Scanning Electron Microscopy Investigation of a Sample Depth Profile through the Martian Meteorite Nakhla

Jan Toporski[*§], Andrew Steele[*†], Frances Westall[‡], and David S. McKay[†]

Classification: Astrobiology special edition

Number of text pages: 20

Number of figures: 6

Number of words in abstract: 135

Total number of characters: 39.058

[*] University of Portsmouth, School of Earth, Environmental and Physical Sciences, Astrobiology Group, Portsmouth, PO1 3QL, UK, Tel.: +44 2392 842258, Fax: +44 2392 842244, e-mail: jan.toporski@port.ac.uk

[†] NASA, Lyndon. B. Johnson Space Center, Mail code SN, Houston, Texas, 77058, USA

[‡] Lunar and Planetary Institute, 3600 Bay Area Boulevard, Houston, Texas, 77058, USA

[§] Corresponding author

Abstract

The ongoing scientific debate as to whether or not the Martian meteorite ALH84001 contained evidence of possible biogenic activities showed the need to establish consistent methods to ascertain the origin of such evidence. To distinguish between terrestrial organic material/microbial contaminants and possible indigenous microbiota within meteorites is therefore crucial. With this in mind a depth profile consisting of four samples from a new sample allocation of Martian meteorite Nakhla was investigated using scanning electron microscopy (SEM) and energy dispersive X-ray analysis. SEM imaging of freshly broken fractured chips revealed structures strongly recent terrestrial microorganisms, in some cases showing evidence of active growth. This conclusion was supported by EDX analysis, which showed the presence of carbon associated with these structures, we concluded that these structures represent recent terrestrial contaminants rather than structures indigenous to the meteorite.

Introduction

As a consequence of the recent announcement of possible microbial activities in Martian Meteorite ALH84001 [1], it became obvious that there was a lack of knowledge as to how microorganisms and/or their chemical remains in extraterrestrial rocks can be identified and their origin ascertained. In recent years the notion that extraterrestrial bodies may become contaminated by terrestrial microorganisms after they fall to Earth has become well established in the meteorite community [2-4]. The sources of contamination within meteorites and the mechanisms of entry of such contamination remains poorly understood. However, recent work is starting to unravel these issues with the discovery of probable recent terrestrial bacterial contaminants in Martian meteorite ALH84001 [5]. Furthermore, microbial contaminants have also been reported from the Murchison meteorite, Allende [6], Tatahouine [7], and several Antarctic chondrites [2, 4, 5]. Adding further interest to the debate on 'life on Mars', McKay *et al.* [8] showed the presence of possible bacteriomorph structures in clays of probable Martian origin on Nakhla [9, 10]. These structures are more suggestive of a bacterial origin than those previously imaged on ALH84001 [1], due to the presence of ancillary structures associated with the bacteriomorphs, which resemble flagellae and extracellular polymeric substances (EPS). However, their Martian origin remains euqivocal.

The "question of living bacteria in stony meteorites" has been discussed as early as in 1935. Roy and Hudson [11] refuted and disproved Lipman's claims [12], that "stony meteorites had brought down with them from somewhere in space a few surviving bacteria, probably in spore form but not necessarily so, which can in many cases be made to grow on bacteriological media". Roy and

Hudson conducted microbial culturing and concluded from the results of their experiments, that the detected bacteria were terrestrial contaminants. Later researchers reported a microbiological examination of carbonaceous chondrites using microscopy techniques [13]. They interpreted findings of small (approximately 10 μm), "organized elements" as microfossils indigenous to the meteorite, although "microbiological contaminants (common airborne bacteria, algae, etc.) in carbonaceous as well as in non-carbonaceous meteorites" had been observed by these authors. More recent work [14-16] reported on microorganisms and their remnants on freshly fractured surfaces in the Murchison, Allende, and Efremovka meteorites. These researchers interpreted cyanobacteria-like structures as indigenous to the meteorite, based on the observation that they are "tightly conjugated" with the mineral matrix. However, no further testing for recent terrestrial contamination has been reported, such as microbial culturing experiments, despite the fresh appearance of some of the organisms [15]. It has been suggested that chemolithotrophic microorganisms (autotrophs), using CO_2 as a sole carbon source [e.g. 17], could live in these meteorites and in igneous rocks. However, the presence of indigenous carbonaceous matter in the same meteorites and rocks could also support growth of heterotrophic microorganisms, as they metabolize organic carbon.

Other studies have shown the abundance of organic substances in Nakhla and other meteorites (e.g. polycyclic aromatic hydrocarbons, amino acids and aliphatic hydrocarbons), suggesting that the abundance of these substances may be a result of contamination from their respective terrestrial environments [18-21]. Glavin *et al.* (1999) [19] concluded that most of the amino acids in Nakhla were derived from terrestrial sources, probably bacteria. However, although these

authors considered terrestrial microbiological activities as a possible source for their findings, no evidence of actual organisms or associated structures has been provided. Clearly, one of the weaknesses of the research completed thus far on Nakhla, as with other meteorites, has been the lack of data correlating the organic chemistry with the morphology of potential sources of the analysed organic material such as microbial contaminants.

With this in mind, we studied four samples of Nakhla (which constituted a depth profile through the meteorite) to ascertain possible microbial contaminants. We aseptically divided each sample into separate aliquots. One aliquot was immediately imaged using Scanning Electron Microscopy (SEM), one in culturing experiments, one for surface analysis and a further one was used for flow cytometry, direct DNA extraction and 16S rDNA studies, to define the types of microbial contamination as per Steele *et al.* (this issue) [6]. The latter analyses are currently under way and only the results of the microscopy examination will be presented in this communication.

Materials and Methods

Four samples of the Nakhla meteorite from a new allocation (curated at the Natural History Museum London, UK, and then taken for allocation to the NASA Johnson Space Center (JSC), Houston, Texas, USA) were analysed. The samples were N45 (containing fusion crust), N46, N47 and N48, constituting a depth profile of Nakhla with N48 being removed from the center of the meteorite. One noteworthy point is that this new allocation was considered fairly pristine due to the presence of a seemingly unbroken fusion crust all around the meteorite.

Upon receipt of the Nakhla samples they were taken from the sample containers in laminar flow conditions and placed into sterile containers. Small pieces of each sample were immediately mounted in laminar flow conditions onto SEM stubs using carbon tape. All handling instruments coming into contact with the meteorite were subjected to alcohol flame sterilization before being used. To increase sample surface conductivity silver dag was sparingly used to connect the base of the samples with the stubs. After allowing 30 hours drying time for the silver dag in a sealed container under laminar flow conditions, the samples were Au/Pd sputter coated for 45 seconds (approximately 10 nm coating). All samples were preliminary imaged using a JEOL SM 6100 Electron Microscope fitted with a light element Energy Dispersive X-Ray Analyser (EDX; Link Analyser 10/85) approximately 48 hours after removal from the sample vials used for transportation. For later studies a field emission gun SEM (Philips XL 40 FEGSEM) was used for high magnification and high resolution studies.

Results

Extensive SEM investigations on Nakhla showed that all four samples contained filamentous structures resembling hyphae-forming microorganisms (Fig. 1).

Figure 1: SEM images representing a depth profile through Nakhla with apparent filamentous hyphae structures A) on N45; B) on N46; C) on N47, and D) on N48. The inset in 1D shows a hypha growing out of ca. 1ìm -sized opening. All scale bars 1ìm except 10ìm for D.

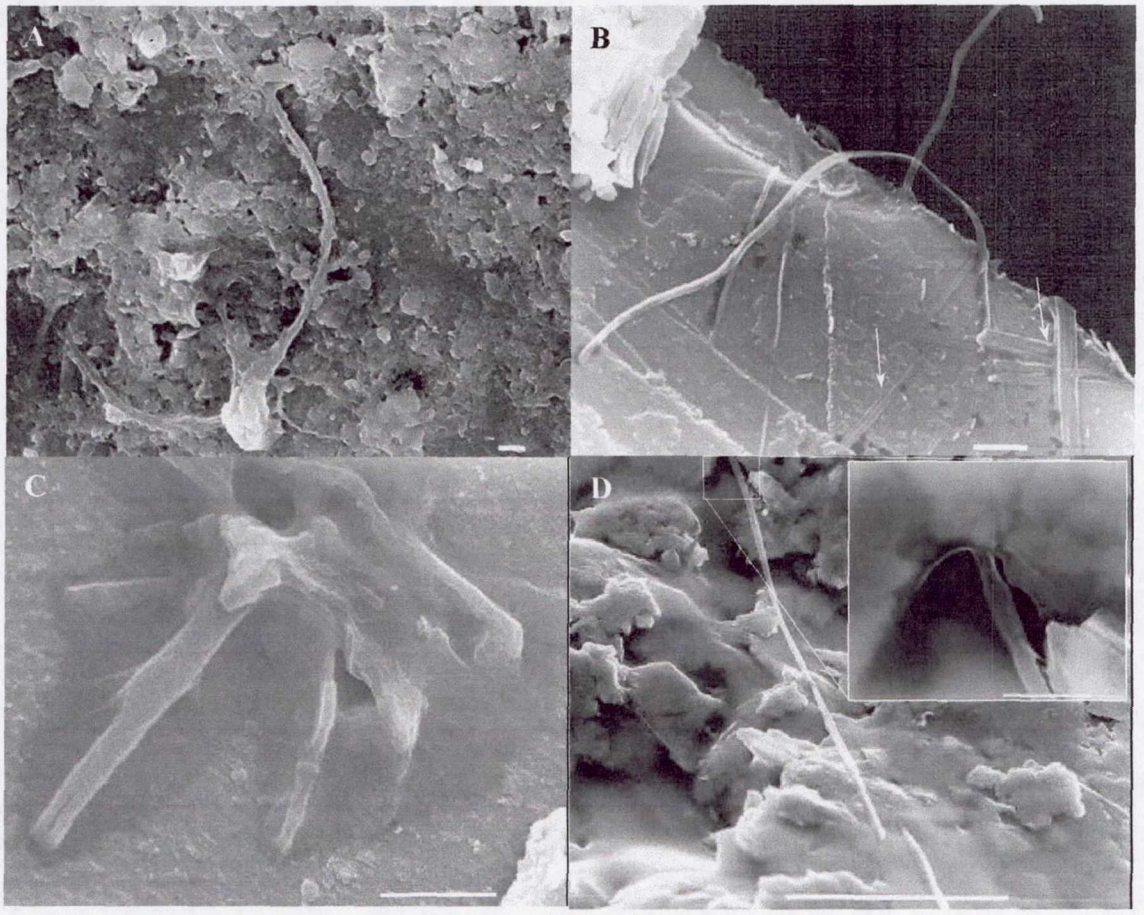
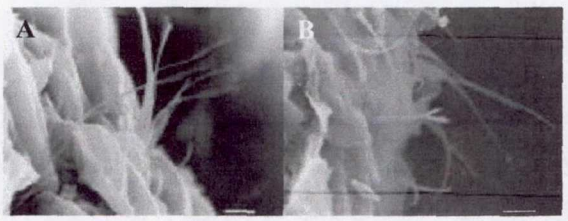


Figure 1A shows what appears to be a filamentous organism (filament diameter approximately 0.5 - 1μm) originating from a spore-like spherical object on the fusion crust of the meteorite (N45). The figures 1B (N46), 1C (N47), and 1D (N48) show apparent filamentous microorganisms, forming both substrate and aerial hyphae, protruding from the underlying mineral surface

(definition in Holt *et al.*, 1994). These organisms exhibit a diameter of 0.2 - 0.4 μm, *i.e.* they are significantly smaller than the features shown on the fusion crust sample (Fig. 1A). Figure 1B shows aerial hyphae as well as flattened, apparently dead and decaying hyphae on the substrate (arrows). The hypha shown in figure 1D was observed on a sample from near the centre of Nakhla (N48). This image shows that the organism is growing out of a small (ca. 1 μm wide) opening. Cracks and fissures as small as ca. 100 nm can be seen in Figure 5 to allow filaments to penetrate the meteorite. Some of the filaments shown in Figure 2A measure only ca. 50nm, indicating that the above mentioned cracks would suffice for penetration. The comparison of hyphae forming organisms on Nakhla (N 48) and a sample with recent cryptoendolithic communities from the Timber Peak (TP) range in Antarctica shows close morphological resemblance (Fig. 2) and provide further evidence of their biogenicity.

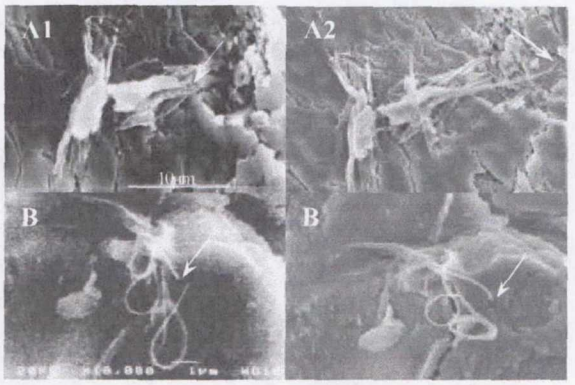
Figure 2: Comparison of two hyphae forming organisms. A) was observed on an internal chip of Nakhla (N48), whereas B) is representing a filamentous organism of the Antarctic cryptoendolithic communities from Timber Peak (TP) range. Scale bars 1ìm.



The images A2 and B2 in Figure 3 were taken 50 days after A1 and B1 on an internal chip of Nakhla (N47) and show significant changes in shape and size of the observed structures. The hyphae of the organism shown in Figure 3A exhibited growth of approximately 10 μm (see arrowheads in Figs. 3A1 and A2), whereas the terminal end of the filament shown in Figure 3B

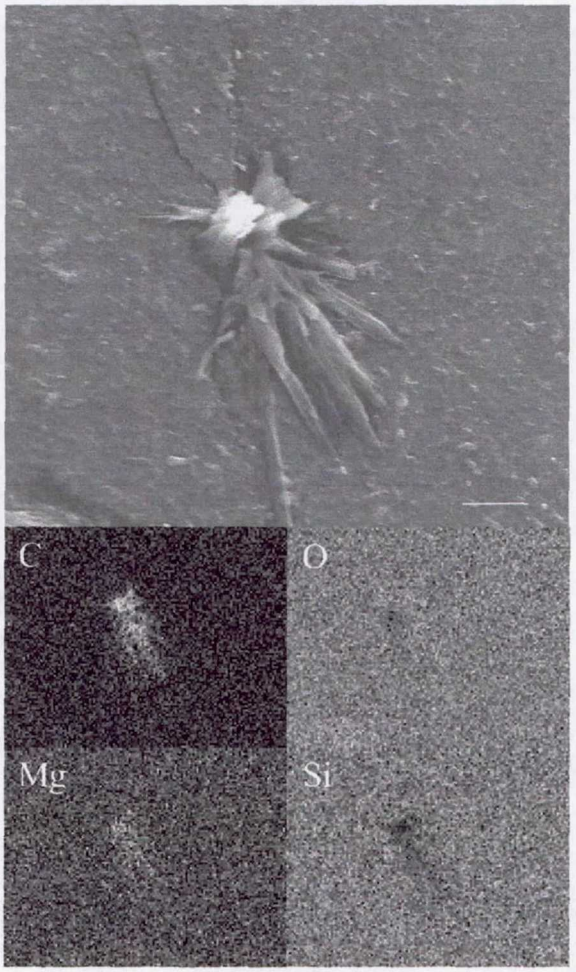
advanced about 1 μm (arrows in Figs. 3B1 and B2). Furthermore, the organism in Figure 3B shows a change of morphology with increased curling of some of the hyphae. Clearly, both organisms were actively growing in the period between analyses, although the samples had been Au/Pd sputter coated and were exposed to the high vacuum of as SEM sample chamber and the electron beam.

Figure 3: Actively growing microorganisms on Nakhla. The images A2 and B2 (imaged on Philips XL 40 FEG-SEM) were taken 50 days after A1 and B1 (imaged on JEOL SM 6100); arrows pointing at areas of maximum growth.



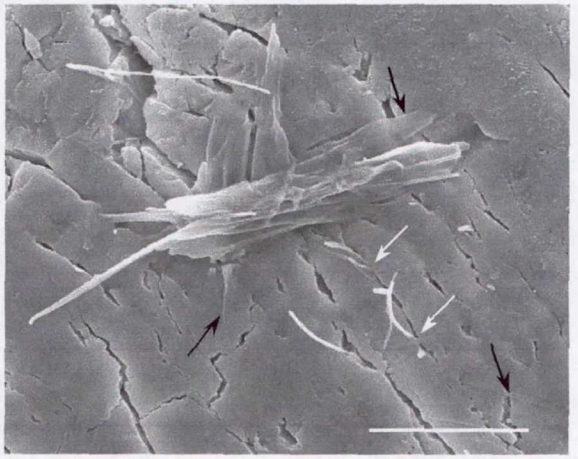
Small filaments (ca. 0.2 μm in diameter), originating from an approximately 1 μm-sized spherical, spore-like object were observed on an internal chip of Nakhla (N47) (Fig. 4). EDX mapping revealed the structure to be carbon and magnesium rich but depleted in oxygen and silicon relative to the mineral background.

Figure 4: Filamentous organism on an internal chip of Nakhla (N47) with hyphae extending out of a spherical, spore-like object. EDX analysis of the spatial distribution of C, Ca, Fe, O, Mg and Si is shown below. Scale bar 1ìm.



The organism in Figure 5 shows evidence of bundling with some filaments appearing flattened (black arrows) and some in close association with the underlying mineral surface (lower black arrow). Furthermore, small filaments can be seen to emerge from cracks and fissures, some as small as ca. 100nm (white arrows).

Figure 5: Hyphae forming organism on N47 showing evidence of bundling. Some of the filaments appear collapsed and flattened (black arrows). Cracks as small as ca. 100nm allow penetration of the minerals (white arrows). Scale bar 5ìm.



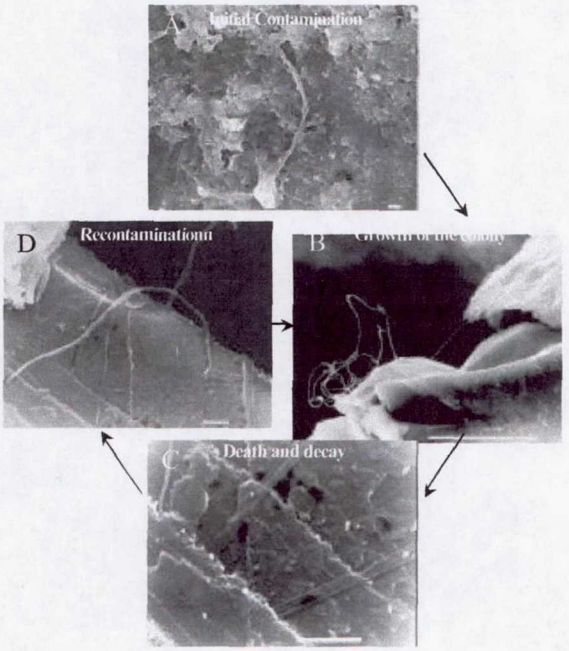
Discussion

From the structures observed throughout Nakhla it can be said that the entire meteorite is contaminated with hyphae-forming microbiota, which in some cases shows evidence of active growth (Fig. 3). The variations in hyphael diameter from only ca. 0.05 µm (Figs. 2A) up to ca. 1 µm (Fig. 1A) may be indicative of the presence of different microbial species, probably filamentous bacteria and fungi. The strong similarity between the organisms in Nakhla and the Antarctic cryptoendolithic organisms (Figure 2) provides additional support for the assumption that the structures observed on Nakhla are recent viable and non-viable terrestrial microorganisms. This evidence clearly emphasizes the potential for microbial growth in rocks, provided that sufficient nutrients are available. These findings support observations that endolithic microbial growth is possible even under unfavorable conditions as in the dry valleys of Antarctica [22, 23]. Most observed organisms appear to be viable, which may be indicative of a phase of recent

successful growth. However, the hyphae of the organisms in figures 3A and 5 are bundled together probably as a result of nutrient and, more specifically, nitrogen limitation [5]. The association of apparently successfully growing and stressed organisms within Nakhla seems to indicate that nutrients either entered this "ecosystem" in an intermittent fashion or are not distributed evenly within the meteorite. Indeed, the coexistence of aerial hyphae associated with apparently dead and decaying hyphae (arrows in Fig.1B and 6A), suggests the following model for a possible course of contamination events (Fig. 6) and may explain the occurrence of active growth:

1. Initial microbial contamination (Fig. 6A) under favorable conditions is followed by
2. Growth of a colony (Fig. 6B).
3. A period of severe nutrient deprivation which eventually results in death of the organism and subsequent degradation and decay (Fig. 6C), followed by
4. Recontamination or revival of dormant spores (Fig. 6D), probably recycling organic remains from a previous contamination event.

Figure 6: Possible course of contamination events. A) initial microbial contamination followed by B) growth of a colony consisting of multiple organisms; C) depicting death of the organisms and in situ decay with subsequent recontamination (D; same area as C) potentially leading to colony growth of a new generation. Scale bars A, B, and C 1ìm, D 10ìm.



The coexistence of organisms in various stages of decay with apparently living organisms (Fig. 1B, 6C and D) illustrates that a dynamic microbial ecosystem may be contained within Nakhla. Viable and dying filaments can even occur within an individual organism (Fig. 5) where phases of growth seem to coincide with decay. EDX mapping on the spore-like structure in Figure 4 revealed carbon and magnesium enrichment in the structure, clearly outlining its shape as seen in the SEM and suggesting that the structure is organic in nature. This region also showed depletion in oxygen and silicon relative to the background which can be attributed to the fact that the organism covers the siliceous substrate below, hence somewhat obscuring the substrate signals.

Generally, minerals and rock surfaces are supposed to be oligotrophic habitats concerning organic carbon [24]. However, cracks and fissures in the meteorite which can be as small as 100 nm (Fig. 1D and Fig. 5, white arrows) seem to allow nutrients and organisms to infiltrate and spread out through the rock. Banfield and Hamers (1997) [25] report that at least a monolayer of adsorbed water can form at mineral interfaces, and Mautner *et al.* (1997) [26] have shown that a thin layer of water enriched in mineral components formed on surfaces within the Murchison meteorite. This may in fact account for sufficient nutrient supply and circulation throughout the meteorites. Furthermore, Sterflinger (1995) [27] developed a model for fungal penetration of rocks, where after initial attachment to the rock surface thin penetration hyphae intrude into the rock along crystallographic weaknesses such as grain boundaries or discontinuities between crystals. Once the penetration hyphae found internal hollows within the rock, new fungal colonies develop. This could be one explanation for some of the small filaments protruding from fissures shown in Figure 5. As mentioned earlier, polycyclic aromatic hydrocarbons, amino acids, and aliphatic hydrocarbons have been found within Nakhla [19, 20]. Fungi are able to metabolise very diverse hydrocarbons, such as aliphatic, aromatic and polycyclic aromatic hydrocarbons [24]. Furthermore, some fungi are extreme oligotrophs and can grow on extremely nutrient depleted substrates [28, 29]. Hence, fungal growth within meteorites is not necessarily limited by lack of organic carbon but may be limited by nitrogen.

A characteristic external feature of most prokaryotic microorganisms [30] and fungi [24] is their production of extracellular polymeric substances (EPS). EPS consist mainly of water and polysaccharides but may have a nucleic acid, amino sugar, and protein component [31] and play an important role in bacterial attachment to a substrate [32]. This is exemplified by the organism

shown in Figure 1C, where EPS can be seen connecting the individual filaments with the underlying mineral.

To what extent the observed organisms represent a suitable source for the fraction of organic matter in Nakhla previously ascribed to terrestrial contamination [18, 20] remains unclear, but it is conceivable that they could influence any organic analyses. Considering the abundance of live microorganisms throughout Nakhla, the detection of bacterially derived amino acids in this meteorite [19] is not surprising. The influence of putative abiotically formed hydrocarbons in Nakhla [33] on organic analyses is subject to speculation. However, the presence of abiotically formed organic matter in Nakhla could provide an additional source of carbon for colonising heterotrophic microorganisms, resulting in a scenario where terrestrial heterotrophs harvest extraterrestrial organic matter. In this scenario the heterotrophic organisms will have very similar carbon isotope ratios to the organic matter they are metabolizing [34].

Present work, including already successful culturing experiments and the classification of the isolated organisms will provide more information towards an understanding of the contamination history of the meteorite. This will be invaluable when considering the crucial requirements for appropriate handling and curation of extraterrestrial bodies on Earth, particularly with respect to future Mars sample return missions.. Analysis of culturing experiments, flow cytometry, direct DNA extraction and 16S rDNA identification of species isolated from Nakhla are currently under way. The results of these studies will provide further details concerning the metabolic characteristics of the observed organisms, although it is probable that they are mostly heterotrophic in nature. Just as with fragments of the Martian meteorite ALH84001, which

became contaminated with recent terrestrial bacteria whilst buried in Antarctic ice [5], Nakhla may have become contaminated during the period of time in the Nile delta sediments, an environment which is certainly extremely habitable for a large variety of microorganisms [14]. However, due to the meteorite's long and partially uncertain curatorial history, it is more than likely that contamination has been introduced during curation and storage.

Conclusions

SEM imaging and EDX analyses have shown that the Nakhla meteorite has become contaminated with terrestrial microorganisms from the fusion crust through to its core. There is overwhelming evidence that the observed microorganisms are solely terrestrial in origin even though in few cases they are in close association with the mineral matrix. However, there is no morphological resemblance between the organisms and their spatial association reported by McKay *et al.* (1999) [8].

Care has to be taken in the interpretation of organics in these meteorites until microbial contamination, which would obscure and distort the results, can be further classified. The challenge is to distinguish between the terrestrial organic material and possible indigenous Martian organics. For many years to come these Martian meteorites will be the only material available for analyses. They contain a wealth of information, which should not be summarily dismissed simply because they may be contaminated, but the issue of contamination should be carefully considered. The characterization of these organisms and their metabolic/diagenetic products will be crucial in the search for biogenic activity in other extraterrestrial samples.

Acknowledgements

The authors would like to thank the DAAD for partial funding of this research, D.J. Hughes and D.M. Martill and R.F. Loveridge at the University of Portsmouth for support throughout this work, M. Barker for valuable comments on the manuscript, D. Wynn-Williams (British Antarctica Survey) for supply of the Antarctic samples, and B. Kessler. This work was supported in part by the NASA Astrobiology Institute.

References

1. McKay D.S., Gibson E.K. Jr., Thomas-Keprta K.L., Vali, H., Romanek C.S., Clemett S.J., Chillier X.D.F., Maechling C.R., & Zare R.N. (1996) *Science.* **273**, 924-930.

2. Steele A., Westall F., Goddard D.T., Stapleton D., Toporski J.K.W., & and McKay D.S. (1999) *Abstracts of the 30th Lunar and Planetary Science conference*, March 15 – 19th . Lunar and Planetary Institute, Houston Texas.

3. Imshenetsky, A.A., & Abyzov, S.S. (1970) Academy Nauk (Moscow) 157 – 166.

4. Toporski, J.K.W., Steele, A., Stapleton, D., & Goddard, D.T. (1999) *Abstracts of the 30th Lunar and Planetary Science conference*, March 15 – 19th . Lunar and Planetary Institute, Houston Texas.

5. Steele, A., Goddard, D.T., Stapleton, D., Toporski, J.K.W., Peters, V., Bassinger, V., Sharples G., Wynn-Williams D.D., & McKay, D.S. (2000a) *Meteoritics & Planetary Sciences*, **35**, 237-241.

6. Steele, A., Griffin, C., Whitby, C., Toporski, J., Westall, F., & McKay, D.S. (2000): , this issue.

7. Gillet, P., Barrett, J.A., Henlin, T., Achoubat, W., Lesourd, M., Guyot, F., & Benzerara, D. (2000) *Earth and Planetary Science Letters*, **175**, 161-167.

8. McKay, D.S., Wentworth, S.J., Thomas-Keprta, K., Westall, F., & Gibson, E.K. Jr. (1999) *Abstracts of the 30th Lunar and Planetary Science conference*, March 15 – 19th . Lunar and Planetary Institute, Houston Texas.

9. Gooding, J.L., Wentworth, S.J., & Zolensky, M.E. (1991) *Meteoritics* **26**, 135-143.

10. Bunch, T.E. & Reid, A.M. (1975) *Meteoritics*, **10**, 303-315.

11. Roy, S.K. & Hudson, N.P. (1935) *Geological Series of Filed Museum of Natural History*, **6**, 179-198.

12. Lipman, C.B. (1932) *Amer. Mus. Nov.*, No. 588.

13. Claus, G. & Nagy, B. (1961) *Nature*, **192**, 594-596.

14. Zhmur, S.I., Rozanov, A.Yu., & Gorlenko, V.M. (1997) *Geochemistry International*, **35**, 58-60.

15. Zhmur, S.I. & Gerasimenko, L.M. (1999): *Instruments, Methods and Missions for Astrobiology II,* Richard B. Hoover, Editor, *Proc. SPIE*, **3755**, 48-58.

16. Hoover, R.B., Rozanov, A.Yu., Zhmur, S.I., & Gorlenko, V.M. (1998) *Proc. SPIE*, **3441**, 203-216.

17. Madigan, M.T., Martinko, J.M., & Parker, J. (eds.) (2000): Brock: Biology of Microorganisms. *Ninth Edition*. Prentice Hall International, Inc., New Jersey.

18. Wright, I.P., Grady, M.M, Gardner, A.F., & Pillinger, C.T. (1998) *Abstract for the Lunar and Planetary Science Conference XXIX*, NASA Johnson Space Center, Houston, Texas, USA.

19. Glavin, D.P., Bada, J.L., Brinton, K.L.F., & McDonald, G.D. (1999) *Proc. Natl. Acad. Sci. USA*, **96**, 8835-8838.

20. Flynn, G.J., Keller, L.P., Jacobsen, C., & Wirick, S. (1999) *Abstracts of the 30th Lunar and Planetary Science conference*, March 15 – 19th . Lunar and Planetary Institute, Houston Texas.

21. Bada J.L. Glavin D.P., McDonald G.D., & Becker L. (1998) *Science.* **279**: 362 – 365.

22. Wynn-Williams, D.D. (1996) *Biodiversity and Conservation*, **5**, 1271-1293.

23. Edwards, H.G.M., Russell, N.C., & Wynn-Williams, D.D. (1997) *Journal of RAMAN Spectroscopy*, **28**, 685-690.

24. Sterflinger, K. (2000) *Geomicrobiology Journal*, **17**, 97-124.

25. Banfield, J.F. & Hamers, R.J. (1997) In *Geomicrobiology: Interactions between Microbes and Mineral, eds.* Banfield, J.F. & Nealson K.H., Reviews in Mineralogy 35, (Mineralogical Society of America, Washington D.C.).

26. Mautner M.N., Conner. A.J., Killham. K., & Deamer. D. (1997) *Icarus*, **129**, 245-253.

27. Sterflinger, K. (1995) PhD thesis, University of Oldenburg, Germany.

28. Parkinson, S.M., Wainwright, M., & Killham, K. (1989) *Mycol. Res.*, **93**, 529-534.

29. Barakah, F.N.I. (1992): PhD thesis. University of Sheffield, UK.

30. Costerton, J.W. J.C. Nickel, & T.I. Ladd (1978) *Scientific American,* 238, 86-95.

31. Platt, R.M., G.G. Geesey, J.D. Davies, & D.C. White (1985) *Can. J. Microbiol.*, **31**, 675-680.

32. Denyer, S.P., Hanlon, G.W., & Davies, M.C. (1993) In *Microbial Biofilms: Formation and control, eds.* Denyer, S.P., Gorman, S.P., & Sussman, M., Blackwell Scientific Publication, Oxford.

33. Zolotov, M.Yu., & Shock, E.L. (1999) *Abstract for the Lunar and Planetary Science Conference XXX*, #1879, NASA Johnson Space Center, Houston, Texas, USA.

34. Steele, A., Toporski, J.K.W., Westall, F., Thomas-Keprta, K., Gibson, E.K., Avci, R., Whitby, C., Griffin, C., and McKay, D.S. (2000) *Abstracts of the 31st Lunar and Planetary Science conference*, March 15 – 19th. Lunar and Planetary Institute, Houston Texas.