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Magnus Ivarsson, Henrik Drake, Anna Neubeck, Oona Snoeyenbos-West, Veneta Belivanova & Stefan Bengtson

To cite this article: Magnus Ivarsson, Henrik Drake, Anna Neubeck, Oona Snoeyenbos-West, Veneta Belivanova & Stefan Bengtson (2021) Introducing palaeolithobiology, GFF, 143:2-3, 305-319, DOI: 10.1080/11035897.2021.1895302

To link to this article: https://doi.org/10.1080/11035897.2021.1895302

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Published online: 27 Jul 2021.

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Magnus Ivarsson*, Henrik Drakeb, Anna Neubeck, Oona Snoeyenbos-West, Veneta Belivanova and Stefan Bengtson

*Department of Palaeobiology, Swedish Museum of Natural History, Stockholm, Sweden; bLinnaeus University Faculty of Health and Life Sciences, Kalmar, Sweden; †Department of Earth Sciences, Uppsala University, Uppsala, Sweden

ABSTRACT
A growing literature of deep but also surficial fossilized remains of lithobiological life, often associated with igneous rocks, necessitates the unfolding of a sub-discipline within paleobiology. Here, we introduce the term palaeolithobiology as the new auxiliary sub-discipline under which fossilized lithobiology should be handled. We present key criteria that distinguish the paleolithobiological archive from the traditional one and discuss sample strategies as well as scientific perspectives. A majority of palaeolithobiological material consists of deep biosphere fossils, and in order to highlight the relevance of these, we present new data on fungal fossils from the Lockne impact crater. Fungal fossils in the Lockne drill cores have been described previously, but here we provide new insights into the presence of reproductive structures that indicate the fungi to be indigenous. We also show that these fungi frequently dissolve and penetrate secondary calcite, delineating the role lithobionts play in geobiological cycles. We hope that the formalization of the sub-discipline palaeolithobiology will not only highlight an overlooked area of paleobiology as well as simplify future studies of endo- and epilithic fossil material, but also improve our understanding of the history of the deep biosphere.

Introduction
Lithobiology is a wide concept involving organisms interacting with, and to some degree depending on, rock substrates during their life cycle. Lithobionts colonize rock surfaces and pore space or metabolically utilizing the rock/mineral (Kuznetsov et al. 1963; Krumbein 1988) (Table 1). Because of its broad definition, lithobiology is not frequently used within geomicrobiology. A more restricted and commonly used term is lithotrophy, from the Greek “lithos” (rock) and “trophi” (consumer). Lithotrophs are usually described as organisms that use inorganic substrates (usually minerals) for biosynthesis or energy conservation via aerobic or anaerobic respiration (Ehrlich 2002). This is a more explicit definition, strictly based on the metabolic pathways of particular microorganisms. As a result, not all organisms living on or in rock substrates are included. In particular, the heterotrophs, an important group of organisms in rock-hosted environments, are excluded from this definition (Sohlborg et al. 2015; Borgenie et al. 2015; Ivarsson et al. 2020). Because of this, lithotrophs are sometimes subcategorised into lithoautotrophs (microorganisms able to use inorganic compounds as energy sources), lithoheterotrophs (microorganisms not able to fix carbon dioxide and must consume additional organic compounds), and photolithotrophs (microorganisms that use light as their energy source). However, by dividing organisms based on metabolism spatial and ecological information is lost. Metabolic pathways can also be difficult to deduce in ancient material, and metabolism is therefore an insufficient benchmark when defining life related to both present and past lithobiology.

Besides metabolic processes, organisms are also classified based solely on their interaction with and affinity to the rock substrate. Epiliths are organisms colonizing the surfaces of rocks while endoliths are organisms colonizing the interior of rock substrates like fractures and vesicles. Endoliths are further divided into subcategories: chasoendoliths – organisms that invade pre-existent fissures and cracks, cryptoendoliths – organisms that invade pre-existent structural cavities, euendoilths – organisms that actively penetrate rock interiors and create habitable cavities, and autoendoliths – organisms that construct the structures in which they reside (Golubic et al. 1981; Marlow et al. 2015; Ivarsson et al. 2020) (Fig. 1). Thus, endoliths, the dominant actors in lithobiology, constitute life that resides in the pore space of rocks, which at depth exclusively involves the deep biosphere.

The deep biosphere extends downwards in sediments, sedimentary rock and igneous rock of both oceanic and terrestrial crust (Orcutt et al. 2011; Heim 2011; Colwell & D’Hondt 2013; Sohlberg et al. 2015; Borgenie et al. 2015; Ivarsson et al. 2020). It represents one-tenth to one-third of all live biomass including both prokaryotes and eukaryotes, which makes it the second largest reservoir of live biomass today (Orcutt et al. 2011; Heim 2011; Sohlberg et al. 2015; Borgenie et al. 2015; Ivarsson et al. 2020). The largest reservoir of live biomass is the surface biosphere, mostly comprised of land plants. However, prior to the colonization of land by land plants at ~400 Ma, the situation was the opposite, and it has been proposed that most of Earth’s live biomass (~80%) was to be found in the deep biosphere (McMahon & Parnell 2018). This suggests that deep environments have played a crucial role in the...
The present paleolithobiology stretches from the present back to ~400 Ma (Ivarsson et al. 2020a) preceded by a 2 Byr gap. Fungus-like mycelium-forming fossils in ocean basalts dated at ~2.4 Ga are an anomaly with their high age but confirms that the ocean floor has acted as a stable microbial habitat throughout most of life’s history (Bengtson et al. 2017).

Most of the fossils are fungal relics but prokaryotes and their structural remains have also been reported (Ivarsson et al. 2020a). The abundance of fossil fungi does not reflect their predominance in the deep biosphere, but rather reflects the differences in cellular properties between fungi and prokaryotes relevant to preservation and fossilization.

Continental paleolithobiological archive

Fossil findings in continental crust are more sporadic than in oceanic crust, but a steadily growing literature over the last decade has established this fossil record as an abundant feature predominantly in fracture networks of Swedish granites (Ivarsson et al. 2013; Drake et al. 2017a, 2017b, 2018a, 2018b) (Fig. 2) but also in German sedimentary rocks (Reitner et al. 2006), Japanese flood basalts (Sakikibara et al. 2014) as well as in igneous crust in the USA (McKinley et al. 2000). Fossil filaments mineralized by oxides, silicates, and clay minerals have been described from various localities worldwide (Hofmann & Farmer 2000), and empty filamentous casts of microorganisms were detected in a quartz-feldspar-calcite vein at a depth of 300 metres in Forsmark, Sweden, dated to 355 ± 15 Ma (Drake et al. 2017a).

Lack of specific morphological characteristics has, however, made the biological affinity of the structures difficult to establish and have led to uncertainties regarding their biogenicity. Even though morphology can be convincing as, for example with the assemblages of ~1 µm sized coccolid and rod-shaped structures from deep mineral veins from the Columbia River basalts (continental flood basalts <10 Myr old), Washington, USA (McKinley et al. 2000), it is not always sufficient to prove biogenicity. Carbonaceous microfossils of colonial (ovo)coccolids cells of 1 µm size were found in calcite veins of unknown age at 207 m depth in Paleoproterozoic diorites in Sweden (Pedersen et al. 1997). The calcite in this fracture had low δ13C values (down to −46.5‰PDB), and in other fractures even lower (down to −125‰PDB) suggesting anaerobic oxidation of methane in the granite fracture network (Drake et al. 2015). At 450 m depth at Äspö HRL, Sweden, a fracture mineral succession in Proterozoic diorite with a thin dark amorphous layer and branched tubular structures was described in a fluorite-calcite-vein (Heim et al. 2012). ToF-SIMS imaging revealed abundant, partly

Table 1. Explanations of related terminology.

| Term                  | Explanation                                                                 |
|-----------------------|-----------------------------------------------------------------------------|
| Lithobiology          | Organisms interacting with, and to some degree dependent on, rock substrates during their life cycle. |
| Lithotroph            | Rock consumer. Organisms that use inorganic substrates (usually minerals) for biosynthesis or energy conservation via aerobic or anaerobic respiration. |
| Geomicrobiology       | The scientific field at the intersection of geology and microbiology.       |
| Epiliths              | Organisms colonizing the surfaces of rocks                                  |
| Endoliths             | Organisms colonizing the interior of rock substrates like fractures and vesicles. |
| Euendoliths           | Organisms that actively penetrate rock interiors and create habitable cavities. |
| Chasmoendoliths       | Organisms that invade pre-existent fissures and cracks.                     |
| Cryptoendoliths       | Organisms that invade pre-existent structural cavities.                     |
| Autoendoliths         | Organisms that construct the structures in which they reside.              |

The fossil record of deep igneous rocks

**Oceanic paleolithobiological archive**

The fossil record of oceanic basaltic crust is mostly based on investigations of IODP (and its precursors) drill cores from the Atlantic and Pacific Oceans (Ivarsson et al. 2020a, and references therein) as well as ophiolites from Germany, Cyprus and South Africa (Peckmann et al. 2008; Eickmann et al. 2009; Bengtson et al. 2017; Carlsson et al., 2019). Extensive reviews of these fossils have been published previously (Ivarsson et al. 2015a, 2019a, 2020b; McMahon & Ivarsson 2019) as well as of ichnofossils in pillow lavas (Staudigel et al. 2008; McLoughlin et al. 2010), thus we refer to those articles for detailed descriptions. Briefly, the fossil record of igneous oceanic crust
functionalized organic moieties, for example $C_xH_y^+$, $C_xH_yN^+$, $C_xH_yO^+$, interpreted to represent the remains of a microbial biofilm that was established much later than the initial cooling of the Precambrian host rock, but the age remains elusive. At Laxemar, fatty acids of putative sulfate reducing bacterial origin was detected in a vein containing pyrite with substantial $\delta^{34}S$ variability ($-19$ to $+132\%_{\text{ppdb}}$) and clay-mineralized remains of a biofilm (dated to 393 ± 15 Ma, Drake et al. 2018b). Thus,
morphology combined with biomarker data and isotopic compositions make a biological interpretation of certain putative fossils more reliable, especially when the fossils represent remains of prokaryotic life.

Similar to fossils from the oceanic crust a majority of the fossilized microorganisms described from the crystalline continental bedrock are fungi. Fossilized fungal hyphae forming mycelium-like networks with possible terminal conidial cells or chlamydomospores were described from the Triberg granite, and from a uranium mine at Krunkelbach, both in Germany (Reitner et al. 2006). In drill core LOC1 at a depth between 171 and 220 m from the Lockne impact crater carbonaceous fungal hyphae occur in brecciated granitoids (Ivarsson et al. 2013). The fossil fungi occur in complex mycelia-like networks stretching out from the fracture walls and display characteristic fungal morphologies such as repetitive septa, anastomoses between branches, and mycelial cords. The fossils co-exist with hydrothermal mineralisation and they were initially concluded
to be contemporaneous with the impact induced hydrothermal system. Recent high spatial resolution Rb/Sr dating of the hydrothermal mineral assemblage has, however, revealed a much younger age of the mineralization (357 ± 7 Ma) compared to the impact (458 Ma) (Tillberg et al. 2019). This means that the fungal colonization took place at least 100 Myr after the impact event, long after the impact-induced hydrothermal activity ceased.

Carbonaceous and partly mineralized fungal fossils were also reported in a Paleoproterozoic quartz vein at 740 m depth at Laxemar, Sweden. The fungal fossils consist of hyphae that formed extensive mycelia covering zeolites and carbonates (Drake & Ivarsson 2018; Drake et al. 2017a). Associated pyrite crystals had isotopic signatures (significantly 34S-depleted; Δ34S down to −53‰) specific for microbial sulfate reduction (MSR). The spatial relationship between the fungi and the pyrite crystals indicates that the fungi and the sulfate reducers likely occurred in a symbiotic-like relationship. Non-Septate and branched fungal hyphae were also reported from Paleoproterozoic granitic gneisses at Forsmark, Sweden (Drake et al. 2018a). The fungal fossils occur at 400 m depth in open fractures containing mineral assemblages of Paleozoic ages (396 ± 7 Ma) and with stable isotopic compositions of C and S diagnostic for microbial activity.

The presence of mycelium-forming fungal hyphae in the subsurface at the time, and prior to the colonization of land by plants (and fungi), challenges the traditional view on the evolution of fungi. Prior to the colonization of land, fungi are believed to mostly comprise flagellated unicellular aquatic organisms. Hypha, the dominant vegetative growth state among fungi, is thought to have developed during the colonization of land to facilitate the symbiotic relationship between plants and fungi called mycorrhiza. New results, however, suggest that hyphae developed in the subsurface long before the mutual colonization of land by plants and fungi (Bengtson et al. 2017; Ivarsson et al., 2020b). The unique apical growth and explorational nature of hyphae is an advantage in deep endolithic environments and enabled early colonization of these deep habitats. Also, the established relationship between fungi and prokaryotes like iron- and manganese-oxidizers (Bengtson et al. 2014; Ivarsson et al. 2015a, 2015b, 2020a) as well as sulfate reducing bacteria (Drake et al. 2017b) indicate complex microbial communities of interacting eukaryotes and prokaryotes in deep rocky settings contemporaneous with the development of other fungal-prokaryotic symbiotic-like relationships such as lichens and mycorrhiza (Peay et al. 2016). Thus, important evolutionary steps of fungi and fungal-prokaryotic interactions were not exclusive to the surficial biosphere but also occurred at depth.

A new branch of the paleobiological tree

A growing literature of fossils in deep igneous rocks identifies a previously unrecognized fossil record that holds novel information of Earth’s second largest biosphere of live biomass and its role throughout life’s history on Earth (Ivarsson et al. 2020a). Of particular interest is the fossil record prior to the plant colonization of land at 400 Ma, as the deep biosphere back then was necessarily the predominant reservoir of live biomass (McMahon & Parnell 2018). Indeed, its importance for the evolution of early life and biogeochemical processes on the early Earth as well as the distinctive nature of the deep endolithic record compared to traditional paleontology call for the creation of a specialized sub-discipline. The introduction of paleolithobiology as a distinct research area within paleobiology is based on the following unique precedents: 1) the fossils are preserved and mineralized in-situ, in their habitats, rather than post displacement in a depositional basin, 2) mineralization is either by inorganically or biologically produced authigenic minerals, or through biomineralization mediated by the metabolism of the organisms, 3) a major bulk of the fossils represent the deep biosphere, a part of Earth’s biosphere not included in the traditional fossil record, 4) paleolithobiology chiefly involves igneous crust in contrast to paleontology that almost exclusively investigates sedimentary rocks, 5) a substantial portion of the organisms are chemosynthetic (or are indirectly dependent on chemosynthesis) commonly preserved by the products of their own metabolism, thus the fossil record involves chemosynthetic (geochemical signatures of life) to a large extent, 6) the fossils are interpreted based on specific biogenicity criteria (Ivarsson 2006), 7) during their life-cycle the organisms were dependent on, and more engaged in interaction with the geosphere compared to organisms investigated within paleobiology as a whole, 8) the deep fossil habitat is uniformly represented through most of Earth’s history in contrast to habitats represented by the sedimentary fossil record (Bengtson et al. 2017).

The above arguments distinguish the paleolithobiological fossil record from the broader discipline paleobiology, and emphasise the need to approach this subfield with unique sampling strategies, different instrumentation and scientific perspectives, yet with the same overall aim as paleobiology: to explore the evolution of life on Earth. Paleolithobiology is strongly cross-disciplinary and bridges fields such as geochemistry, microbiology, and geobiology, but on a broader scope also geology and paleobiology.

A majority of the fossils involve endoliths, but the boundary between epibi- and endolithic habitats is fluid and not distinct (see Fig. 1). A similar life style as well as taphonomic and preservational similarities between epilithic and endolithic fossilized life call for a mutual treatment. The lifestyle and preservation of epilithic microorganisms responsible for the formation of desert rock varnish, for instance, is similar to endoliths in terrestrial rocky environments (Gorbushina et al. 2002; Perry et al. 2004; Gorbushina 2007). Microorganisms forming iron-manganese crusts/nodules in marine environments engage in a similar lifestyle and become preserved in a similar manner as subseafloor endoliths (Connell et al. 2009; Orcutt et al. 2020; Ivarsson et al. 2020b). Despite epiliths being, to a large extent, dependent on phototrophy, in contrast to endoliths, the community structures, colonization, interaction with the rock substrate and preservation are in many ways similar, and the different habitats should be treated under the same paleolithobiology umbrella. This also highlights the key difference between paleolithobiology and paleontology. In the first, the habitat is the same as the preservation environment, while the habitat in the latter is commonly not the same as the preservation environment (Fig. 3), important exceptions being trace fossils, fossil organisms attached to hard bottoms, and reefs. There are, of course, cases where the distinction is not clear. An example is the in situ preservation of organisms by silica-saturated fluids in association with hot springs and volcanic activity (Dong et al. 2019). The most famous example of
this type of preservation is the Rhynie chert in Scotland with its extraordinary plant-, fungi- and animal fossils (Garwood et al. 2020). Silica-rich water from volcanic springs rose rapidly, which led to instantaneous petrifaction of the early terrestrial ecosystem with the result of exceptional ultrastructure preservation (Rice et al. 1995). However, with a few exceptions of lichens, the organisms of the Rhynie chert were not rock-living, thus, despite their mode of preservation they should not be included in the paleolithobiological archive.

The in situ fossilization makes the paleolithobiological archive not only valuable for ecological reconstructions of the community structures, but also regarding the interaction between the organisms and their physical and chemical environment, for example in mineral weathering and precipitation. Detailed investigations of the fossil composition (elements, isotopes and biomarkers) as well as the composition of associated minerals will enable insight in mobilization and immobilization of bio-available elements, metals and nutrients (Ivarsson et al. 2020a). High spatial resolution techniques like SIMS, LA-(MC)-ICP-MS, ToF-SIMS, and ion microprobe enhance the level of the data collection and assist in placing the fossils in a larger context that discloses subsurface-surface exchange of hydrocarbons and organic matter (Drake et al. 2015, 2017a, 2019) or the influence of biogeochemical budgets.

Biogenicity

Because of the microscopic nature of most rock-hosted fossils, extensive investigations and discussions regarding biogenicity are usually required. Identification of fossils and evaluation of their possible biogenicity are complex tasks beyond the scope of this paper, especially since the paleolithobiological archive is made up of three different fossil categories that require separate identification and biogenicity criteria. For further reading we suggest following papers for each type: 1) Body fossils (Ivarsson 2006; Bengtson et al. 2014; McMahon & Ivarsson 2019; McMahon 2019; Ivarsson et al. 2020a), 2) trace fossils (Staudigel et al. 2008; McLoughlin et al. 2007, 2009, 2010; McLoughlin & Grosch 2015; Fisk & McLoughlin 2013), and 3) chemo-fossils (Drake et al. 2015, 2017a, 2017b, 2019; Ivarsson et al. 2020a; Heim et al. 2012). However, the need to distinguish biological fossils from abiotic micro-structures or biomorphs is fundamental in microfossil investigations and many times a challenge. Paleolithobiological fossils always represent colonization of existing rock, as compared to microorganisms preserved in sediments that subsequently lithifies to sedimentary rocks. The question of indigenousness is therefore crucial. Additionally, rock-hosted fossils typically occur in cracks, open vugs, and vesicles, and on rock surfaces, which makes indigenousness and syngenetic with authigenic minerals difficult to establish. Once indigenousness is established the weighing procedure for or against biology versus abiogenic explanations begins. Numerous lists of biogenicity criteria have been formulated with the aim to rule out abiogenic possibilities (Wacey 2009, and references therein). Biogenicity criteria for microfossils have, in general, been based on findings in sedimentary rocks, but modifications to suite igneous rocks have been formulated (Ivarsson 2006; McLoughlin et al. 2007, 2010; McLoughlin & Grosch 2015). Even though the lists differ slightly they are usually...
boiled-down to the following criteria: 1) The geological context of the sample should be known; is it compatible with past life? 2) Is the putative microfossil indigenous to the rock rather than being a modern contaminant? Is the putative microfossil syngentic with secondary mineralization? 3) Does the fossil-like structure resemble known microbiological morphologies? 4) Does the fossil-like microstructure contain chemical evidence for life such as molecular biomarkers or stable-isotope signatures diagnostic for life? 5) Is there evidence for structural remains of colonies or communities within the sample?

Even though the bulk of the criteria is still valid, there is a risk in “locking” oneself to fixed biogenicity criteria that simply need to be checked off a list. The distinction between biological morphologies and abiotic mimicking is a field of constant progress, where the borders are pushed forward and, at times, overlap. Recent advances have shown that the complexity of biomorphs sometimes matches “undoubted” biological morphologies (McMahon 2019), which calls for flexibility in the biogenicity criteria. Indisputable fossil assemblages do occur and have been described but a substantial portion of the fossil findings is ambiguous and needs to be approached with caution. One common strategy is to treat non-biological “possibilities” as “null hypotheses” in the sense that an ambiguous microbial-like structure “should not be accepted as being of biological origin until possibilities of their non-biological origin have been tested and can be falsified” (Brasier et al. 2004).

There is a risk in this reasoning since the available “abiotic null hypotheses” very well could be inappropriate null hypotheses. In other words, many fossil-like microstructures are likely to be the products of abiotic processes that have not yet been discovered or brought to the attention of palaeontologists; the right “null hypotheses” are thus not yet available (McMahon et al. 2021). At the same time, we cannot take into account all unpublished biomorph and abiotic possibilities, and need to relate the studied forms to known biomorphs.

One way to avoid this deadlock is to construct hierarchical evaluation systems where criteria need to be satisfied at a given category before the candidate biosignatures can be evaluated at the next level (McLoughlin & Grosch 2015). The hierarchical scheme is formulated to give increasing confidence of a biogenic origin by systematically checking off a list of criteria. Even though such a model is designed to secure a confident biogenicity of putative bio-structures it is yet limited by pre-defined and fixed criteria, and has no room for flexibility. It is limited by presumptions on what type of microorganisms are expected and what such microorganisms leave behind, like trace fossils or biominerals. For example, if the scheme is based on knowledge of prokaryotes and the studied micro-structure is of euarchytic origin with complex morphologies and growth patterns that falls out-side the given range of criteria for prokaryotes the outcome will be wrong. We may compare it with the commonly used taxonomic keys based on sequential binary choices. A taxonomic key is useful to identify forms within a known spectrum of taxa but collapses when additional taxa are present that were not available for the construction of the key. Also, such a hierarchical model is only designed to confirm or reject biogenicity, it does not involve the aim to reach a deeper understanding of the investigated structures. In the end, this may lead to unnecessary rejections on both ends – possible biomorphs as well as fossils.

As an alternative, we suggest a more neutral approach where both biogenic and abiotic criteria need to be fulfilled independently. Instead of asking “Can we prove biogenicity of these structures?” we should ask “Can we propose a mechanism for the formation of these structures?” We advocate a model of parallel genesis evaluation, where a possible biogenic and a possible abiotic genesis are investigated in parallel. With such a strategy biogenicity criteria are not formulated to exclude abiotic explanations and vice versa, but to gather and evaluate information in favour of both possibilities independently, and in the end, to reach the most likely explanation. Morphology, as an example, is investigated independent of the chemical composition, and categories do not have to be satisfied before moving on to the next level. Absence of biomarkers diagnostic for life does not mean that complex morphology of a fossil is considered less important and in the end results in rejection of a possible biological interpretation. There are numerous examples of morphological traits that are known only from biology and not from abiotic biomorph production, like different stages of reproduction structures (Ivarsson et al. 2020a).

Even in a model like this, basic requirements such as indigenuousness and syngenecity need to be satisfied before moving on with investigations of morphology and chemical composition. However, it is not always as straight-forward as with microfossils in sedimentary rocks. Fossilized communities in open vesicles, for instance, are preserved by authigenic mineralization and not embedded in secondary minerals. This means that morphology, authigenic mineralization and chemical composition are interlinked, and to understand indigenuousness the physical and chemical nature of the fossils need to be resolved.

The strategies to test biogenesis versus abiogenesis differ between the three main types of fossils, and also within each category. Figure 4 is an outline for parallel genesis evaluation in general terms and includes the criteria that need to be addressed. Depending on the nature of the microstructures or signatures that are investigated the criteria vary and can be more specified, but there is no hierarchical order between them. Certain criteria like abiotic or biological isotope signatures and syngentic biomarkers unique to life are, if satisfied, considered strong and compelling arguments for either an abiotic or a biological interpretation. When all lines of evidence have been investigated and evaluated the most probable explanation will be reached. In cases where it is difficult to determine the origin of a putative structure it will be termed dubiofossil (Hoffmann, 1972).

**Exploration of the paleolithobiological fossil record**

For further exploration, drilling campaigns dedicated to exploring the paleolithobiological record should be prioritized. Until now, drill cores have only been available through other projects where the drilling sites have been selected on premises other than paleobiology. Future drilling campaigns where the paleolithobiological record is one of the major targets would enable better suitable site selections based on aspects such as variations in rock composition, tectonic regimes, age, hydrologic regime, depth and geographic spread. Also, existing mines are an under-used but important
Figure 4. Scheme showing parallel genesis evaluation with criteria for both biological and abiotic interpretations. The outcome of the evaluation will be either a biological or abiotic origin, or if ambiguous, classification as a dubiofossil. The scheme is designed to fit body fossils, trace fossils, and chemofossils in general terms, but criteria may need to be modified, added, or removed for each group depending on the nature of the fossils. For both biological (green) and abiotic (red), evaluation of the geological and geochemical context, as well as the indigenousness with the sample need to be resolved. The conditions need to correspond to known conditions for abiotic processes and/or be compatible with life. The category “Biological mineral textures” involve minerals with potential biological origin such as framboidal pyrites, microstromatolites, or carbonates with biological microfabrics. Morphology, and chemical composition, including organic content and isotopic composition, will all be treated individually as single lines of evidence for either a biological or abiotic interpretation. Thick lines indicate particularly important criteria. “Complex biological morphology” refers to morphologies only known from biology that have not been mimicked by abiotic processes such as certain stages in reproduction cycles. “Organic content” is not necessarily of biological origin but the term “Syngenetic biomarkers” indicates complex biomolecules that are known only from biology, and not to be formed abiotically. Hence, it is of highest importance that the biomarkers are syngenetic with the fossil structure and not introduced to the sample at a later stage like, for instance, being adsorbed on metals or minerals. This is the reason for the term “Adsorption of biomolecules” within the red area of the chart. “Biological isotopic signatures” includes compound specific isotope signatures of both minerals and organic molecules.
resource that can be utilized for sampling and as platforms for future drilling operations. This would give access to specific depths and rock types, as well as provide more geographic spread than existing deep drilled bore holes presently grant us.

Another strategy is to investigate existing collections. Since most of the paleolithobiological record is to be found in igneous rather than sedimentary rocks the search for fossils in collections should focus on petrological and mineralogical rather than paleontological collections, which predominantly consist of sedimentary rocks. A particular focus should be carbonaceous material, which may reveal exotic details of the deep biosphere when studied with modern methods. The finding of the Lockne fossils is an example of this. The presence of bitumen in the drill cores was reported by Sturkell et al. (1998), and its source was explained as downward migration of hydrocarbons from overlying organic-rich schists heated at impact. Detailed investigations showed that a majority of the carbonaceous material and bitumens in the Lockne drill cores were in fact fossilized fungal communities (Ivarsson et al. 2013). Fungi had formed a basal biofilm that covered the entire mineral surfaces in cavities in the brecciated granite (Fig. 5A,B). From the biofilm a mycelium of interconnected fungal hyphae had grown out into the pore space, where it was preserved as carbonaceous material (Fig. 5C). These features however, were only visible at micro-scale investigations using SEM.

The Lockne fungal fossils have been described in detail by Ivarsson et al. (2013), and age determined by in-situ Rb/Sr dating of secondary calcite-albite-feldspar (357 ± 7 Ma) (Tillberg et al. 2019). Here, we report new observations of the fungal fossils that deepens the complexity of these communities.

**Samples**

The filamentous structures were found between 171.30 and 219.90 m depth in the drill core. The entire fossiliferous section is thus 48.6 m in length. Nine samples were selected from this section at various depths. The selection was done with respect to abundance of cavities such as fractures and vugs, their size and depth, as well as where these were observed to contain filamentous structures. The fragile and exposed nature of the filamentous structures demanded relatively open vesicles, thus, deep and narrow vugs and fractures were excluded. Each sample contained usually more than one cavity with filamentous structures. The filamentous structures were found and studied under microscopy in situ in the drill core samples. For the ESEM analysis the cores were sawed to cubes of centimetres in diameter. A few filamentous structures were removed with forceps of stainless steel and placed on a plate with silver glue for ESEM and EDS analyses.

**ESEM**

An XL30 environmental scanning electron microscope (ESEM) with a field emission gun (XL30 ESEM-FEG) was used to analyse the minerals and the filamentous structures. The ESEM was equipped with an Oxford x-act energy dispersive spectrometer (EDS), backscatter electron detector (BSE) and a secondary electron detector (SE). The acceleration voltage was 20 or 15 kV depending on the nature of the sample. The instrument was

![Figure 5. Fungal fossils from Lockne impact crater. A. An euhedral calcite crystal covered by acarbonaceous biofilm from which hyphae protrude and creep along the surface. Branching and anastomoses occur in between hyphae. B. A biofilm has come loose from a crystal. C. An entangled and complex mycelium of hyphae and incorporated minerals.](image-url)
calibrated with a cobalt standard. Peak and element analyses were made using INCA Suite 4.11 software. Since most EDS analyses were performed in situ and subject to variations in focal depth the EDS analyses should be regarded with caution. However, consistency throughout the analyses and similar results from analyses of filamentous structures removed and placed on silver glue indicate that the analyses are reliable.

**Synchrotron-radiation X-ray tomographic microscopy (SRXTM)**

Synchrotron-radiation X-ray tomographic microscopy (SRXTM) was carried out at the X02DA TOMCAT beamline at the Swiss Light Source, Paul Scherrer Institut, Villigen, Switzerland. For optimal penetration the energy of 22 keV was applied. A total of 1501 projections were acquired, during rotation of the specimen over 180°, post-processed and rearranged into flat- and darkfield-corrected sinograms. Exposure time per single projection was 450 ms. During the scanning process a LuAG:Ce 20 um scintillator was used. The obtained tomographic volume were visualized using Avizo 9.5.0 (FEI Company). The object was segmented in the software’s segmentation editor by threshold segmentation which allows less manual interaction, but requires the higher quality images. With the 20x lens used, the resulting voxel size was 0.325 µm.

**Results and discussion**

Overall, the fungal mycelium consist of a basal film laid down on the mineral surfaces from which hyphae and other growth structures propagate (Fig. 5). Besides the entangled mycelial network of interconnected hyphae, abundant and irregular swelling structures also occur that are of various size and morphologies. Not all can be related to known fungal morphologies, to some extent owing to diagenesis, but some clearly possess morphologies known from fungi (Webster & Weber 2007), not least endolithic fungi (Golubic et al. 2005; Beuck & Freiwald 2005; Radtke et al. 2011). Within the entangled mycelium, club-shaped hyphae with lengths between 20 and 50 µm and diameters between 5 and 10 µm occur with terminal swellings with diameters between 10 and 20 µm (Fig. 6). The hyphae are distinctly septated with the swelling on top. These structures correspond to known morphologies of fungal conidiospores (Webster & Weber 2007).

There are also larger swellings that develop into cone-shaped structures with a base diameter of 50 to 70 µm and a top diameter of 70 to 100 µm. Different stages of growth are observed starting with a relatively flat elevation from the basal film (Fig. 7A), followed by growth perpendicular to the film forming a swelling (Fig. 7B) that eventually develops into a cone-shaped body (Fig. 7C). The top-side of the cones is characterized by globular lumps about 5 to 10 µm in diameter (Fig. 7B,D,E). These relatively large structures are similar to sporangia among sac fungi (Ascomycota) (Webster & Weber 2007). Similar swellings with cone-shaped features have been reported from endolithic marine fungi producing trace fossils in shells and corals (Golubic et al. 2005; Beuck & Freiwald 2005; Radtke et al. 2011). The cone shaped swellings were described as *Saccomorpha clava* most likely produced by the fungus *Dodgella inconstans* Zebrowski (Golubic et al. 2005). The presence of extensive reproductive structures clearly indicates that the fungal communities were indigenous to the deep habitats where they existed in vital colonies, rather than having

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**Figure 6.** ESEM images of conidiophores. A. Conidiophore-like structures with terminal swellings. B. Conidiophores protruding from a hyphae.
Figure 7. ESEM images of sporangia. A–D. Sporangia at various growth stages. The sporangia protrude perpendicular to the underlying biofilm in a cone-shaped fashion and develop a bulgy top. E. Close-up of the bulgy top of the sporangia.

been randomly introduced by downward migrating fluids from the surface.

The fungal hyphae also display frequent penetration of the associated calcite. Bundles of hyphae drill and infiltrate the minerals, sometimes straight through which makes it difficult to distinguish between sites of entry and exit (Fig. 8A–E). In places where hyphae have been removed from the mineral surface marks of negative pits are left behind indicating chemical dissolution of the mineral (Fig. 8B). Fungi are known to etch carbonates chemically from both subsurface environments (Bengtson et al. 2014; Ivarsson et al. 2015b) and more surficial environments like soils (Gadd 2010). It could be argued that instead of actively dissolving the minerals the hyphae have been passively entombed by later mineral precipitation. However, the hyphae normally creep along the mineral surfaces and at a certain point change growth direction straight into the mineral substrate; this indicates the mineral was already formed when the hyphae penetrated it (Figs. 8, and 9). Also, all hyphae originate from a basal film that is laid down on the mineral surfaces (Fig. 5), which excludes the possibility of the hyphae only being encapsulated by mineral growth, and supports the fact that the hyphae have been actively boring into the carbonates.

The reasons for penetration of minerals could be explorational, in the sense of searching for, or acquiring, new habitats. There could also be trophic reasons. The fungi may obtain necessary elements like Ca, C or trace elements from dissolving the carbonates. It is obvious that the fungi are responsible for mobilizing elements in the deep settings, which in a larger context could influence the exchange of elements between the geosphere and the hydrosphere, and be of importance for geobiological cycles.

A similar connection between bitumen and microbial bio-signatures in the form of extreme variability of C isotopes in calcite and preserved microbial molecules (such as fatty acids) was possible in mineral coatings at the Siljan impact structure (Drake et al. 2019). Micro-scale U-Pb dating of the calcite crystals showed that the microbes lived 80–22 million years ago, and a connection between previously descended bitumen of sedimentary origin and methanogens was presented (Drake et al. 2019). The connection between bitumen and subsequent microbial colonization in bedrock fractures therefore seems important for understanding initiation and energy sources for the microbial activity in the deep biosphere.

Outlook

At the Swedish Museum of Natural History, Stockholm, there are numerous bitumen samples, which have been collected from mines from different locations in Sweden over the course of more than 100 years. We have now initiated studies of these bitumen samples in the search for ancient microbial life. We focus on the search for morphological evidence of microorganisms, mineralized microorganisms, isotopic composition of co-occurring calcite and pyrite, and preserved organic remains, in
Figure 8. ESEM images of fungal borings in calcite. A. Overview of fungal hyphae interacting with calcite surface. B. Close up showing dissolution marks made by a hypha. C. Close up of the point where several hyphae penetrate the calcite surface. D. A hyphae penetrating a calcite right through. E. Close up showing the point of entrance or exit of the fungal boring.

Figure 9. ESEM images of fungal borings in calcite. A. Point of penetration in calcite by several parallel hyphae. B. Point of penetration in calcite by several parallel hyphae. Note the alteration in hyphal appearance towards the penetration, from smooth to bulgy. C. A hyphae creeping along a calcite surface partly dissolving the mineral.
Figure 10. Photographs of calcite-bitumen specimens from cavities from A. Gåsgruvan (NRM specimen #20 140 162). Euhedral rhombohedral calcite occurs to the left and anhedral black coloured solid bitumen to the right. B. Dannemora mine specimen showing euhdral calcite (scalenohedral habit) covering a fracture surface. Solid bitumen (black spheres), Fe-oxides (reddish fine-grained coating) and pyrite (fine-grained cubic crystals, visible as fine-grained spots with metallic luster in the photograph) occur together with the calcite (NRM specimen #22 202). C. SEM image (NRM specimen #22 202) of aggregates of radiating Fe-oxides on solid bitumen.

Figure 11. SEM- A and SRXTM B image of calcite crystals from the Dannemora sample in Figure 9B. A. Back-scattered SEM-image showing two partly altered scalenohedral calcite crystals with a partial coating of bitumen (dark) and spherical dissolution pits with pyrite on the rims (bright). B. Volume rendering of a scalenohedral calcite crystal with pyrite precipitates highlighted (in yellow). Fe-oxides occur as aggregates of radiating in crystals (bright grey) and calcite is dark.
order to shed more light on the fossil deep biosphere in the Baltic shield and of the connection between bitumen and subsequent microbial processes (Figs. 10 and 11). The timing of colonization of the deep biosphere in the Baltic shield can also be assessed by utilization of micro-scale radiometric dating of minerals formed in relation to microbial activity. It is noteworthy that the most promising samples for detection of fossilized life in the igneous crust are not in the fossil collection, but in the mineral collections.

We conclude that a formal definition of a paleolithobiological framework including sample- and analytical strategies is long overdue, and that such a schema as we have outlined herein, will not only enhance, but also enable more effective future exploration of deep and surficial fossilized lithobiological material.

Acknowledgments

We thank Marianne Ahlborn (SU) for assistance with ESEM, Andreas Karlsson (NRM) for access to the NRM mineral collections, and Stephanos Kilias, National Kapodistrian University of Athens, for discussions. We acknowledge the Paul Scherrer Institut, Villigen, Switzerland for provision of synchrotron radiation beamtime at the TOMCAT beamline X02DA of the SLS and would like to thank Federica Marone for assistance.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

This work was supported by the Carl Trygers Stiftelse for Vetenskaplig Forskning [18:167]; Svenska Forskningsrådet Formas [2017-0076]; Vetenskapsrådet [2013–4290,2017-04129,2017-05186].

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