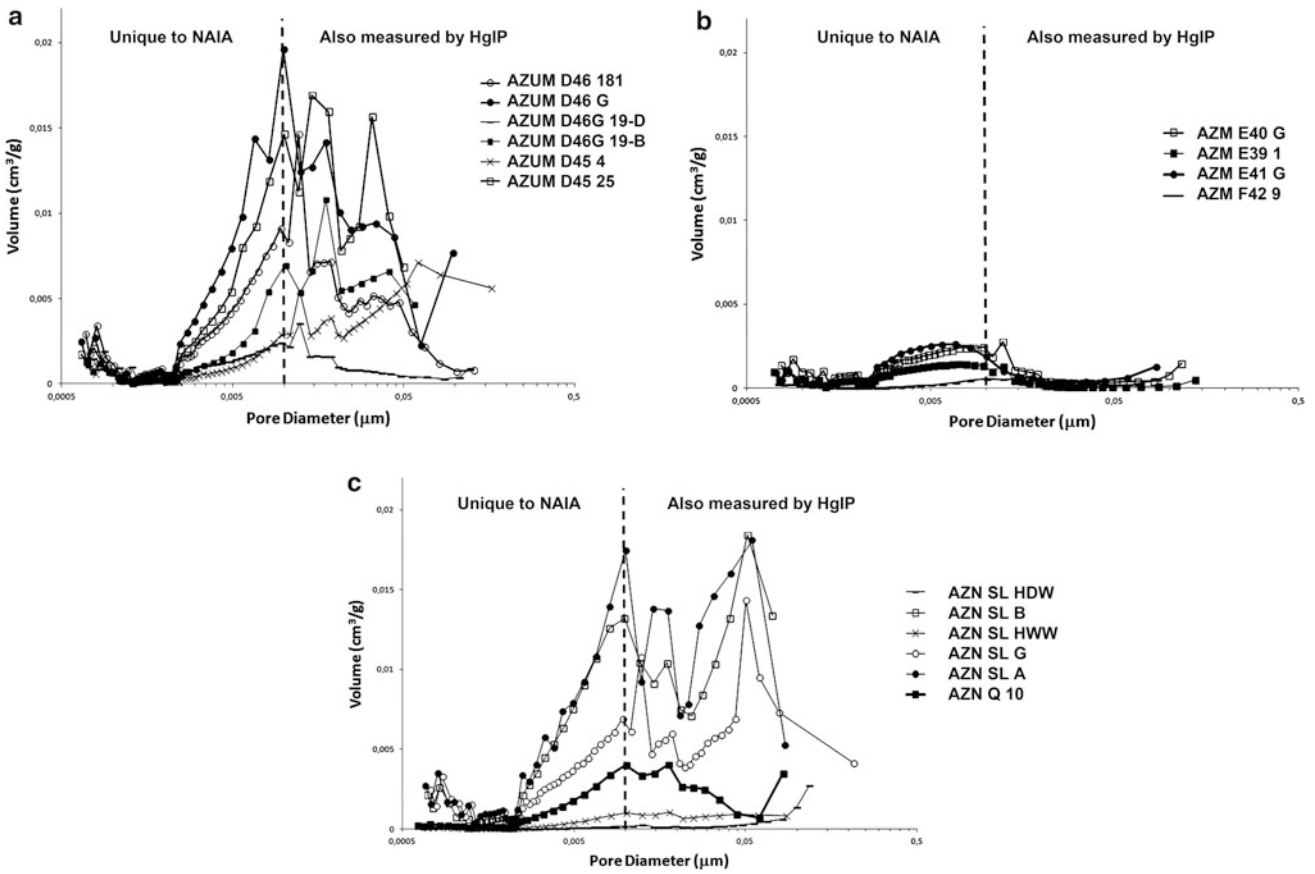

Fig. 11.3 Pore size distributions of Azokh fossil bone samples measured by Nitrogen adsorption isotherm analysis. **a** Azokh 1, Units II–III, **b** Azokh 1, Unit Vm, **c** Azokh 2

has been used to analyze archaeological bone porosity on numerous occasions (e.g., Nielsen-Marsh and Hedges 1999; Smith et al. 2002, 2008) but NAIA has not been used as comprehensively.

In this data set, when measured using HgIP most of the bones have either a large pore space associated with collagen loss or have little collagen but lack this pore space. Presumably, in the latter case, this pore space has been opened with the loss of collagen but subsequently re-filled by exogenous mineral. A similar pattern is true for the pores measured by NAIA in the 0.001–0.1 μm pore diameter range; with bone from Azokh 1 Units II–III (Fig. 11.3a) having the largest NAIA pore volume, and the heavily in filled and fossilized bones from Unit Vm showing low NAIA pore volumes (Fig. 11.3b).

There is a strong relationship between the pore volumes measured by the two techniques in the smallest pore range (Fig. 11.4), with both measurements responding in the same way to the diagenetic processes in the bone. There is some overlap in the two pore ranges measured by the different methods (HgIP in the smallest pores is approximately 0.01–0.1 μm but with NAIA from 0.001 to 0.1 μm), but this common pore volume measured does not appear to be completely responsible for this relationship. It is clear that the sub 0.01 μm pores measured *only* by NAIA are mimicking what is happening in larger pores. The sub 0.01 μm pores are increasing in volume with collagen loss (Fig. 11.3a, c) and also being infilled (Fig. 11.3b).

Although the pore space measured by NAIA in samples from Azokh 1 Vm is small in comparison to other samples, where large amounts of collagen have been lost, there is some evidence that this small pore volume is indeed what has been suggested above: the pores opened by collagen loss have subsequently been refilled. Figure 11.5 shows the same data as Fig. 11.3b with a smaller *y-axis* to accentuate the pore volume. In addition the "well preserved (collagen rich) bones" from Azokh 2 are included and AZUM D46G 19-D is included for comparison, as the sample from Units II–III with the smallest volume in this pore range. At this scale it is clear that the pore volume in the Unit Vm bones is significantly larger than that found in the "well preserved bones" that have >20% collagen, with the exception of AZM F42 9. AZUM D46G 19-D has a similar pore size distribution to the Unit Vm material and can clearly be seen to be a filled in bone. Interestingly AZM F42 9 has a low pore volume in this pore range, similar to the "well preserved bones"; the reason for this is not clear, and it was noticed that many of the haversian structures and other pore spaces visible under ESEM were infilled with mineral, indicating extensive infilling.

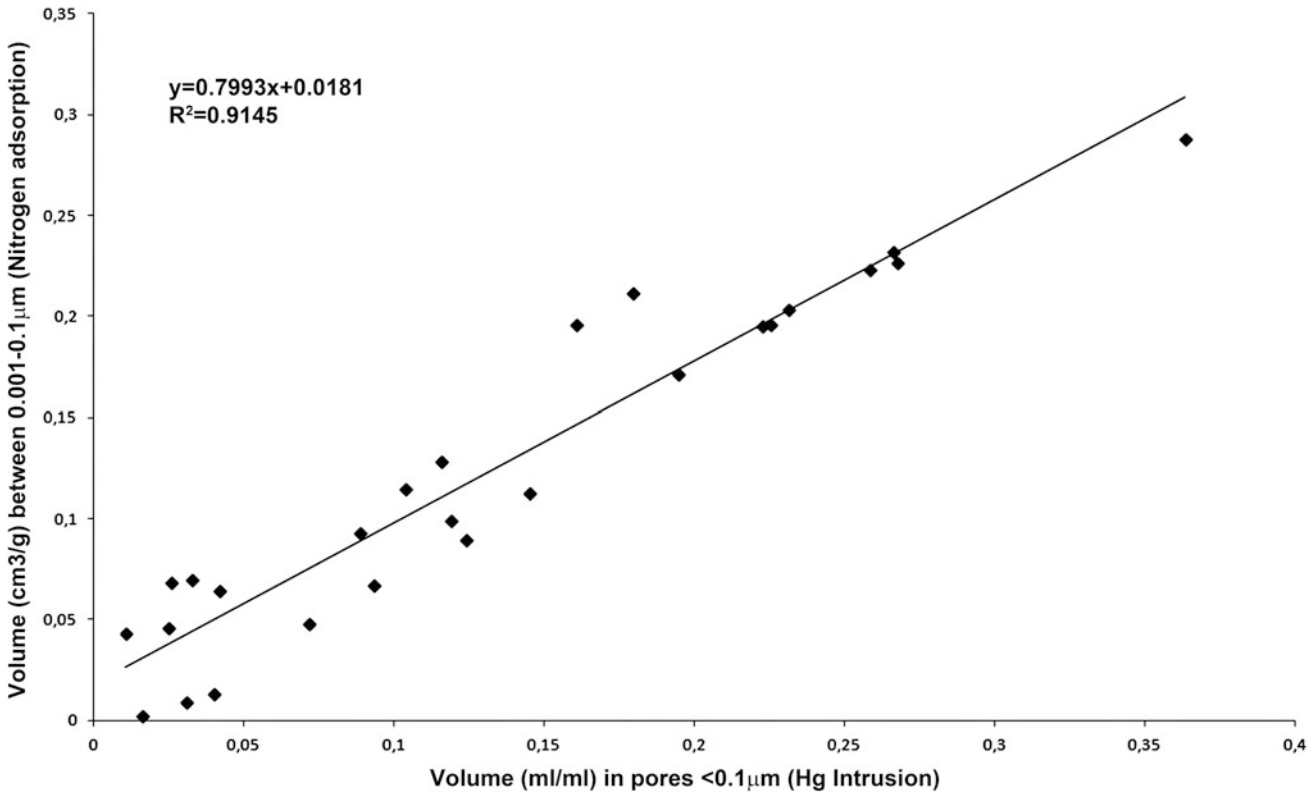

Fig. 11.4 Pore volume comparison: nitrogen adsorption isotherm analysis volume versus mercury intrusion porosimetry volume on the same bone specimen for pores <0.1 μm

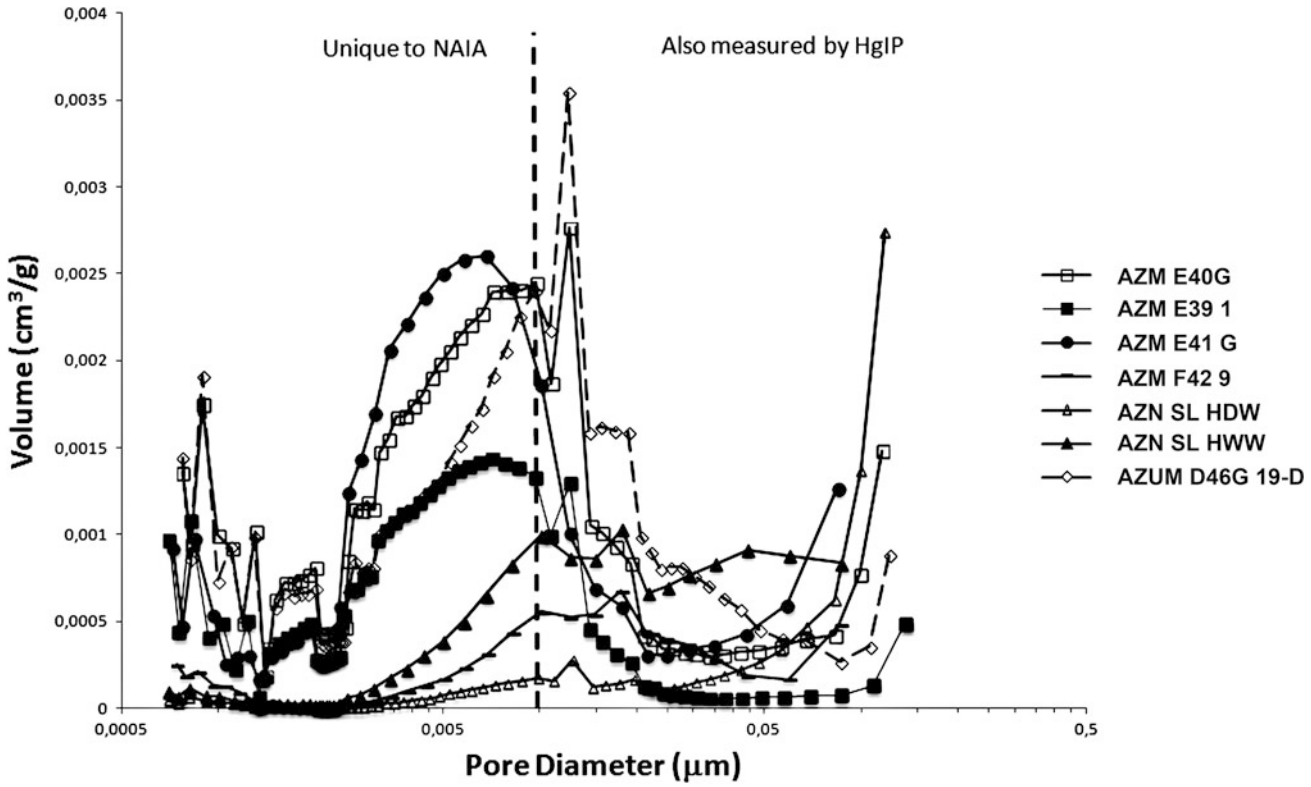

Fig. 11.5 Detailed pore size distributions of Azokh fossil bone samples measured by nitrogen adsorption isotherm analysis. Note that the *y-axis* is much smaller than in Fig. 11.3

When observing the pore structures at this fine scale there is certainly evidence to suggest that this pore space is opened via collagen loss (like the 0.01–0.1 μm range observed using HgIP) and subsequently (although not completely) refilled. This process generally leaves a different porosity pattern to "well preserved bone" that has not lost collagen. Further studies are needed to make this pattern clearer, but from the data from the Azokh material we can suggest that this is the case. It appears that in this data set NAIA is providing similar information to that given by HgIP, as the smaller pores seem to reflect the loss of collagen from the bone. In this sense it appears that NAIA could be used as a non-destructive tool to investigate non-microbial collagen loss in archaeological bone. However, as NAIA cannot be used to measure the larger pores that indicate microbial loss it cannot provide all the information that HgIP can.

Of note is the role of infilling of the pores at this site and how this obscures some of the interpretations that might easily be made using HgIP. Previous studies have suggested that HgIP can be used to identify distinct types of preservation; i.e. ACH, microbially attacked bone, "well preserved bone" and bone undergoing mineral dissolution (Nielsen-Marsh et al. 2007; Smith et al. 2007). In this data set, although microbial attack has been identified in some bones, the characteristic pore structure caused by this (porosity in the 0.1–10 μm pore diameter range) is not obvious. Similarly many of the bones analyzed here have undergone collagen loss without microbial attack and we might expect to observe pore increases in the <0.1 μm pore diameter range. Again this information has been obscured by infilling. In data sets where pore infilling is prevalent it becomes imperative to do histological examinations to determine the role of microbial attack in the diagenetic histories of the bones, as HgIP cannot be used to make distinctions between bones with and without microbial damage. Moreover, NAIA should be used to investigate porosity changes in such data sets as it can provide some information on collagen loss and infilling and would be non-destructive.

A Model of Bone Diagenesis at Azokh Caves

Bones from dead animals that enter the fossil record start out in the "well preserved bone" category (i.e. recently living tissue). From the surface exposed bones tested from Azokh 2, we can presume that bone can remain relatively unmodified for at least a few years or decades. Some changes do

occur, in initial diagenesis at the site with the modern material showing weathering and in some cases substantial mineral degradation, and some microbial attack.

Fossil bone in Units II–III at Azokh 1 is most similar to bone described by Brock et al. (2010) recovered from Etton Causewayed Enclosure. This appears to be similar to the ACH bone found in European Holocene sites (Smith et al. 2002, 2007; Nielsen-Marsh et al. 2007), but with infilling of the pore space (evident in the Azokh material from the porosity and histological analysis). Bone from Unit Vm is heavily fossilized and the pore structure is extensively in-filled, so that the bone is not porous but dense. This latter type of preservation is not typical of those described in European Holocene deposits (Smith et al. 2007; Brock et al. 2010), because of the extensive infilling of the pore structure.

Given the main features of the ancient material in Azokh 1 (predominantly ACH or Etton Causewayed Enclosure type bone in Units II–III and heavily infilled bone Unit Vm), it seems reasonable to suggest a model of bone diagenesis at Azokh Cave proceeding as follows. The initial phases of degradation at the site lead to some ACH bone and microbially attacked bone, with the cave providing a relatively stable environment where the pore space is infilled with exogenous or authigenic mineral over time. The evidence from the measurements suggests that this process takes hundreds of thousands of years at Azokh as the material from oldest layers measured here (Unit Vm) is heavily mineralized, where as the younger bones (Units II–III) still retain some pore space, although there is evidence that this has been partially filled. The rate of the initial collagen loss cannot be known at Azokh, but it has been observed within 700 years at Apigliano in Italy (Smith et al. 2002), and we can speculate that at Azokh it could have occurred over a similar time span. Afterwards, the process of pore infilling was probably gradual and the conditions for bone preservation were generally benign. In this model it seems that the type of preservation found in Unit Vm is the natural progression of bone that has passed through an early stage like that in Units II–III.

Alternatively, it is of course quite possible that both units had quite different modes of diagenesis, as the initial conditions are thought to be very important in determining the later stages of diagenesis (Trueman and Martill 2002; Jans et al. 2004; Smith et al. 2007; Nielsen-Marsh et al. 2007). The two strata measured here are separated by 100–200 ka, and environmental conditions (for example temporal variation in precipitation) could have been different for bones at these two different times, or subsequent burial depth could play a part in the differing diagenetic pathways. Thus we could speculate on a model where ACH occurred only in Units II–III, while in Unit Vm diagenesis could have occurred without an ACH phase, but with a slow rate of collagen loss and slow rate of pore infilling.

It is interesting to note is that the conditions in the cave deposits appear to be benign for both ACH bone and microbially attacked bone, with both types of bone appearing in the deposits and both undergoing infilling, although it should be noted that there is only sparse evidence of microbial attack (some bones with histological index 4–5) in Azokh 1 Unit Vm. This indicates that once bone passes through the initial phases of degradation, the Azokh sediments provide a stable and largely benign environment for bone preservation, at least macroscopically.

Prospects for Molecular Preservation

The ancient bone material from Azokh Caves presents the characteristics of heavily altered bone, with or without mineral infilling in the pore spaces. Collagen preservation is exceptionally poor in all the ancient material, with low ‘collagen’ yields and none of the acid insoluble material recovered giving good collagen C:N ratios. Previous studies have indicated that the best preserved material (i.e. with higher collagen levels, and less microbial attack) is the best material for DNA amplification (Colson et al. 1997; Haynes et al. 2002; Gilbert et al. 2005). Pruvost et al. (2007) showed that DNA could be retrieved from fossil bones heavily attacked by bacteria, suggesting that bacterial attack may not be the only reason for DNA degradation. The age of the fossils studied by these authors, however, is much younger (Holocene) than those of Azokh. Given the poor organic preservation observed in Azokh Caves sites, even in modern (Holocene) bones, it seems likely that ancient DNA preservation will be equally poor in the Azokh material. One proposed mechanism of DNA survival in ancient bone is via adsorption to the surface of the bone mineral crystals (Tuross 1993; Götherström et al. 2002) or molecular ‘niches’ within the histological structure (Geigl 2002), but given the highly altered mineral of the bones at Azokh Cave, survival of ancient DNA via these mechanisms also seems unlikely.

Conclusions

1. The fossil bone from the site of Azokh Caves is in general poorly preserved with no collagen preservation observed and in most cases with extensive mineral alteration.
2. Histological examination reveals that some bones have undergone microbial attack and that many show evidence of exogenous minerals embedded in the histological structure. Using collagen as a guide for organic preservation it is unsurprising that aDNA preservation at

the site is so poor; moreover the heavily altered mineral of the bones would also provide little hope for aDNA preservation.

3. There are distinct types of preservation for the bones in the three areas analyzed. Modern material from the surface of Azokh 2 shows diagenetic parameters characteristic of "well preserved bone", although this material is mixed with poorly preserved material (Holocene), that in general has ACH like preservation.
4. The material from Azokh 1 Units II–III shows typical ACH bone and microbial attacked bone, but both types have some infilling of the pore space with the ACH type bone giving similar diagenetic parameter and HgIP traces to bone from Etton Causewayed Enclosure (Brock et al. 2010).
5. Material from Unit Vm is heavily fossilized with extensive pore infilling and high density values. This kind of heavily infilled fossil preservation has been observed in Dinosaur fossils previously (Trueman and Tuross 2002) but not in archaeological material, so Azokh Caves represents the first time this type of preservation has been observed in Pleistocene material.
6. Azokh also presents two variables that were not present in previous studies of bone diagenesis using this diagenetic parameter approach (e.g., Smith et al. 2007). One factor is the cave environment and the other is that the material in Azokh is much older than that measured by Smith et al. (2007). One or both of these factors could be important in creating the type of bone preservation at Azokh Unit Vm and making it different from those of previous studies.
7. The use of nitrogen adsorption isotherm analysis and mercury intrusion porosimetry to measure the pore structure of the bones at Azokh was particularly successful, especially as the collagen preservation was so poor that it enabled the samples to be dried and outgassed easily. This aided the comparison of the two techniques when applied to the same bone sample and revealed that the two techniques appear to be measuring similar aspects of bone degradation. HgIP shows an increase in porosity in the small pores when collagen is lost from the bone non-microbially i.e. ACH bone). NAIA shows a similar pattern and that small pores below the range of HgIP are also affected by this non-microbial collagen loss. In Azokh 1 Unit Vm HgIP shows no increase (presumably because the pores that were opened through collagen loss have been filled in with mineral). The pores measured using NAIA, do show extensive infilling, but this is not complete. When observed at a finer scale, there is a difference between the pore structures of the Unit Vm material that has undergone chemical collagen loss and collagen rich bones, even when there has been some infilling of the pores in the first group. It appears that the pores measured by both techniques (HgIP and NAIA) are responding in the same manner to the same processes, in that pore space is opening with collagen loss and becoming infilled.
8. The study of pore structures at Azokh also provides a cautionary tale for the use of mercury intrusion porosimetry. Whilst this technique has provided a powerful way to distinguish between different early taphonomic bone types based on characteristic pore size distributions (Smith et al. 2007); the infilling of pores (e.g., in Azokh Unit Vm) obscures this detail, making such distinctions impossible. Thus when analyzing such heavily fossilized bone it becomes imperative to analyze histological sections to determine the role of microbial attack in the role of bone degradation at the site.

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