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Hidden in the coat: Geomicrobiological investigations of rock art ecosystems and challenges for their sustainable preservation

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Chapter 1

Introduction

1.1. Preservation of rock art

Rock art is a precious heritage that prevailed from prehistory into the modern times, it holds significant implications to past cultures and climate changes and are found in most of the world. Records of rock art span from the Pleistocene to the modern times, it indicates the development of human cognition and modern behavior (Aubert et al. 2019), while recording the fauna and flora that were long gone due to shifting landscape and climates (Guagnin et al. 2016). Rock art is also increasingly fragile, under the threats of human development, vandalism, looting, inadequate tourist management and natural weathering (Watchman 2005; Whitley 2005; Agnew et al. 2015; Cerveny et al. 2016; Agnew et al. 2022). There is ongoing research in remote regions of Africa and Asia to discover rock are sites and assess their significance as well as their preservation. Rock art is endangered in Africa by climate change and human activities. For instance, many North African rock art is enduring strong deterioration mostly due to the onset of climatic conditions different from the time of its creation (Cremaschi et al. 2008). Hall et al. (2007) proposed that solar radiation causing thermal stresses may lead to separation of pigment and paint of Southern African rock art (a sort of thermoclastic effect). The impact of moisture and temperature on rock surface is also noticed and monitoring is suggested for preventive measures (Meiklejohn et al. 2009; Sumner et al. 2009).

In recent decades archaeologists developed new lines of research into the wealth of knowledge rock art holds, as well as concern for its preservation state, especially open-air rock art that is exposed directly to natural and human weathering. Rock art is a complex and dynamic system: its investigation and preservation require a holistic perspective including contributions from Archaeology, Earth Sciences, and Microbiology. The aim of this thesis is to combine interdisciplinary methods to investigate the status of open-air rock art from case studies and what this means for their long-term preservation. The importance of natural weathering on rock art is acknowledged but much more could be further studied. Rock decay has been investigated among the disciplines of geology and geomorphology, in terms of physical, chemical and biological issues, but a holistic approach to combine all-natural impacts would provide significant insights into all open-air sites (Darvill and Batarda Fernandes 2014). Rock art, including the pigments and underlying stone, is subject to physical, biological and geochemical changes in the natural environment (Dorn 2013). Such processes often happen simultaneously and sometimes the consequences of bio-geochemical weathering could be observed. The decay of rock art includes the deterioration of pigments and the alteration of rock supports. Weathering of the rock surface involves complex interactions between minerals and microorganisms, in which the contribution of microbes to rock surface decay has long been noticed (Gorbushina 2007). Biotic weathering caused by lichen and other biological organisms on rock art and stone materials is well known in literature (Warscheid and Braams2000; Doehne and Clifford 2010) and could be explained through biogeophysical and biogeochemical processes. Biogeophysical steps include the penetration of fungal hyphae that cause mechanical damage to the substrate. Biogeochemical weathering involves the dissolution of the substrate and precipitation of new minerals formed by producing acids such as oxalic acid and carbonic acids (Aubry et al. 2012; Marques et al. 2016).

However, recent research suggests a more articulated scenario; besides as an agent of deterioration to rocks, there is the possibility that some biological growth on rock surfaces is not related to decay, in some cases they are even suggested as beneficial to rock preservation (Pinna 2014; Gulotta et al. 2018). Microorganisms do not exist as single planktonic cells on lithic surfaces, instead they form aggregations bound together by self-secreted extrapolymeric substances (EPS) and survive at the air-rock interface as subaerial biofilms (SABs). SABs protect the microbial communities within and affect the rock both biochemically and physically (Villa et al. 2016), which have been noticed by geologists and rock art researchers. (Dorn 2013) pointed out that biological organisms can hold fragile rock surfaces in place and produce protective coatings of oxalic crusts. (Dorn 2009) also suggested that research on rock coatings is important to understand the weathering and stabilization of rock art. For example, rock varnish in arid lands is a type of rock coating that contributes to the integrity of lithic surfaces thus hampering the effects of the physical disaggregation (Dorn 1998; Zerboni 2008).

In this research we propose to consider components of the rock art, including pigments, rock substrate, microorganisms and the surrounding environment as a “rock art ecosystem” with intertwined interactions that result in the alteration of the rock art (Zerboni et al. 2022). This thesis will utilize an interdisciplinary approach with case studies that generated several papers both published and in progress. The major case study is Yabelo in southern Ethiopia, where a recent archaeological investigation confirmed that the rock art in this region is of great cultural significance, and requires further research as well as actions for its preservation (Gallinaro et al. 2018). Fieldwork in Ethiopia was supported by the (H)ORIGN Project (PI: E. Spinapolice of the Sapienza, University of Rome), funded by the Italian Ministry of Education, University and Research (MIUR) and is part of “Tracing the First Herders in East Africa: Cultural, Symbolic and Biological Trajectories” (PI: M. Gallinaro of the Sapienza, University of Rome). The second case study is Salut in Oman where M. Cremaschi and colleagues performed rock art survey of the area surrounding the citadel supported by the local Ministry of Heritage and Tourism (MHT) and the Italian Ministry of Foreign Affairs (MAECI). Finally, the research was supported by the University of Milano through the SEED2019 grant (CHROMA Project) and MUR through the project "Dipartimenti di Eccellenza 2018–2022" awarded to the Dipartimento di Scienze della Terra "A. Desio" of the Università degli Studi di Milano and benefited by the instrumental innovation acquired through this funding. The PhD of Ying-Li Wu was funded by the Ministry of Education, Taiwan.

1.2. Analysis of rock art and sustainable research

The first part of this thesis includes chapters 2 and 3, where we discuss the processes affecting rock art stability, how to minimize harm during investigations of rock art and the application of scientific analyses for producing and dating rock art. It is fundamental to understand how rock art was made in the first place before we can proceed with any preservation issues. The materials and techniques used all influence the robustness of rock art and how it will age. Technical analysis of pigments and ochre investigate ancient technologies and provides clues for the manufacture of rock art that could also relate to the cultural choices and available resources of the people that made them (Sillar and Tite 2000; Martinon-Torres and Killick 2015). Pigments are usually made with inorganic mineral chromophores, iron oxide being the most common mixed with organic binders that could be proteinaceous products of domestic animals. Mineral pigments are generally referred to as ochres, but this term likely includes a large range of Fe and Mn oxy-hydroxides. Iron ochres such as hematite produce red colors, while charcoal and soot provide black, and gypsum and clay minerals produce white, to name a few (Domingo and Chieli 2021).

Inorganic and organic ingredients could be identified by a variety of analytical instruments (Dayet 2021), but concerning the long-term preservation of rock art, non-invasive or micro-invasive investigations have priority over invasive sampling that will result in ultimate loss. Thanks to the development of portable instruments, it is possible to make preliminary examinations of rock art in situ with non-invasive spectroscopy such as portable XRF (X-ray fluorescence) and portable Raman; they provide elemental and mineral information for the pigments, base rock, and mineral coatings overgrowing the rock art. Hernanz et al. (2014) analyzed mineral crusts and pigments of open-air rock art in Spain in situ with Raman and diffuse reflectance infrared Fourier transform spectroscopy, they identified dolomite and calcite in the rock substrate, oxalate, gypsum, calcite, clay, dolomite, α-quartz, anatase and hematite on the surface of the painting panel or in surface accretions. Hematite and amorphous carbon are found as pigments for red and black pictographs. However, these analyses only reflect surface conditions and a combination of both in situ and invasive laboratory methods are used for understanding the coating-paint-rock stratigraphy and organic binders (Dayet et al. 2022).

Laboratory-based investigations could achieve results to further define rock art in terms of production and preservation state, depending on the method there are different sample requirements. Mineral composition could be recognized by thin section petrography, X-ray diffraction (XRD), Raman spectroscopy and Fourier transformed infrared spectrometry (FTIR). The semi-quantitative elemental analysis could be carried out under scanning electron microscopy coupled with dispersive X-ray spectroscopy (SEM-EDX) and X-ray fluorescence (XRF), while quantitative elemental analysis could be achieved by inductively coupled plasma and atomic emission spectroscopy (ICP-AES), neutron activation analysis (NAA) and laser ablation inductively coupled mass spectroscopy (LA-ICP-MS), in which NAA and LA-ICP-MS are the most sensitive methods for trace elements (Dayet 2021). For organic binders, it is possible to apply gas chromatography-mass spectrometry (GCMS) and liquid chromatography and tandem mass spectrometry (LC-MS/MS) for protein analysis. (Domingo et al. 2021; Domingo and Chieli 2021; Dayet 2021).

The preliminary investigation with portable equipment could serve as a basis to continue planning if the research requires further sampling, as well as finding locations for effective sampling that would minimize the impact on the integrity of the rock art. However, for remote rock art sites that are difficult to export portable instruments, we propose geomorphological observations on site and sampling nearby rock coatings that are representative of crust and patinas on the rock art for destructive analysis. For a direct sampling of the rock art, using adhesive strips could minimize the damage while obtaining enough material for pigment and biofilm analyses under a scanning electron microscope (SEM) and confocal laser scanning microscope (CLSM). SEM provides both high resolution microscopic images and semi-quantitative elemental analysis, while CLSM reconstructs 3D images of the biofilm. These two different sampling approaches can provide information on the preservation state and manufacturing technology while sustaining the integrity of the rock art. Small rock samples are important because they allow observation of the surface and inner profiles, while on the contrary portable tools only allow surface analyses. Moreover such samples make visible the interaction between rock, SABs and pigments under high-resolution microscopic techniques (Zerboni et al. 2022).

Another important research line of rock art involves dating, which could imply who made them and explain the events depicted in chronology. The dating of rock art could also provide information for the associated archaeological deposits. Techniques for dating have also evolved greatly in the past decades, in addition to relying on style and relative archaeological context, the advancement of accelerator mass spectroscopy AMS-^{14}C radiocarbon dating allowed direct dating with only a few milligrams of the sample, which require organic carbon for example charcoal. To remove contaminating carbon sources including oxalates, samples usually undergo chemical pretreatment such as acid-base-acid (ABA) before combusting the remaining target organic carbon for AMS-^{14}C dating. Preliminary examinations with microscopic and spectroscopy instruments are crucial to achieving precise sampling of organic carbon and removing contamination (Bonneau et al. 2017a; Bonneau et al. 2017b). In another approach, two independent methods were used to date rock art in Eagle Cave, Texas. In the first one, organic carbon was directly isolated from the paint layer with the plasma oxidation technique instead of combustion and AMS-^{14}C dated. Direct plasma oxidation of the organic carbon could avoid the risks of losing organic carbon during intensive ABA washing. In the second one, oxalates precipitated over and beneath the rock art were dated for relative *ante quem* (minimum age) and *post quem* (maximum age). Once formed, oxalate has no significant carbon exchange with the atmosphere and the carbon isotope measured indicates the time of its formation. In this case the stratigraphy of oxalate-paint layer-oxalate must be confirmed, and organic carbon is removed with low-temperature plasma oxidation technique to obtain purified oxalate samples. Dating results from both methods support each other in presenting an oxalate minimum age, direct organic carbon age from the painting layer and maximum oxalate age (Steelman et al. 2021). However it should be kept in mind that oxalates are unstable and easily undergo recrystallization thus reopening the ^{14}C system.

In the case of Sulawesi, Indonesia, uranium series dating was used to confirm the oldest hunting scene discovered so far. Thin films of water running over cave walls precipitated calcium carbonate to form coralloid speleothems overlying rock paintings. The calcium carbonate contains small amounts of uranium, which could be used to date carbonate formation because ^{238}U and ^{234}U eventually decay to ^{230}Th at a known rate. The results showed the hunting scene was at least 43.9 ka years old, the earliest evidence of narrative storytelling with figures in action, which is of great significance because inventing fiction could be a critical stage in the evolution of human language and modern cognition. (Aubert et al. 2019). Shortly before this revelation, the same team (Aubert et al. 2018) applied uranium-series dating to suggest the oldest figurative rock painting (at that time) of a wild bovid to be made at least 40 ka years ago, also in Indonesia.

Rock art is a valuable resource of cultural heritage that reserved implications of past anthropological and environmental data, with overlying crust as a source of dating while providing stability to the rock surface. Because rock art is a fragile heritage of high value, we proposed a sustainable approach toward its analysis and preservation in chapter 2. In chapter 3 (Zerboni et al. 2021) puts forward an interesting case by radiocarbon dating of organic carbon trapped inside rock varnish, thus providing a closed system that avoided later contamination. The dark varnish was identified on petroglyphs in Oman, which enhanced the original picture while the rock substrate was weathered down. Instead of sampling directly from the petroglyph, rock varnish elsewhere on the same rock was taken thus preserving the integrity of the rock art. In these two papers we suggested sampling protocols that are sustainable for the rock art, while generating crucial data for dating and analysis, proving the methods worked favorably in terms of both preservation and academic research.

1.3. Rock coatings and the microbiome at rock/atmosphere interface

In the second part of the thesis we present geomicrobial investigations of rock art coatings in chapter 4 and further discuss microbial bio-pigments in chapter 5. After we gain information on how rock art was manufactured, and the possibilities of acquiring chronology by dating and developing best practices for sampling, we then take a step further to research preservation issues. It is important to investigate the coatings formed on rock art sites, because the analysis of crusts and patinas on rock art is crucial to understanding the rock art preservation (Domingo et al. 2021; Russ et al. 1999).

Coatings are characteristic of open-air rock art sites and display a variety of forms, Green et al. (2017) analyzed mineral depositions in rock art shelters of Kimberly, northern Australia, and defined four different systems: polychrome fringes, dispersed wall coatings, floor glazes, silica stalagmites and skins. In these systems mineral phases alternate and no single source or process was identified, but the major minerals in accretions were sulfates, oxalates, and phosphates with a minor proportion of nitrates. In this research, mineral crusts, patinas, accretions…etc. will be referred to collectively as rock coatings. Rock coatings are found in different geological and climate settings, their source of formation is a combination of abiotic and biotic processes. According to Dorn (2013), there are many different types of rock coatings, such as mineral crusts of carbonate, nitrate, phosphate, sulfate, oxalate, rock varnish, and heavy metals and silica glaze. The reality is that coated rock surfaces are much more common than uncoated ones. Coatings come in a variety of textures and colors, they influence the appearance as well as weathering of the original bedrock. The formation of rock coatings could be seen because of elemental transportation during landscape geochemistry processes, when elements reach physical, chemical, and biological barriers they are precipitated as coatings. Physical and chemical barriers often work simultaneously to create inorganic rock coatings, for example soluble Al-Si complexes on silicate minerals are mobilized during wetting, when the transition state is crossed and the wetting film ruptured, silica precipitates as silica glaze. Another example is the black-colored gypsum crust caused by air pollution, where sulfur from fuel combustion reacts with the carbonate in limestone and forms gypsum sulfate crusts.

On the other hand, microorganisms are associated with the biomineralization of biological rock coatings, for example oxalate crusts are often formed by lichens and fungi (Carter and Viles 2005; Carter and Viles 2003; Gorbushina 2007; de la Rosa et al. 2013; Marques et al. 2016; Gadd 2017). Overflowing water could carry oxalate from the lichens and precipitate crusts at another location after evaporation. Desert rock varnish occurs under physical, chemical and biological barriers. First clay minerals and dust are aggregated on the rock-air interface, then the Fe and Mn are concentrated by metal fixing bacteria (Northup et al. 2010). Fe and Mn will then be physiochemically cemented to clay minerals and the rock to form dark varnish. In the case of phosphate skin, bird droppings or microorganisms provide the phosphates that are mobilized and precipitated (Dorn 2013).

The role of the microbiome in rock art preservation has been gaining attention. Dark rock varnish that contains Mn and Fe from dust and biomineralization was used as a canvas to carve petroglyphs in The Burrup Peninsula, Western Australia, but local industrial development could induce acid rain that changes the pH and microbiome of the rock surface and lead to the dissolution of the rock art (Gleeson et al. 2018). From the same region, Green et al. (2021) investigated dark-colored accretions associated with petroglyphs and rock paintings, finding mostly calcium oxalate and gypsum and in some cases a significant amount of phosphates. The laminated nature of the accretions and SEM images of microbes suggest a biological origin, and the possibility of using the oxalate material for dating. Because of the complicated interactions between rock substrate, pigments, and microbiology during physical and biogeochemical transformations described above, the preservation of open-air rock art galleries requires a multidisciplinary approach (biogeoscience).

The lithic surface can be an extreme environment for microbial survival because of the limited nutrients it provides. The surface of rocks is often exposed to excessive UV radiation and fluctuation of temperatures, and in some cases, the same rock could endure severe temperature changes between day and night. These difficulties make the rock surface inhabitable to most organisms, and the microorganisms that could adapt usually survive under the protection of biofilms. Microbes produce SABs for water retention and mitigate harmful solar energy with pigments (Gorbushina 2007; Gorbushina and Broughton 2009). They form communities with microbial cells embedded in an extracellular polymeric substance (EPS) of proteins and polysaccharides that provide existential resources and protection from desiccation or radiation (Villa et al. 2016). The biofilm could be made of different autotrophs and heterotrophs, being endolithic or epilithic according to their method of colonization. Microorganisms in SAB produce a wide range of pigments for multiple functions. Bacteria and algae produce chlorophylls, carotenoids and phycobiliproteins as photosynthetic pigments to harvest light (Pagels et al. 2019; Maoka 2020). Cyanobacteria in terrestrial habitats are exposed to UV radiation and synthesize scytonemin, carotenoids and mycosporine-like amino acid compounds (MAAs) for photoprotection (Asencio and Hoffmann 2013). Melanins are polymeric substances that appear black or brown to red and are photoprotective against UV in microorganisms including bacteria, fungi and protists (Gao and Garcia-pichel 2011).

Another consequence of SAB that is considered a major concern in the conservation of stone monuments is discoloration due to bio-pigments. Biofilms can appear green from photosynthetic pigments produced by algae, black due to pigmentation of fungi, or show a variety of colors including brown, orange and pink induced by cyanobacteria, archaea and bacteria, to name a few (Gaylarde 2020). The color of biofilm on stone could be influenced by environmental factors such as water and CO_2 (Prieto et al. 2020). Besides discoloration, the microbiome can transform the lithic surface by both disintegrating the minerals and precipitating new ones. Although biofilm-forming organisms are well known for taking part in deterioration (Warscheid and Braams 2000), little is understood about their interactive mechanisms on the substrate which is crucial in determining the role they play (Gulotta et al. 2018). Investigation of SABs will offer the opportunity to better understand how they transform rock surfaces in the “rock art ecosystem”, hence improving our knowledge of the preservation of rock art.

This second part of the thesis consists of chapters 4 and 5. Chapter 4 investigated microbial communities and rock coatings found on rock art panels. Research in rock coatings is important to the preservation of rock art, and microorganisms play an important role in changing both the composition and color of rock art surfaces. We visualized the biofilm structure with CLSM and extracted genomic material on rock art samples with different colored coatings from Ethiopia, then identified bacterial communities by amplifying and sequencing the 16S rRNA gene. Bacteria with mineralization potentials that could form patinas were identified, as well as animal microbiome that could result from herding activities at the site. The results imply that different colored coatings from the same rock art panel could harbor microbial communities with different compositions and functions (Wu et al. 2020). Cultural heritage made of stone is often discolored by pigments from biofilms, therefore a review was made to discuss microorganism-produced pigments concerning their role in the broader ecological context in chapter 5.

1.4. Researching microbial participation in rock coatings formation

As described above, understanding rock coating is an important part of rock art preservation. The formation of rock coatings is a complicated issue that needs expertise in both geology and biology, therefore we presented the latest research in this thesis with chapter 6 by combining interdisciplinary methods in geochemistry, molecular biology and bioinformatics to further understand how microbial communities are involved in the formation of rock art coatings.

We are aware that abiotic and biotic formation of mineral deposits and coatings both occur in nature, for example calcium sulfate (gypsum) minerals in abiotic pathways (Sauro et al. 2014; Van Driessche et al. 2019) and sulfur oxidizing microbiome on stone (Ding et al. 2022) both contribute to global geochemical cycling of the element sulfur. Meanwhile researchers continue exploring microbial biogeochemical cycling of major elements on earth, for instance both quartz weathering and the mobility of amorphous silica deposit in an orthoquartzite cave are influenced by chemotropic bacteria (Sauro et al. 2018), and biomineralization played a role in forming calcite cave speleothems (Dhami et al. 2018).

Microorganisms are known to build structures through mineral precipitation. Biomineralization occurs by two different methods, “biologically induced biomineralization” and “biologically controlled biomineralization”. Biologically induced mineralization is where the cell has no control over the process, precipitations are byproducts of metabolism and interactions with the surrounding environment that form amorphous to poorly crystalline minerals. On the other hand, biologically controlled biomineralization develops minerals with intracellular or epicellular devices that control the intake of specific ions to create mineral supersaturation states. Induced mineralization results in the production of iron hydroxides, magnetite, manganese oxides, clays, amorphous silica, carbonates, phosphates, sulfates, and sulfide minerals. Controlled mineralization forms magnetite, greigite, amorphous silica and calcite (Konhauser 2006). This research intends to explore the role of biomineralization/microbiology in the formation of rock coatings on Ethiopian rock art. The first step is to identify the microbial community, then investigate if the microbial community contains functions to precipitate the mineral coatings.

The ability to research non-culturable microbiome had vastly advanced in the past two decades, making extraction of DNA from environmental samples more successful, lowering costs for sequencing technologies, and generating new programs that make phylogenetic and functional analyses available (Hershey and Barton 2018). However, identifying the microbial community present on the samples does not necessarily inform us of their actions. Within the ‘omics’ technology, metagenomics derived from DNA explores taxonomic features by defining ‘who is there’ (Franzosa et al. 2015; Mihajlovski et al. 2015; Hugerth and Andersson 2017). Transcriptomics studies changes in community transcription, while metabolomics study community metabolic products, and metaproteomics study community protein expressions; these data could help us understand how geochemistry influence microbial community structure (Hershey and Barton 2018). Amplicon sequencing using bacterial 16S ribosomal RNA (rRNA) as a marker gene is common in microbial research for phylogenic and diversity analysis, but functional analysis of the genes usually requires whole shotgun metagenome sequencing. Shotgun metagenomic analysis was applied to suggest the contribution of algae, cyanobacteria and other bacteria phyla to calcium carbonate precipitation in coastal areas of marine and hypersaline lakes (Saghaï et al. 2015; Walter et al. 2021). Nir et al. (2021) also used shotgun metagenomics on petroglyphs in the Negev desert (Israel) to analyze the functions of the microbial community. Ninety-six percent of the identified sequences were assigned to bacteria, and the metabolic pathways suggested the potential role of bacteria in participating in the solubilization and mineralization of mineral substrates. However, it is possible to predict metagenome functional content from marker gene (e.g., 16S rRNA) surveys using software such as PICRUSt (Langille et al. 2013). For example, Gonzalez-Pimentel et al. (2021) used PICRUSt2 in a geomicrobiology research of cave basalts, and predicted genes encoding enzymes involved in C, N, and S biogeochemical cycling, as well as microbial-induced carbonate precipitation.

In the last part of this thesis (chapter 6) we took a step further from chapter 4 by not only identifying the bacterial community on rock art coatings, but also investigated bacterial genes with the potential to precipitate minerals and revealed new insights into microbial diversity and functions related to the formation of rock coatings.

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Chapter 2

The sustainability of rock art: preservation and research[1]

Abstract
Rock art is a widespread cultural heritage, representing an immovable element of the material culture created on natural rocky supports. Paintings and petroglyphs can be found within caves and rock shelters or in open-air contexts and for that reason they are not isolated from the processes acting at the Earth surface. Consequently, rock art represents a sort of ecosystem because it is part of the complex and multidirectional interplay between the host rock, pigments, environmental parameters, and microbial communities. Such complexity results in several processes affecting rock art; some of them contribute to its destruction, others to its preservation. To understand the effects of such processes an interdisciplinary scientific approach is needed. In this contribution, we discuss the many processes acting at the rock interface—where rock art is present—and the multifaceted possibilities of scientific investigations—non-invasive or invasive—offered by the STEM disciplines. Finally, we suggest a sustainable approach to investigating rock art allowing to understand its production as well as its preservation and eventually suggest strategies to mitigate the risks threatening its stability.

Keywords: rock art; sustainability; ecosystem; surface processes; non-invasive sampling; scientific analyses

2.1. Introduction

Rock art is widespread worldwide, from hyperarid deserts to remote islands, and represents one of humans' most fascinating cultural manifestations. Pictograms and petroglyphs are part of the archaeological record and can be found within caves and rock shelters or in open-air contexts. In all cases, rock art is directly connected to its past and present environmental settings for multiple reasons (Cremaschi et al. 2008; Zerboni and Cremaschi in press). It is an immovable element of the material culture, created on natural rocky supports (boulders, vertical/flat rock outcrops, rock walls of caves and rock shelters) embedded in the landscape. Furthermore, rock art often depicts motifs representing past environments/ecosystems (e.g., Cremaschi et al. 2008; David et al. 2013; Guagnin et al. 2016), and can serve as proxy data to reconstruct past biomes. Finally, the tight nexus between rock art, its natural support, and the surrounding landscape led paintings and engravings to suffer the same surface processes affecting rock surfaces along the Earth Critical Zone (Gallinaro and Zerboni 2021; Zerboni and Cremaschi in press), the outer part of the planet spanning from groundwater to vegetation top that supports life on the Earth's surface (Richter and Mobley 2009).

Consequently, physical, chemical, and biochemical weathering and erosion menace the preservation of world rock art (Darvill and Batarda Fernandes 2014; Clottes 2008; Agnew et al. 2015). Surface

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[1] Zerboni, A., Villa, F., Wu, Y.-L., Solomon, T., Trentini, A., Rizzi, A., Cappitelli, F., and Gallinaro, M., 2022. "The Sustainability of Rock Art: Preservation and Research." *Sustainability* 14 (10). https://doi.org/10.3390/su14106305.

processes include a large variety of events and dynamics that can destroy rock art or, in a limited number of cases, preserve it. Such processes act at different scales of resolution – from the macro- to the micro-scale – but the most common of them work at the micro-scale, thanks to the interaction between the lithosphere, atmosphere, hydrosphere, and biosphere. The interaction between the rock surface, pigments (in the case of pictographs), and microorganisms produces effects that are often detectable to the naked eyes in terms of degradation of the artwork (dismantling, exfoliation, change/fading in color, formation of crusts of biofilms), but in some cases, they contribute to stabilize surfaces and thus preserve rock art. However, their complete comprehension needs specific interdisciplinary laboratory investigations (Fig. 2.1). At the same time, human activities may increase the rate of deterioration of rock art or cause its destruction. Rock art is a dynamic system at the edge between many compounds of the near-surface Earth Critical Zone. Therefore, to understand its formation and preservation become mandatory to investigate the composition of rock art, the host rock, and the microbial communities (Fig. 2.1), as much as for most of cultural heritage (Liritzis 2018).

Figure 2.1. Today, rock art research is extremely interdisciplinary and requires skill from humanities as much as from STEM disciplines.

In this contribution, we explore the possibility offered by archaeological science to investigate the rock art, going beyond the mere characterization of pigments and binder, and dating. We offer an overview on different approaches, including the description of the host rock, the role played by the local microbial community and the alteration that the rock art ecosystem suffered since its creation. Because surface and near-surface rock art contexts (rock shelter and open-air sites) are the most endangered, we focus our work on them. We discuss the sustainability of scientific analyses in terms of sampling and preservation. We suggest that a specific approach to sample pictographs and petroglyphs, while reducing the impact of sampling, and maximising information to mitigate ongoing or future threats is mandatory.

2.2. The fragility of the rock art ecosystem and the challenge of sustainability

From the perspective of rock art fragility and its interaction with environmental processes menacing its stability and preservation, we can distinguish three major rock art sites conditions. In fact, if one considers rock art sites (i) inside a cave, (ii) in the atrial part of a cave or in rock shelters, or (iii) in completely open contexts, then a growing interaction with surface processes is evident. Such categories are merely related to topographic and geomorphological factors tuning the stability of environmental processes in correspondence of rock art sites and have no cultural, anthropological, or artistic implication.

Cave sites (Fig. 2.2) are often isolated from the surface dynamic, and their micro-environmental conditions are generally steady up to the discovery of rock art when pristine climatic and biological conditions are perturbated by humans visiting the site for scientific and/or touristic purposes (Hoyos et al. 1998; Mangin et al. 1999; Albertano and Bruno 2003; Denis et al. 2005; Bastian and Alabouvette 2009; Saiz-Jimenez et al. 2011). In such conditions, for instance, variations in humidity, light, and the colonization of microorganisms represent a potential threat to rock art (Grieken et al. 1998; Malaurent et al. 2006; Dupont et al. 2007; Lacanette et al. 2013), as much as for the whole cave ecosystem (Hong 2006; Moldovan et al. 2018). The complex ecology of communities living in natural caves has been known and explored for a long time (e.g., Hershey and Barton 2018; Moldovan et al. 2018), but specific investigations have also disclosed several microorganisms interacting with pigments in cave sites (Schabereiter-Gurtner et al. 2002; 2004; Dupont et al. 2007; Bastian et al. 2010), whose metabolic processes are possibly critical for the preservation of rock art. Cave rock art sites thus appear to be substantially stable and – apart in the case of perturbation of their climatic and microbiological settings – they are substantially conservative.

Figure 2.2. Some examples of caves illustrating different types of deterioration of the rock walls potentially involving rock art. (A) Green biofilm, karst dissolution, and modern graffiti on the wall of a cave from northern Italy. (B) Mn-bearing coatings on the wall of a cave from the Italian Apennines. (C) A cave in southern Italy with speleothems covering the rock walls and obscuring the pristine surface.

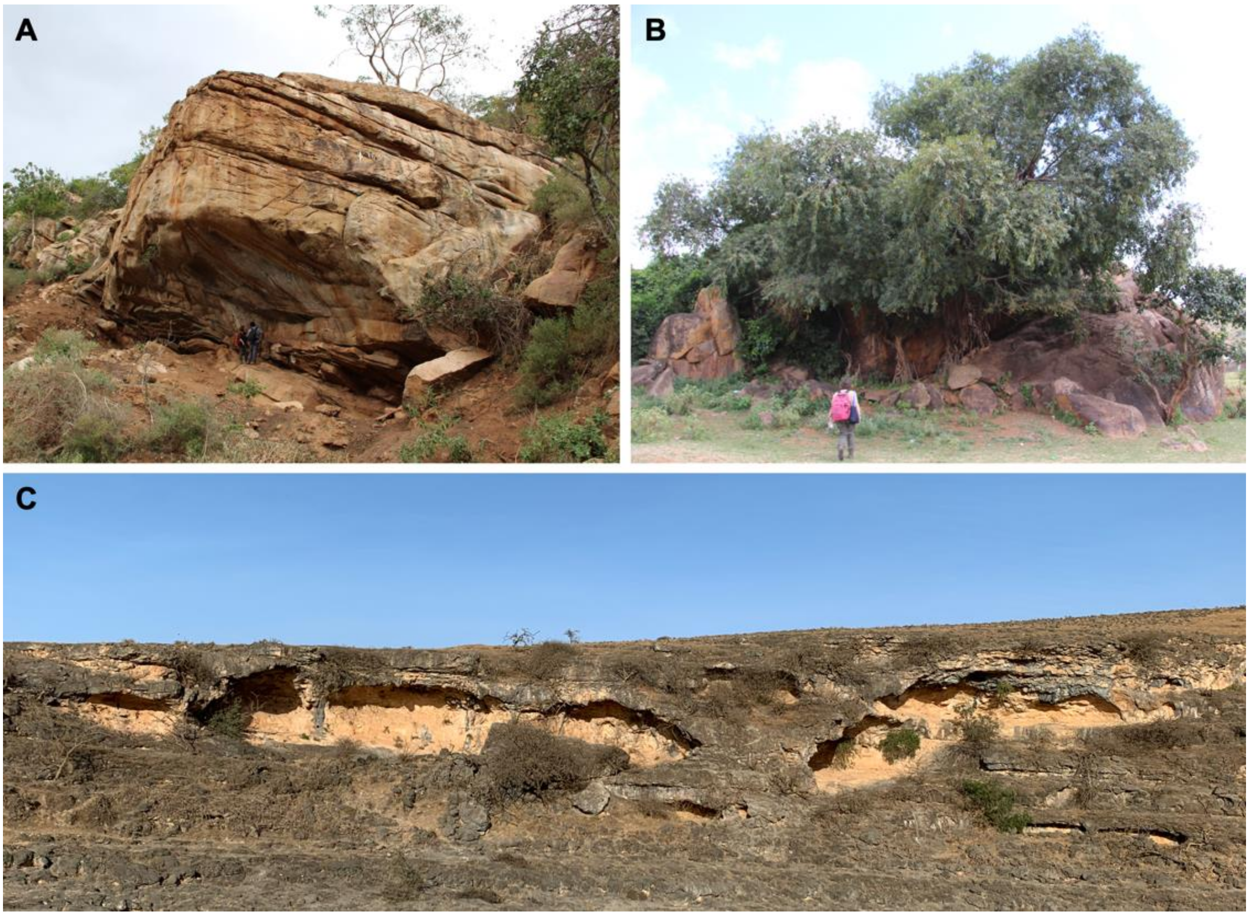

Figure 2.3. Some examples of rock shelters with rock art from (A, B) Ethiopia and (C) the Sultanate of Oman. In each case, the lithology of the bedrock and environmental processes oversees their formation and preservation along time.

The case of rock art sites along the walls of the atrial part of cave or rock shelters is different. From the geomorphological point of view, we consider in this category all the locations that are close to the Earth's surface, but they are, at least in part, sheltered under a rock roof (Fig. 2.3). These include the atrial part of deep karst or solutional galleries and various types of rock shelters, formed after deterioration/erosion of rock walls or related to the collapse of rock cliffs. For the sake of brevity, we refer to this category of rock art locations as rock shelters. Due to their proximity to the surface, rock art site in rock shelters deeply interacts with physical, chemical, and biological surface processes. For the same reason, rock art galleries on bare rock walls or boulders in open-air contexts (Fig. 2.4) are tightly related to the complex dynamic of processes acting at the interface between the lithosphere, hydrosphere, atmosphere, and biosphere. From our point of view, pictograms and petroglyphs found in rock shelters and open-air contexts share similar contexts and are affected by the same processes. Moreover, such processes oversee the preservation of rock art as much as its rocky support (Gallinaro and Zerboni 2021); for that reason, we cannot distinguish between the stability and decay of the host rock and the preservation of rock art pigments.

The host rock, mineral, and organic elements of pictographs and the biological community living at the interface between the two (Wu et al. 2020; Nir et al. 2021) interact in terms of biogeochemical cycles and thus belongs to the same system. From this perspective, we must introduce the concept of ‘rock art ecosystem’ (Fig. 2.5), meaning the complex and multidirectional interplay between the host rock, pigments, environmental parameters (humidity, light, pH, Eh, alkalinity, etc.), and the biological community that at the microscale tunes the preservation of rock art. Defining rock art as an ecosystem implies the existence of multiple interactions between a group of living organisms (the rock art and rock surface biome) living in a specific environment, represented by the rock/air interface and pigments.

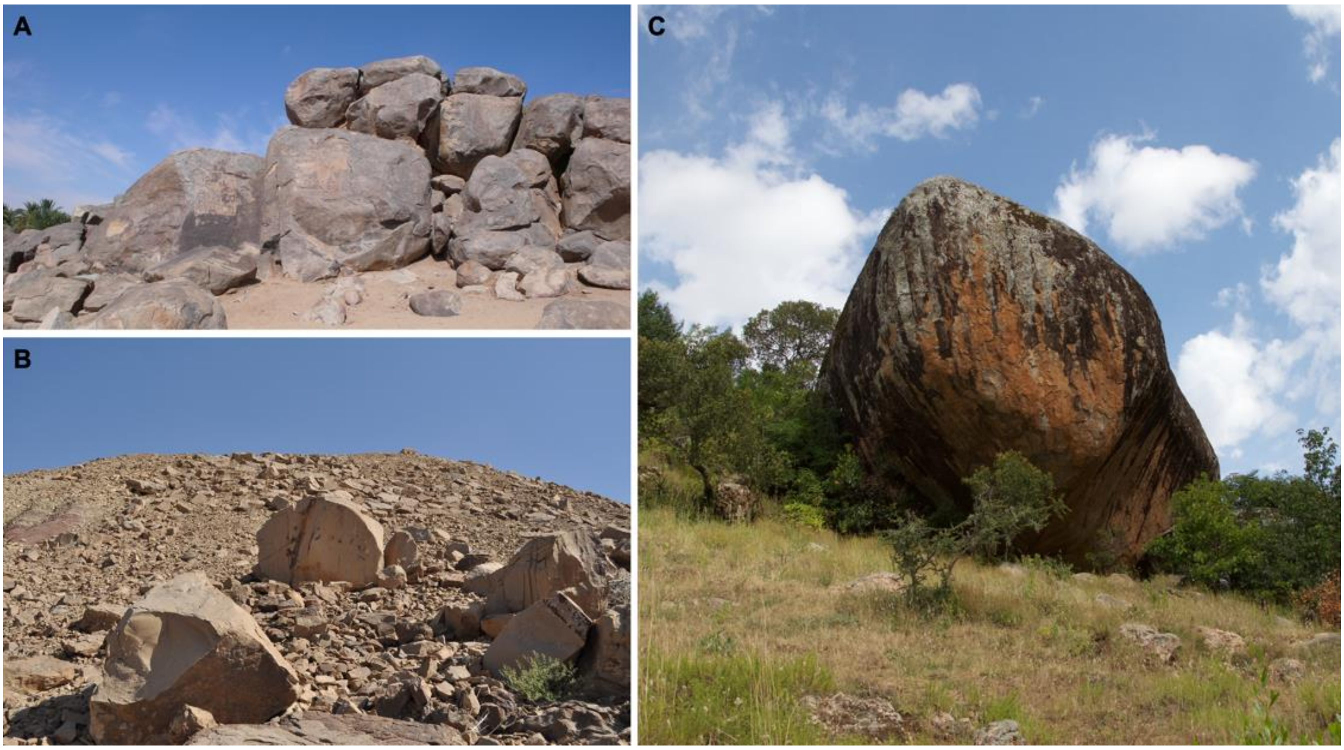

Figure 2.4. Some examples of open-air contexts with tock art: (A) rock wall with engraved hieroglyphs from Sudan, (B) a boulder with engravings in the Sultanate of Oman, (C) a granitic boulder (tor) with paintings in southern Ethiopia.

To understand the processes controlling the preservation or destruction of rock art is therefore mandatory to investigate a complex ecosystem, including all the mineral and biological components, postulate strategies for its preservation and identify the way for its sustainability. Various multifaceted analyses on the rock are required to understand active and inactive processes at each site, assess the ongoing dynamics, and suggest future scenarios, including mitigation strategies for specific threats.

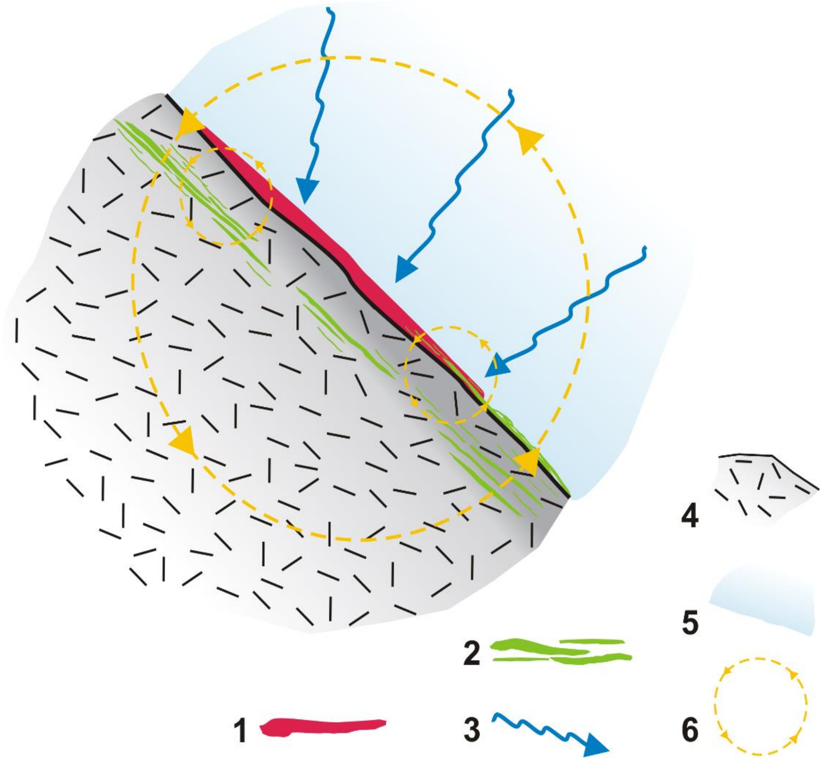

Figure 2.5. Theoretical sketch illustrating the concept of 'rock art ecosystem' as the result of the interaction between the rock substrate, pigments, the biological community and external environmental processes. Key: (1) pigment; (2) SABs (epilithic or endolithic); (3) exogenous forcings; (4) rock; (5) external environment; (6) biogeochemical exchanges between the components of the system.

2.3. Processes affecting rock art stability

In this section, we offer a brief overview of natural and human-induced menace threatening rock art sites in rock shelters and open-air contexts; the same processes are summarized in Table 2.1.

Table 2.1. Summary of major processes affecting rock art sites.

Process	Natural/Human	Scale	Location	Effect
Slope instability	Natural	Macro-scale	Rock shelter and open-air contexts	Destruction of rock substrate; slope deposit covers rock art sites
Cryoclastism **Thermoclastism** **Haloclastism**	Natural	Meso-scale	Rock surface along rock shelter and open-air contexts	Exfoliation; granular disaggregation; breakage; spallation; surface rejuvenation
Biological weathering	Natural	Meso-scale to macro-scale	Rock surface along rock shelter and open-air contexts	Rock surface desquamation and disruption; ecofacts (e.g. invertebrate nest) cover rock art
SABs growth	Natural	Meso-scale to micro-scale	Rock interface along rock shelter and open-air contexts	Promoting desquamation and granular disaggegation; stabiliziation of rock surface; formation of case hardening
Atmospheric agents	Natural	Micro-scale to meso-scale	Pigments	Decoloration, degradation, erosion
Continuos human occupation	Human	Macro-scale to micro-scale	Rock shelter and open-air contexts	Deterioration of rock surface; decoloration; destruction; rubbung of surfaces; alteration of chemical composition
Intensive land use	Human	Macro-scale to meso-scale	Rock shelter and open-air contexts	Destruction
Uncontrolled tourism	Human	Meso-scale to micro-scale	Rock surface along rock shelter and open-air contexts	Destruction; deterioration of surfaces; alteration of chemical composition; decoloration; vandalism
Inadequate investigation/restoration	Human	Meso-scale to micro-scale	Rock surface along rock shelter and open-air contexts	Destruction; decoloration; acceleration of the deterioration of surfaces

2.3.1. Natural processes

The origin of rock art dates to the Upper Pleistocene when parietal representations popped out at many world locations (e.g, Aubert et al. 2017). Since that time, the location of sites and rock art itself underwent several, major, rapid, or long-term climatic and environmental changes tuned by global climatic dynamics and local forces. For that reason, many representations depicted in rock art refer to specific environmental conditions and constitute an archive of proxy data for paleoenvironmental reconstruction complementary to the natural hydroclimatic archives commonly explored in Quaternary sciences (Zerboni 2012; Guagnin 2015; McDonald 2015).

A further implication of the initial statement of this section is that manifestations of rock art arose over a very long period under climatic and environmental conditions that often are no longer in balance with those of today (e.g., Dorn et al. 2008; Gallinaro and Zerboni 2021; Zerboni et al. 2021; Zerboni and Cremaschi, in press).This has been primarily explored in arid lands of the Old World, where rock art representations dating to the latest Pleistocene and the Early and Middle Holocene preserve evidence of a fauna assemblage not compatible with the present-day biome of the Saharan and Arabian deserts (e.g., Cremaschi et al. 2008; Huyge et al. 2011; Jennings et al. 2013; Guagnin 2015; Guagnin et al. 2015; 2017; 2018), thus suggesting the occurrence of major, regional climatic shifts. Such significant climatic and environmental changes involved all the components of the landscape. Consequently, the rock art ecosystem thermodynamically evolved towards new equilibria in terms of physical and chemical modifications of the host rock and pigments, and the evolution of the microbial communities. Moreover, a major climatic transition occurred globally since the Middle Holocene (Mayewski et al. 2004) and one of the main consequences was the activation of several geomorphological processes that increased the possibility to damage archaeological sites and destroy rock art galleries (Dorn et al. 2008).

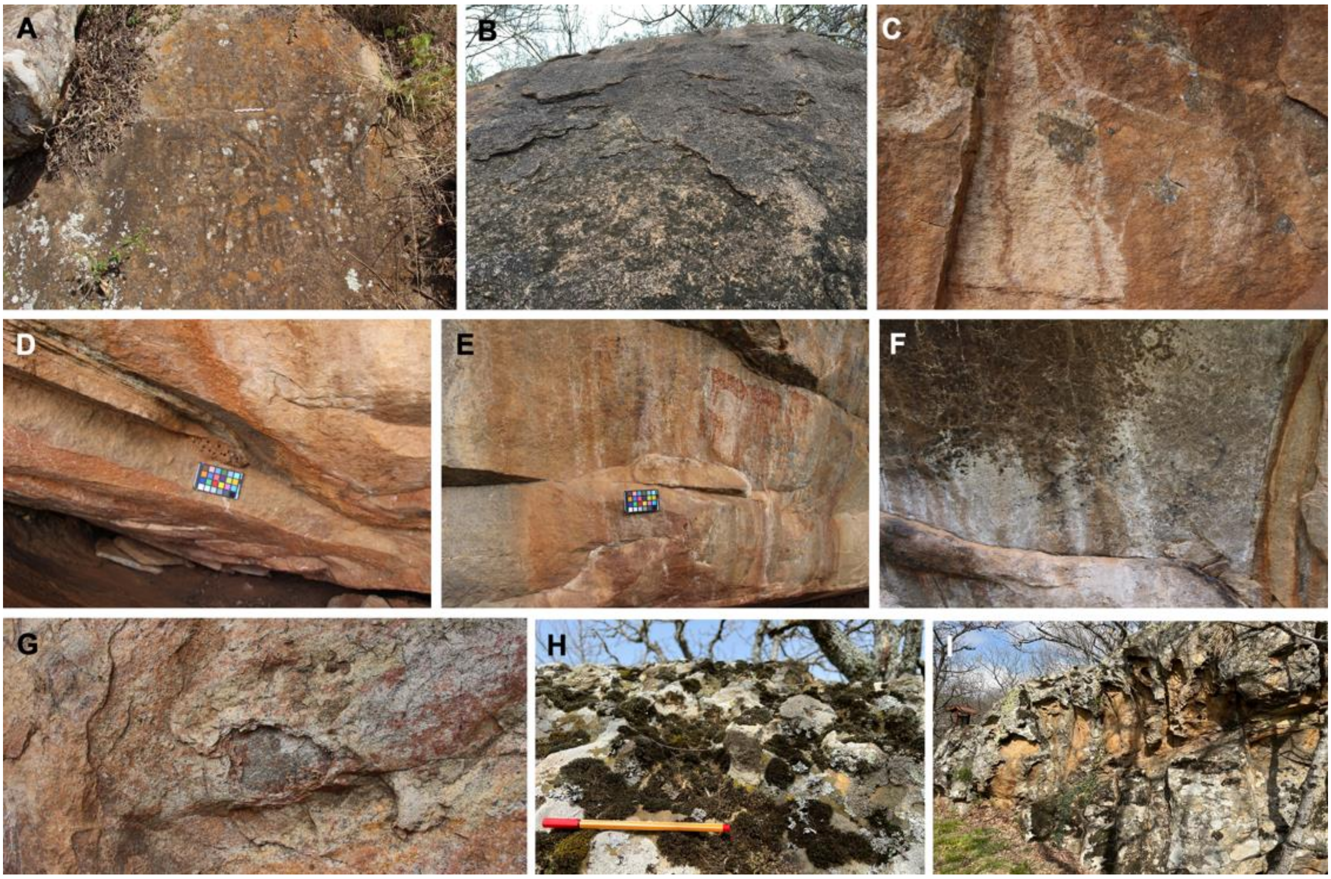

Figure 2.6. A variety of examples of rock surface decay from rock art sites or nearby them. (A) Lichens growing on engravings in an open-air site of Ethiopia. (B) Exfoliation of a granite surface; notice also the dark SAB covering most of the surface. (C) Exfoliation over a small area of paintings from a rock shelter in southern Ethiopia. (D) Hymenoptera nest in a rock shelter from southern Ethiopia. (E) Whitish crusts covering paintings in a rock shelter from Ethiopia. (F) Whitish crusts and dark SAB covering paintings in a rock shelter from Ethiopia. (G) Exfoliation and accumulation of reddish Fe-rich oxides on the surface of a rock shelter from Ethiopia. (H) Biological weathering on a limestone from southern Italy. (I) Effect of dissolution on a limestone from southern Italy.

From the geomorphological viewpoint, surface processes acting at the micro-, meso- and macro-scale, influence landscapes and ecosystems (Slaymaker et al. 2009; Dorn et al. 2013), including the rock art ecosystem (Fig. 2.6). Among geomorphological processes, slope and fluvial processes are the most important actors in the preservation or destruction of pictographs and petroglyphs at the macro-scale. In open-air contexts, the degradation by the gravity of rocky slopes may destroy or cover rock art sites. Similarly, the aggradation of sedimentary deposits along riverbeds or the lateral erosion of rivers may obscure rock art sites. Such processes are tuned by environmental changes, and ongoing climate change amplifies the dynamicity of natural systems thanks to the acceleration and increase of intensity of several geomorphic processes (Allen 1997). Extreme weather events, for instance, fuel catastrophic flooding that are even more severe in arid lands, where the absence of vegetation cover hampers the possibility to protect the soil and reduce the times of concentration of rivers. More in general, we may refer to the concepts of biostasy and rhesistasy proposed by (Erhart 1956), suggesting that phases of instability push towards the obliteration or destruction of rock art and phases of biostasy may promote the rejuvenation of rock surfaces. Climatic instability oversees the increase in the intensity of slope failure and rockfall that involves rock art sites. The collapse of the roof of rock shelters, for instance, occurred at the transition towards more cold or arid conditions, and it is related to large-scaled cryoclastism or thermoclastism. In some cases, rockfalls involved rock art galleries. Along river catchments, paroxistic events of floods, including the rapid increase in fluvial load, triggers significant soil erosion, mobilization of blocks and boulders, and deposition of sediments. This is particularly severe in arid lands, where occasional and localized rainfall may result in destructive flash floods. Occasional rainfall also promotes runoff along slopes and sometimes along the walls of rock shelters, leading to the deposition of thin coatings of mud and calcite that occasionally cover paintings and engravings and promotes the microbial colonization.

At the meso-scale, thermoclastism, cryoclastism, and aloclastism induce large fractures on rocks that are evident on cliffs, walls, and single boulders, as much as – at the micro-scale – flaking, spalling, exfoliation, and granular disintegration. Such processes are critical along the walls and roofs of rock shelters, and most likely, this process oversees the loss of many rock art galleries. For instance, in arid lands, seasonal or daily temperature excursions trigger thermoclastism. Even though the true effects of this process are still debated, it appears evident that on granular and polymineral rocks continuous expansion and contraction of grains promote the rejuvenation of surfaces through flaking (Vincente et al. 1993; Peña-Monné et al. 2022). A further process acting in arid regions is wind abrasion acting at the very rock surface; impacts of sandy particles amplify granular disaggregation and surface polishing including consequences on engravings (Zerboni et al. 2015). Humidity is a further factor that can promote the rate of the dismantling of rock shelters. In fact, capillarity allows water to promote solutional weathering and weakens the outer part of rock walls. In the Sahara, it has been observed that this process was favoured within rock shelters with organic deposits and in the proximity of joints between permeable and impermeable rock strata, because both factors promote the permanence of water in the proximity of the rock surface (Cremaschi et al. 2014; di Lernia et al. 2016).

Various weathering processes act at the interface between the atmosphere and the rock (Hall et al. 2012), thus also involving paintings and engravings. At the micro-scale, it is hard to distinguish between physical, chemical, or biological weathering and most processes are a combination of them (Carroll 1970; Bland and Rolls 1998). Physicochemical, biophysical, and biochemical weathering are the processes observed worldwide, and among them, biogeomorphological processes are the most evident. Insects and other invertebrates are very active on rock surfaces; for instance, wasps use the rock surface as a substrate to build up nests or excavate the rock leading to its mechanical disruption (Zerboni and Nicoll 2019). Nevertheless, the most effective biogeomorphological processes acting at the rock/atmosphere interface are those promoted by microorganisms (bacteria, fungi, and algae) living above or inside the rock surfaces (Fig. 2.5): epiliths and endoliths, such as criptoendoliths, and chasmoendoliths (Golubic et al. 1981). Thus, it is not surprising to observe a thin veneer of densely packed microorganisms bound together by a secreted extracellular polymeric matrix (EPM), which formed complex communities called sub-aerial biofilms (SABs) (Villa et al. 2016). Biofilm-dwelling cells interact intimately, influencing each other's evolutionary fitness through cooperative and collective behaviours. This social behaviour confers cells substantial advantages compared to their planktonic counterpart in terms of increased resistance and resilience against external threats including desiccation and antimicrobial agents (Villa and Cappitelli 2019).

The growth of SABs on stone heritage has long been associated with a threat to conservation called biodeterioration, an undesirable change in material properties caused by the microorganisms' activity.This is not surprising given the widespread evidence of biogeomorphological (e.g., secondary mineral formation, EPM swelling and contraction) and biogeochemical processes (e.g., production of organic and inorganic acids, metal-complexing EPM), which are vital to pedogenesis (Negi and Sarethy 2019; Liu et al. 2020). While SABs on stone heritage imply current or past interactions with the lithic substrates, their presence does not necessarily have a biodeteriorative role as is frequently thought (Dorn et al. 2012; Pinna 2014; Gadd and Dyer 2017). A growing body of literature has reported the SABs' neutral or even bioprotective effects on stones under certain conditions (Viles and Goudie 2004; Viles and Cutler 2012; Coombes et al. 2018; Gulotta et al. 2018; Sanmartín et al. 2020) . Such findings, combined with the advances in biomineralization studies with indigenous carbonatogenic bacterial communities (Rodriguez-Navarro et al. 2015; Jroundi et al. 2017; 2020), have strengthened the concept of SAB-based protection as a sustainable strategy for stone heritage conservation (Favero-Longo and Viles 2020; Ortega-Morales and Gaylarde 2021). SABs oversee many surface processes acting at the microscale on rock surfaces. Some of them are destructive and contribute to the rejuvenation of rock surfaces because they promote disaggregation and exfoliation, others contribute to surface stabilization. In both cases, SABs action is relevant for rock art studies (Fig. 2.7).

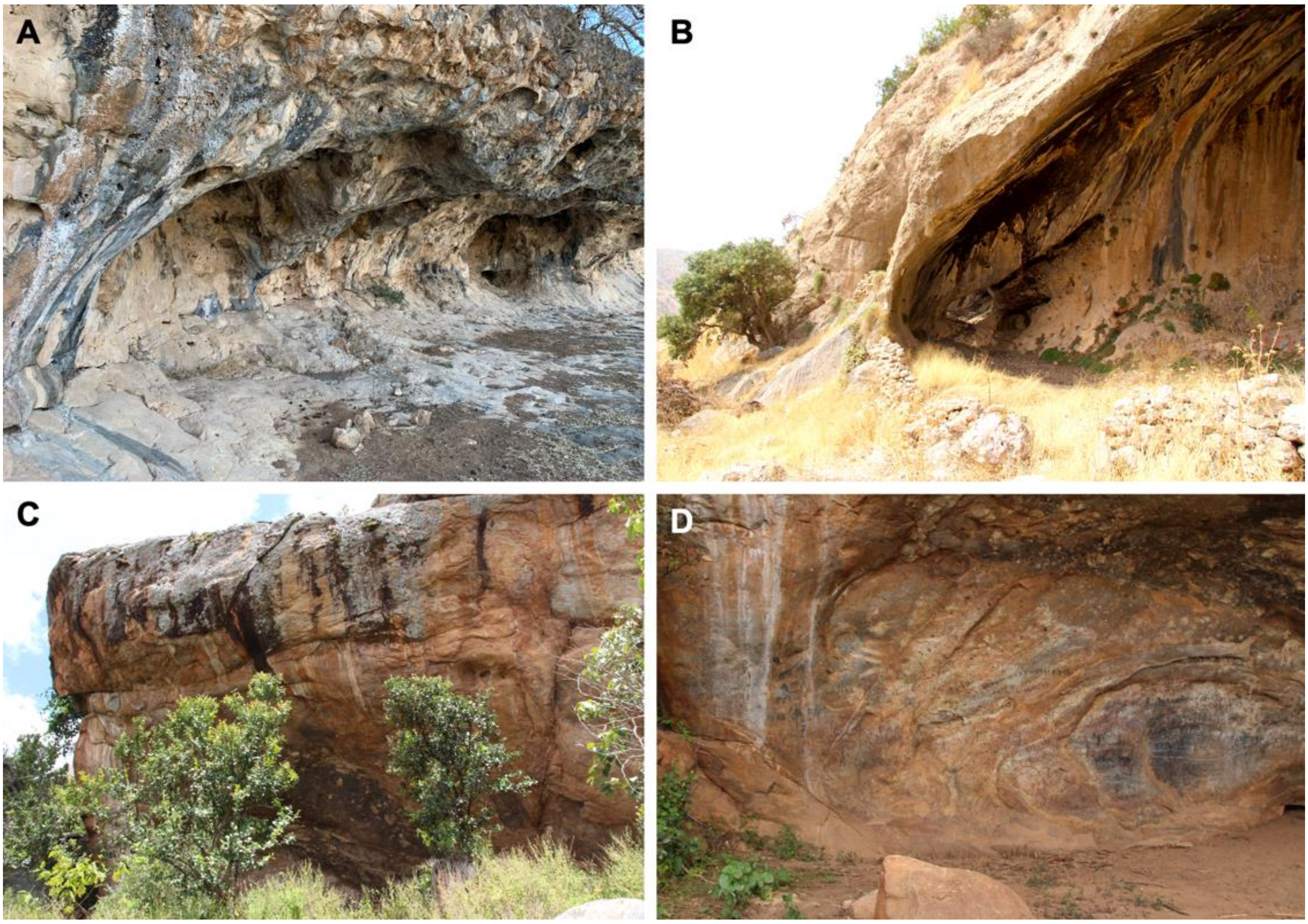

Figure 2.7. Examples of rock shelters displaying different types of SABs and other kinds of rock alteration from (A) the Sultanate of Oman, (B) Kurdistan Region of Iraq, and (C, D) southern Ethiopia. In each picture the different is evident between the pristine rock surface and those where darkish to whitish SABs developed.

SABs flourish along the rock walls of open-air contexts and rock shelters (Fig. 2.7), and their development is related to local environmental settings; many parameters such as environmental humidity, sunlight, and wind tune their formation and evolution. SABs forming on the rock surface may obscure pictographs and petroglyphs, and, in the case of paintings, SABs can interact with their mineral and organic constituents, leading, for instance, to their decomposition or removal. Biogeochemical cycles between rock art pigments and SABs/microorganisms (Fig. 2.5) are poorly investigated but likely oversee the diagenesis of organics binder and prevent obtaining reliable radiocarbon dating of rock art. The case of endoliths may represent a great threat to the preservation of rock art sites. At many sites, paintings suffer severe damage due to granular disaggregation and desquamation, which can be promoted by chemical and physical stress triggered by the metabolic processes of microorganisms. The factors controlling desquamation are many and their action and interaction are never entirely investigated. It seems that desquamation is the combined result of physical weathering and biological activity (Chen et al. 2000; Hall et al. 2012; Mergelov et al. 2012). The accumulation and efflorescence of solutes (mostly gypsum, halite, oxalates) sometimes of microns below the rock surface promotes physical weathering and the detachment of the uppermost rock layers thanks to the continuous growth of crystals (haloclastism). Solutes have a twofold origin: they are accumulated from the local alkaline aerosol, or biomineralized (as a by-product) by different microorganisms living at and within the rock surface (endolithic organisms).

Microbial communities contributed to the formation of rock coatings (Dorn 1998), which sometimes play a sheltering effect on rock surfaces (Zerboni et al. 2021). The most recurrent example of coatings formed after biogeochemical weathering and relevant in rock art research is Mn- and or Fe-rich rock varnish. Rock varnish is the biochemical accumulation of manganese (and/or iron) oxyhydroxides thanks to the mediation of microorganisms (Potter and Rossman 1979; Cremaschi 1996; Dorn 1998; Zerboni 2008). The formation of rock varnish has been observed in different world environments, but it is noteworthy to notice that its preservation is favoured in arid environments. Mn/Fe biomineralization likely occurs under semi-arid environmental conditions, and climatic transitions towards arid environmental conditions hamper the rejuvenation of rock surfaces; this has been reported in many deserts of the world (Dorn 1998). From the geomorphological point of view, rock varnish plays a twofold role, being sometimes a canvas for engravings, in other cases the dark veil covering and protecting surfaces (Fig. 2.8). When rock varnish is a relict landscape feature or the rate of varnish formation is too slow to re-cover engravings, it represents a non-renewable canvas for ancient artists and the rate of varnish (Cremaschi 1996; Martínez-Pabello et al. 2021). However, the partial regrowth of rock varnish is a valid relative dating method for engravings superimposition (Cremaschi 1996; Eisenberg-Degen and Rosen 2013), as well as offering the opportunity to estimate petroglyphs' age via chemical measurement of elements and areal density of Mn and Fe (Macholdt et al. 2019; Andreae et al. 2020; 2021; Guagnin et al. 2022; Andreae and Andreae 2022). From a different point of view, the formation of continuous and some tens of microns thick Mn-rich coating represent a case-hardened shell (Dorn et al. 2012) protecting rock surfaces against wind abrasion. Where deterioration processes are particularly severe, the development of a biomineralized Mn- and Fe-rich rock varnish inside the grooves of the engravings hampers the effect of rock dismantling, sheltering petroglyphs and promoting their preservation (Zerboni et al. 2021).

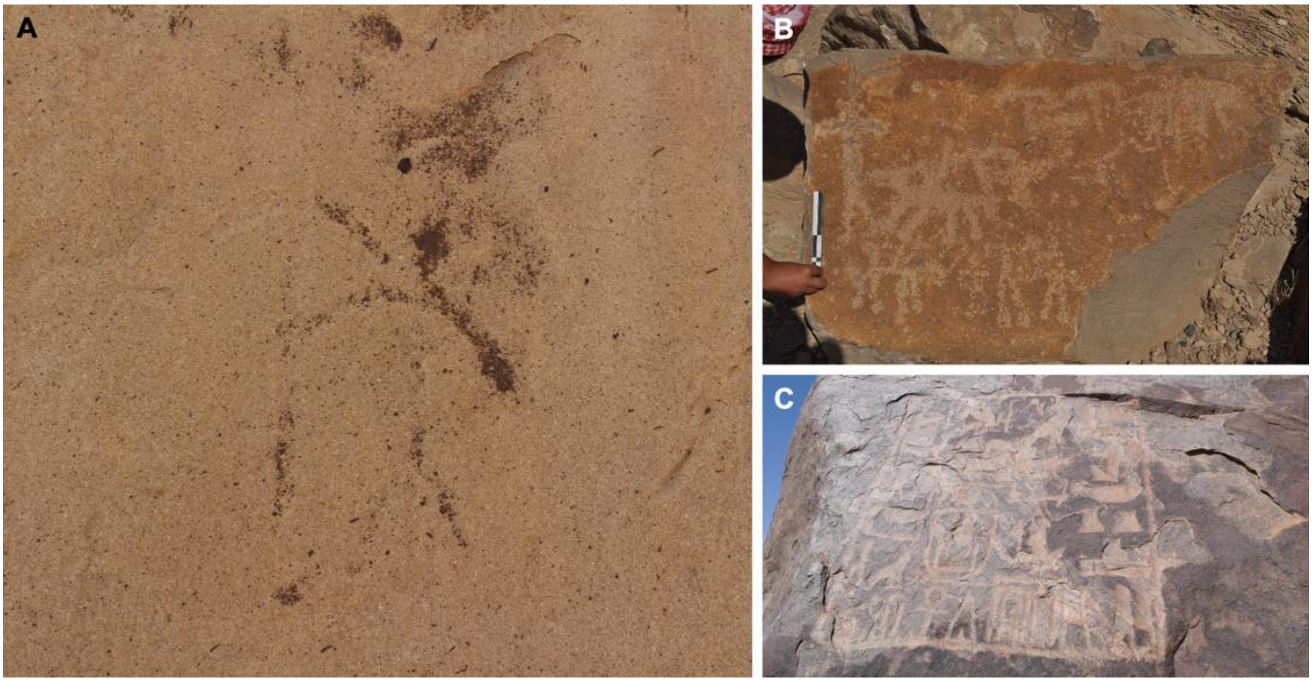

Figure 2.8. Mn and/or Fe rock varnish is relevant for engravings because it can preserve engravings from destruction as reported from the Sultanate of Oman (A). Moreover, rock varnish is also a sort of natural canvas for petroglyphs, as evident from (B) engravings on a limestone block covered with Fe-rich varnish from the Sultanate of Oman and (C) engraved hieroglyphs on a rock wall of granite covered by a Mn-oxyhydroxides bearing coating found in Sudan.

Finally, it has been reported that biogeomorphological processes leading to the destruction of rock art are more abundant when – as in correspondence of many rock shelters – a thick archaeological deposit, rich in organics, is present (Cremaschi et al. 2014). Likely, this is the consequence of the micro-environmental conditions created in the rock shelter due to the accumulation of organics that increases local moisture and promotes microbial activity. However, the state of conservation of paintings is excellent if they have been buried for a long time in a sterile environment and only recently exhumed (Huyge et al. 2011).

Paintings have been subjected to perhaps even more severe degradation than engravings, due to their intrinsic lower resistance to atmospheric and biological agents and surface processes. They have been preserved on the sections of shelters protected from the action of rainfall, wind, and direct sunlight, but other processes are involved in their progressive degradation (see below).

2.3.2. Human-related processes

Anthropogenic processes, ranging in intensity and severity, represent a critical threat to rock art preservation. Deliberate damages related to vandalism have the most immediate and destructive effects. Spray paint or engraved graffiti covering rock art are worldwide reported with different damage intensity (Higgins 1992; Di Lernia et al. 2010; Agnew et al. 2015; Taruvinga and Ndoro 2003). The removals and thefts of portions of rock art panels have been frequently reported, as done by collectors, and sometimes in the past also by scholars (Hachid 1985; Bednarik 2008; Yates et al. 2022). To these deliberate damages, we should also add acts of iconoclasm that can target and destroy specific motifs (Červíček and Kortler 1979; Holtorf 2006; Suková 2015).

Furthermore, it is possible to identify at least four other sets of anthropogenic processes that can (more or less) accidentally menace rock art, such as (i) the daily use of rock art shelters and caves through the millennia, (ii) the unsustainable and uncontrolled economic development, (iii) the inadequate management of rock art heritage, and (iv) the improper and unprofessional research study.

i. The rock art sites are often places where people live or at least spend part of their life, conducting activities that can have long-term and cumulative negative impacts on rock art. The use of fire and the continuous touch or rubbing of humans and/or animals on rock art panels result in the formation of patination that may cover the original painting or contribute to their deterioration (Smith et al. 1999; Gunn 2011). Also, the presence of domestic animals can disturb rock art, influencing the humidity and/or chemical composition of the atmosphere in correspondence with the rock wall.

ii. Many different processes related to economic development can negatively impact rock art sites with different degrees of damage. Oil exploitation, mining, building, and infrastructures are only a few examples of the anthropogenic processes that can accelerate the degradation or even cause the destruction of rock art contexts in the open air. The effects of oil prospections can accelerate the cracking of the host rock surfaces, whereas the mining, buildings and infrastructures can cause the removal, displacement, and destruction of rock art panels (Anag et al. 2002; Klemm and Klemm 2012).

iii. The lack of adequate management of rock art sites associated with an unsustainable tourism can also dramatically impact rock art. The absence of protections distancing visitors, visitor centres or informative panels open the way to inappropriate site visits, increasing the risk of touching, wetting, and vandalism to the rock art (e.g., Di Lernia et al. 2010).

iv. Improper recording and restoring/conservation processes can significantly impact rock art. Invasive techniques of recording by direct contact and rubbing of paintings and engravings surfaces conducted by researchers contribute to the fading and vanishing of painted motifs, the alteration of carvings, and the damage of rock surfaces (Loendorf 2001; Di Lernia and Gallinaro 2009). These actions can also be combined with other processes, such as enhancing the contrast between a pictograph and its rock substrate by wetting the rock surface with water or other liquids (Chaffee 1994) or the inadequate attempts to obtain cast of the petroglyphs. The former causes the fading and vanishing of paintings, whereas the latter results in the deterioration of the rock surface, the partial removal of the original rock varnish and possibly the permanent littering of the rock. Furthermore, the use of chemical products (e.g., Paraloid B-72) to consolidate rock and painting surfaces can result in the turn of a darker tone in the color of the painting and rock surface and a crust effect, increasing the risk of surface spalling (MacLeod 2000).

2.4. Methods for investigation of rock art

From this brief review on processes acting in rock shelters and open-air contexts, it is clear how many varied factors represent potential threats to rock art. Some of the physical and chemical processes that cause the degradation of the rock surfaces on which there are the manifestations of rock art are very evident and trigger huge loss. Climatic and environmental factors promote processes, and ongoing climate changes amplify their effects. Yet, biophysical and biogeochemical processes acting at the very rock-air interface and related to the existence of SABs are much more challenging to identify, quantify and interpret. Likely, most of them oversee the loss of rock art, but in some cases – especially in the case of SABs – they can hamper the efficiency of specific destructive processes. In such a complicated scenario, mitigating the effects of natural processes with targeted interventions and eventually planning *in situ* restoration are the only possibilities for preserving rock art. To accomplish the task of threatening identification, a specific diagnostic approach that includes monitoring of each site and scientific analyses of rock art and the bedrock is required. We believe that the two components (rock art and rock substrate) must be investigated together, as they represent a unique ecosystem.

Starting from this consideration, we suggest a biogeomorphological approach on weathering processes based on *in situ* and laboratory analyses of rock and pigments, and the multidisciplinary investigation of SABs (Liritzis and Korka 2019; Wu et al. 2020; Gallinaro and Zerboni 2021; Zerboni et al. 2021). As most the rock decay processes act at the microscale – thus representing a sort of ‘phantom menace’ – it is almost unrealistic to identify and describe threatening processes without sampling SABs, pigments, and the rock surface. An *in situ* characterization of the components of the rock art ecosystem is not entirely informative, as it is impossible to fully understand the proper dimension of the biological community and ongoing biogeochemical cycles. Physical, chemical, mineralogical, and biological analyses on rock surfaces and rock art have different degrees of disturbance, from non-invasive to considerably invasive, depending on the applied sampling strategy and the amount of material sampled for analyses. The scientific community is aware that rock art sampling is controversial, because, in many cases, it leads to great damage (e.g., Dayet et al. 2022) , but recent approaches demonstrate that strategies of limited destruction and almost non-invasive sampling are possible (Gallinaro and Zerboni 2021). Besides that, we are also aware that scientific investigation is mandatory to understand many cultural traits of rock art (Domingo and Gallinaro 2021), including ancient technologies and the choice of ingredients for pigments (Sillar and Tite 2000; Dorn 2006; Martinon-Torres and Killick 2015; Domingo and Chieli 2021), and for dating (Watchman 1993; Bednarik 2002; 2021). Scientific analyses in the recent two decades revealed diverse mineral colorants in pigments, and in rare cases investigated organic binding agents that are more prone to deterioration.

We briefly review the major possibility offered by scientific investigations on rock art, which are reported in Table 2.2. Non-invasive methods are today currently applied to record rock walls hosting rock art (Jalandoni et al. 2018; Peña-Villasenín et al. 2019; Degli Esposti et al. 2021) and to assess their state of preservation, including to identify ongoing processes of rock decay (Mol and Clarke 2019). Different instruments (including low-cost) instruments and methodologies have applied to perform Structure-from-Motion photogrammetry on rock art panels. Moreover, several indexes exist to scientifically estimate the stability of rock art and they are based mostly on biogeomorphology observations and *in situ* measurements (Dorn et al. 2008; Groom et al. 2019). Stability indexes offers the first effective tool to plan investigation and preservation strategies, as in the case of the Rock Art Stability Index (Cerveny et al. 2016; Cerveny 2005).

A wide range of geochemical techniques have been used to characterize the composition of rock art and ochre materials (Domingo and Chieli 2021; Popelka-Filcoff and Zipkin 2022 and references therein), in which the mineral composition could be identified by thin section petrography, X-ray diffraction (XRD), Raman spectroscopy and Fourier transformed infrared spectrometry (FTIR). Elemental analysis could be carried out by semi-quantitative methods such as scanning electron microscopy coupled with dispersive X-ray spectroscopy (SEM-EDX) and X-ray fluorescence (XRF), or the concentration of elements could be determined by inductively coupled plasma and atomic emission spectroscopy (ICP-AES), neutron activation analysis (NAA) and laser ablation inductively coupled mass spectroscopy (LA-ICP-MS), in which NAA and LA-ICP-MS are considered most sensitive for trace elements (Dayet 2021). Among these, the portable version of XRF and Raman are non-invasive methods that could be executed without sample removal and preparation, while the attenuated total reflectance (ATR) mode of FTIR and LA-ICP-MS require tiny volumes or micro-samples. It is important to highlight that portable instruments can be easily employed in the field but with the limitation of use only on external surfaces. To preserve the integrity of the rock art, non-invasive spectroscopic analyses are recommended for a preliminary inspection to thoroughly understand the current situation *in situ*. Based on preliminary analyses, plans could then be laid out for sampling that is crucial to answering research questions, thus minimizing the risk of damages. This strategy requires the use of portable instruments that could be eventually transferred to the site

(Domingo and Chieli 2021), but not in remote rock art contexts. The most applied devices are portable XRF (pXRF) and Raman. Portable XRF can read elements heavier than Al or Si; it is, therefore, possible to identify pigments in rock art palettes such as Fe from red hematite ochre and Mn in black manganese oxides (Dayet et al. 2022). Raman identifies both minerals and organic substances but encounters difficulties when identifying organic binders *in situ* due to fluorescent background materials; moreover, the possible diagenesis of organics may hamper the possibility to distinguish between primary constituents and neoformations. FTIR can act as a complementary vibrational spectroscopy method in some cases for characterizing organic binder residues. However, sampling is usually required for further details, and such is the case when using gas chromatography-mass spectrometry (GCMS) that effectively identifies organic molecules in works related to cultural heritage (Domingo et al. 2021; Domingo and Chieli 2021). Non-invasive spectrophotometric and colorimetric methods have been occasionally applied to rock art research to characterize paintings and engraved rock surfaces based on color gradations and spectroradiometers, colorimeters and mobile platform apps for recording of color have been tested (Ruiz and Pereira 2014; Molada-Tebar 2020). Such methods have been developed to improve the characterization of colors for recording of rock art sites (Molada-Tebar et al. 2019) and sometimes include the development of low-cost software allowing the colorimetric recording from complete image scenes with commercial cameras (Molada-Tebar 2020). *In situ* microfading spectrometry has been tested for mapping color degradation of rock art paintings (Carrión-Ruiz et al. 2021), and colorimetric analysis testing the possibility that color gradations might be quantifiable for purposes of dating has been experimented on petroglyphs (Bednarik 2009).

Table 2.2. Summary of scientific analyses carried out on rock art illustrating their aims, limitations, and invasiveness of sampling.

Analytical method	Sample requirements	Research question	Information provided	Limits
Structure-from-Motion photogrammetry	Non-invasive	Rock art recording	3D models or rock art sites	
Stability indexes	Non-invasive	Define the preservation of rock art	Quantitative data on rock surface stability	
Optical microscopy	Enough sample to manufacture thin section	Mineral composition and texture	Identify minerals and their interaction	Difficult to identify organic constituents
XRF	Portable: in situ, no sampling Benchtop: sampling required	Inorganic pigment, bed rock, crust, accretions	Qualitative elemental analysis	Detect elements heavier than Al or Si; only surface analyses
Raman	Portable: in situ, no sampling Benchtop: sampling required	Organic and inorganic pigments, bed rock, crust, accretions	Identify minerals, organic and inorganic molecules	Background noise and fluorescence affect results in situ; only surface analyses

XRD	**Small sample to produce powder**	**Crystalline structure**	**Quantitative mineral analysis**	**Difficult to identify pristine and newly formed minerals**
FTIR	Small sample to produce KBr powder pellet or micro-sample	Mineral and organic residues	Identify minerals and organic molecules	More proficient with amorphous and organic materials
SEM-EDX	Non-destructive to sample but require carbon coating and sometimes polished surface; alternatively very small samples	Surface morphology, stratigraphy and composition of pigments	High resolution images and semi-quantitative elemental analysis	Analyses are semi-quantitative
Confocal laser scanning microscopy	Non-invasive sampling procedure through adhesive tapes	SAB architecture and interaction with the mineral substrate	3D images and semi-quantitative analyses of the SAB components	Analyses are semi-quantitative
Molecular investigations	Small samples, destructive	Structure and function of the SAB community	Qualitative and quantitative data about the identified microorganisms and their activity.	Difficult to recover genetic materials from SABs on rock art.
ICP-AES	Small samples, destructive	Concentration of elements	Quantitative analysis of major elements	
NAA	Small samples, destructive	Concentration of elements, provenance studies	Quantitative analysis from major to trace elements	
LA-ICP-MS	Small samples, micro-sample	Concentration of elements, provenance studies	Quantitative analysis from major to trace elements	
GCMS	Small samples, destructive	Organic binder	Identify organic molecules	
LC–MS/MS	Small samples, destructive	Organic binder	Identify proteins	
AMS ^{14}C	Small samples, destructive	Chronology	Age of painting	Require a preliminary assessment of organic content
Uranium-series dating	Drilling microcores	Chronology	Relative age of painting (limit ante or post quem)	Possible gaps between carbonate deposition and rock art production

In recent decades, archaeological dating methods improved precision and minimized the amount of sample needed (Bednarik 2002; 2021). These developments also benefit the dating of rock art, which is more challenging than other archaeological issues due to the limited material available for dating. Radiocarbon dating remains the most exploited methodology because colors are generally obtained by mixing mineral pigments and organic binder (milk, egg, honey, resin). Unfortunately, the organic remains of microorganisms are also present within pigments; their occurrence suggests that the organic binder may offer a good substrate for bacteria, fungi, and algae, but also implies that the organic fraction of painting underwent degradation and diagenesis across time (Zerboni 2012; Gallinaro and Zerboni 2021). This is one of the reasons why radiocarbon dating on paintings is very difficult and requires accurate identification and characterization of the organic compound submitted to measurements (Bonneau et al. 2017a; 2017b). The main component of rock art – namely the organic pigment, charcoal, carbon black or soot – is scarce and available to limited sampling due to its value and conservation issues. Moreover, what will be analysed for each individual sample must be understood to interpret the dating result correctly. With the accelerator mass spectroscopy (AMS) ^{14}C dating, only a few milligrams of sample are required. Bonneau et al. (2017a) employed an extensive protocol on south African rock art using SEM-EDX, Raman spectroscopy and FTIR analysis to determine organic carbon in the samples before treating them with a modified acid-base-acid (ABA) treatment. These investigations helped ensure enough organic carbon from the paint source while ABA pre-treatment removed contaminating calcium oxalates before combustion and graphitization for radiocarbon dating. Steelman et al. (2021) used a different approach at Eagle Cave in Langtry, Texas, with plasma oxidation to isolate organic carbon directly from the paint layer, and avoided loss of dating material during an acid pre-treatment. An alternative to dating pigments is to date oxalate accretions over and beneath the painting. Calcium oxalates are often associated with microbial activities that precipitate minerals over the rock art, obscuring the art while preventing further deterioration. Oxalates formed under the painting provide the maximum age while accretions formed over the painting give a minimum age, suggesting a possible chronology for the painting. In this case, carbonate and organic carbon were removed with plasma oxidation, and pure oxalate samples were dated. Caution is required in dating oxalates as they are highly soluble and they can suffer multiple recrystallizations (Gallinaro and Zerboni 2021), and consequent re-opening of the carbon system. The same stratigraphic concept described for oxalates could be employed for coralloid speleothems formed from thin water running over the surface (Hoffmann et al. 2016). In this case, micro-drilling or scratching of calcium carbonate developed above/beneath rock art can be dated, thus offering not a direct age for rock art manufacture (as in the case of radiocarbon dating) but a limit *ante quem* (speleothem covering rock art) or *post quem* (rock are above speleothem) for its production. Samples of speleothems with paint in between were taken from caves for uranium-series dating on rock art from Europe (Pike et al. 2012; Hoffmann et al. 2018a; 2018b) and Asia (Aubert et al. 2018; Bednarik 2021; Wu et al. 2022). Carbonates are more stable than oxalates and U-series dating results are more reliable than other dating systems. Attempts have been made to determine the age of petroglyphs and weathering crusts and varnishes efficiently support the substrate for radiocarbon (Zerboni 2012; Zerboni et al. 2021;) and chemical measurement of elements (Macholdt et al. 2019; Andreae et al. 2020; 2021; Guagnin et al. 2022; Andreae and Andreae 2022). In this case, the required sample is small, and it can be collected, after a careful geomorphological assessment of surfaces, on the rock substrate of engravings but not directly on them. Luminescence methods have been attempted in some cases; in fact, surface luminescence dating of rock surfaces and Optical Stimulated Luminescence (OSL) have made some considerable progress and can be applied to engraved rock surfaces (Liritzis et al. 2017; 2019). In this case, sampling can be made not directly on engraved surfaces, but such studies must carefully also consider the degree of deterioration of the host rock surface (Liritzis et al. 2019).

The analysis of organic residues and SABs is a new development of heritage science and offers perspectives on how microorganisms are changing the art and its surrounding lithic environment. Early research was conducted in Atlanterra shelter (south Spain) using cultured methods (Gonzalez et al. 1999), while in recent years, next-generation sequencing allowed the identification of non-culturable bacteria, which are the majority in environmental samples. These include research from Ethiopia that identified bacterial communities from rock art panels by 16S rRNA gene sequencing, which found bacteria with mineralization potentials that could form patinas, and animal microbiomes possibly resulting from herding activities at the site (Wu et al. 2020). The researchers used confocal laser scanning microscopy (CLSM) to reconstruct the 3D images of the SABs colonizing the colored outer coatings from the Ethiopian rock art gallery. The samples were collected using the adhesive tape strip technique, which allows obtaining the mirrored image of the SABs. This technique is easy to apply, inexpensive, reproducible, and safe for the rock surface. In addition, it is possible to obtain information on the morphology and taxonomy of microorganisms, and their relationships with the colonized material surfaces. The 3D SAB images showed the organization of the microbial communities, highlighting the differences between the two samples investigated. The sample close to the bottom of the rock art panel with whitish patina presented a more diverse SAB with higher phototrophs, chemotrophs, and EPM than the sample taken from the lower right with red patina on the surface. Another research used shotgun metagenomics combined with microscopic investigations to reveal the structure and function of the SABs colonizing petroglyphs in the Negev desert (Israel) (Nir et al. 2021). 96% of the identified sequences were phylogenetically assigned to the Bacteria, suggesting the predominance of this domain in the SAB community of petroglyphs. The SABs showed metabolic pathways involved in elements cycles and uptake processes, providing evidence of their potential role in the solubilization and mineralization of the mineral substrates. Interestingly, Roldán et al. (2018) applied both 16S rRNA sequencing and proteomics (protein analysis with liquid chromatography and tandem mass spectrometry (LC–MS/MS) on Levantine rock art to reveal bacterial communities and possible protein binder. Moreover, metagenomics is often applied to the investigation on the decay of building materials (Villa et al. 2016; Cappitelli et al. 2020; Delegou and Karapiperis 2022). Traditionally, to know genera and species of lichens, symbiotic organisms formed by fungi and algae or cyanobacteria, identification tools were paper published as dichotomous keys (Clauzade et al. 1985;

Wirth 1987; Smith et al. 2009). More recently, digital keys are also available (Nimis and Martellos 2020). However, we should keep in mind that either cultural or molecular techniques only provide the current snapshot of the colonization. Thus, previous rock art phenomena (e.g., mineral precipitation or dissolution) cannot be associated with the current biological activity with high fidelity, especially if the time gap is significantly large.

2.5. Non-invasive or micro-invasive methods?

The delicate equilibrium between sampling and quality of collected data is crucial in understanding rock art in terms of dating, composition, and assessment of its preservation. In the case of rock art dating, sampling is mandatory apart from the very few relative dating cases, such as luminescence dating applied to sediments covering rock art sites (see, for instance, Huyge et al. 2011). State-of-the-art radiocarbon and U-series dating methods require few milligrams of organics or carbonate, respectively, thus resulting in minimum invasive sampling. In any case, sampling points fall on representations; only for U/Th dating, the sampling can be done besides rock art representations, after an accurate assessment of the lateral continuity of speleothem related to rock art. The same minimal invasive approach is rarely applicable for radiocarbon dating, except in the case of engravings covered by rock varnish or other kinds of crusts/coatings (Cremaschi 1996; Huyge et al. 2001; Zerboni et al. 2021).

Non-invasive methods such as photogrammetry and the definition of stability indexes are mandatory to assess the preservation of rock art sites. Moreover, non-invasive analytical methods can help to trace a preliminary assessment of the characteristics of rock art and its support, but their application is limited to the surface. Yet, the major biogeomorphological processes occur within pigments and/or below the rock surface; this suggest that sampling is required to fully understand the relationships between the many components of the rock art ecosystem and assess the threats to rock art preservation. Microsamples also offer the opportunity to conduct laboratory experiments on rock substrate stability after artificial aging (Abd El-Hakim et al. 2019). In a recent experiment with pXRF, Dayet et al. (2022) observed that the heterogeneity of paintings system and the variability in primary and secondary mineral hamper the possibility of investigating rock art only following a non-invasive approach and concluded that the best solution for rock art research is a combination of *in situ* and laboratory analyses. From this perspective, a micro-invasive approach that guarantees rock art preservation and makes available a small quantity of pigments and/or rock substrate is mandatory.

In our recent experience in rock art research in a variety of remote locations, we tried to establish a procedure to reduce sampling and, at the same time, increase the possibility of mining information using different methodological approaches (Wu et al. 2020; Gallinaro and Zerboni 2021; Zerboni et al. 2021; Villa et al. in press). In remote locations, it is hard to transport (or export) portable instrumentation, thus we decided to proceed with several steps: (1) biogeomorphological survey of the rock substrate to assess the potentially many and diversified types of surface rock decay and SABs formation at the rock surface and/or below it; (2) micro-sampling (using small sterile chisels) of each type of deterioration evidence on the rock surface, far from rock art representations; (3) very micro-invasive sampling of painting (pigments) or rock decay evidence related to representations. The latter point is the most critical of the procedure, and to guarantee the sustainability of a micro-invasive sampling we decided to use sterile tape for sampling. The sterile tape removes minimal part of pigments, SABs, and weathering surfaces from a rock art gallery, allowing a quantity of sample sufficient for microbiological investigation and chemical and mineralogical characterization. Tape samples are divided into subsamples and sent to the different analytical lines, starting with the observation under optical and scanning electronic microscopes. SEM imaging and semiquantitative analyses guarantee the first assessment of the composition of rock art and intensity of rock decay, highlighting the occurrence of organics and the interaction between the components of the rock art ecosystem. Such procedure is, in our opinion, the best compromise between sampling and preservation and it allows us to understand the major surface processes affecting rock art.

2.6. Rock art between sustainability of research and responsible tourism

World rock art constitutes a significant archive on the past that can help to understand tangible and intangible aspects of ancient societies. It represents at the same time an extraordinary tool to fill the gap between the past and present, raising the awareness of civil society about the outstanding value of this heritage. Furthermore, rock art can represent a significant source of economic development (e.g., to promote tourism), particularly in remote areas of the world. However, rock art is one of the most fragile elements of the cultural heritage, a non-renewable resource that need to be properly investigated, managed, and preserved. As evident from this contribution, rock art is a complicate ecosystem, and the understanding of its many interactions requires an interdisciplinary approach. Many natural and anthropogenic factors largely damage paintings and petroglyphs as well their rocky support. In many cases, there is little to do, and prevention is the most effective mitigation strategy, through education and training of local people, especially in areas where continuous monitoring is not possible. Yet, recent developments in the research allow to minimize the impact of recording and sampling techniques, highly reducing the damages related with the research.

At the same time, physicochemical and microbiological analyses are opening new perspective for the preservation of rock art, that, if combined with efficient development and management plans can favour and support programs of sustainable tourism. Rock art has an extraordinary potential for development of local communities, being highly appreciated by tourists (Deacon 2006; Duval et al. 2019). Today, rock art tourism ranges from well-designed and controlled visitor centers and full fee-paying tourism ventures run by commercial operators to unrestricted visitation of archaeological areas promoted by amateur local guided tours (Duval et al. 2019). The different approaches have consequently different results in terms of economic growth of local communities and in the standards of rock art site preservation and maintenance. As suggested by Deacon (2006), the promotion of rock art as touristic resource requires shared strategies aimed at understanding the interaction of key elements that affect the long-term conservation of frequently visited rock art sites as much as their original environmental and cultural (e.g., ethnoarchaeological) settings. In fact, specific tourism practices need to be adequate to local circumstances and local stakeholders (Little and Borona 2014; El Menshawy 2017; Duval et al. 2019) and the successful touristic development of rock art sites must include the development of local economy and the promotion the local cultural landscape (Bollati et al. 2018; Gallinaro et al. 2018; Filippo et al. 2019). In such contexts of potential human-triggered threats to rock art sites, several successful examples of touristic accessibility exist, combining preservation and cultural and economic promotion. In some places there are on-site visitor centers; elsewhere tourist can freely visit a site without any form of supervision. In the case of caves (Duval et al. 2019), successful strategies include the complete closure of the original site, the allocation of a limited number of visitors, and the definition of restrictions. Occasionally, impressive on-site three-dimensional replicas of cave sites have been built (e.g., at Lascaux, Altamira, Pont d’Arc) or replicas are located within archaeological museums. Controlling the access to rock shelter and open-air sites is more difficult (Di Lernia et al. 2010; Duval et al. 2019) and requires infrastructure, including physical barriers, walkways, and information panels. For remote areas where it is difficult to control the access to rock art sites, a common strategy is to not divulge the exact location of the sites to allow the access to such sites only through the services of local guides (Duval et al. 2019). Finally, new technologies (e.g., immersive virtual reality) can provide powerful tool to increase the potentiality of virtual visits to rock art sites.

In conclusion, rigid (and standardized) protocols for sampling and analyses are required (Hadda et al. 2021) to obtain comparable results, and a general more ethical approach to the sustainable study of rock art can no longer be postponed. Sustainability of rock art in terms of scientific investigation and its exploitation as a touristic attraction is the challenge for the future.

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Chapter 3

Age, palaeoenvironment, and preservation of prehistoric petroglyphs on a boulder in the oasis of Salut (northern Sultanate of Oman)[2]

Abstract

The preservation of rock art in open-air contexts is a global issue controlled by several environmental processes, which are less investigated than the cultural significance of engravings and paintings. For that reason, we discuss the age, preservation, and palaeoenvironmental context of petroglyphs discovered on the flat, almost vertical face of a large boulder fallen along the western slope of Jabal Hammah, a rocky hill that borders the oasis of Salut (northern Sultanate of Oman). Geoarchaeological investigation highlighted that, in the region, the preservation of petroglyphs is due to the interplay of two contrasting weathering processes. On one hand, karst dissolution – even if it is a very slow process in arid and semi-arid lands – gradually levels the surface of boulders. On the other hand, a biomineralized Mn- and Fe-rich rock varnish has developed inside the grooves of the engravings, thus sheltering them from extreme dissolution and promoting the preservation of the pristine shape of the representations. Moreover, organics trapped within the rock varnish have been radiocarbon dated to 2600 ± 60 uncal. years BP. This result allows establishing a limit *ante quem* for the production of these specific engravings and to root it to the Bronze or Iron Age exploitation of the area. This result is of particular relevance in a region where well-dated rock art is virtually absent. Today, the biogeochemical processes leading to the formation of the protective crust are almost inactive, and not consistent with the present dry environmental settings. Their occurrence is in accordance with other local palaeoclimatic record, and suggests Bronze and Iron Age climatic conditions wetter than today. A broader implication of our work is that it shows how a multidisciplinary approach to the study of rock art provides the opportunity of understanding the age of rock art and its paleoenvironmental significance. We demonstrate that physical, chemical, and biological weathering processes are in charge of the preservation and/or destruction of rock art; such processes have to be seriously taken into account in projects of rock art field assessment.

Keywords: Rock art; Rock weathering; Microscopy; Climate change; Radiocarbon; Sultanate of Oman

3.1. Introduction

The preservation of rock art in open-air contexts – as much as other cultural heritages (Howard 2013) – is tricky, especially in locations where extant climatic and environmental settings are different respect to those present at the time of the production of rock art galleries (Darvill and Batarda Fernandes 2014; Giesen et al. 2014; Groom 2017). This is especially true in arid and semi-arid regions, where Holocene climatic changes have deeply and repeatedly reshaped the environment. In such contexts, open-air rock art galleries are, at the same time, a record for past climates as much as a feature threatened by new environmental conditions (Cremaschi et al. 2008; Zerboni 2012). As a consequence, rock art (paintings and engravings) may suffer strong weathering and taphonomic processes – including physical, chemical, and biological weathering – and in some cases can be

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[2] Zerboni, A., Degli Esposti, M., Wu, Y.-L., Brandolini, F., Mariani, G. S., Villa, F., Lotti, P., Cappitelli, F., Sasso, M., Rizzi, A., et al. 2021. Age, Palaeoenvironment, and Preservation of Prehistoric Petroglyphs on a Boulder in the Oasis of Salut (Northern Sultanate of Oman). *Quaternary International 572*, 106–119. https://doi.org/https://doi.org/10.1016/j.quaint.2019.06.040.

completely erased. For these reasons, the understanding of the processes leading to rock art preservation and/or destruction and its paleoenvironmental context are as important as its cultural, archaeological, and artistic implications (Whitley 2005; Dorn 2006; Dorn et al. 2008; Allen et al. 2011; Bednarik 2012; Giesen et al. 2014).

Studies promoting the understanding of preservation of rock art preservation and its environmental context remained scanty for a long time. Recently, however, an increased awareness that the understanding of rock art and its decay requires multidisciplinary approaches promoted the development of some projects focused on the assessment of rock art preservation and the study of the interface between rocks and pigments. Some studies considered the many processes affecting rock art and their rock supports (Cerveny 2005; Hall et al. 2007, 2009; Mol and Preston 2010; Groom 2016, 2017; Gallinaro et al. 2018), and applied indices describing the stability of rock art (Dorn et al. 2008; Allen and Lukinbeal 2011; Groom and Thompson 2011; Allen and Groom 2013). In other cases, studies are dedicated to understanding the age and paleoenvironmental significance of rock art (Cremaschi 1996; Dorn 2006; Zerboni 2008, 2012; Bednarik 2012; Macholdt et al. in press). For that reason, in this paper we examine some engravings recently discovered in the northern part of the Sultanate of Oman (oasis of Salut), with the main aim to decipher their age and significance in relation to the local and regional archaeological background, and to reconstruct the main environmental processes leading to the decay or preservation of petroglyphs.

Several petroglyphs' panels were discovered in the region and the most of them show the same slightly varnished, usually dotted pecking technique, and the same iconographic repertoire commonly witnessed in other parts of the Arabian Peninsula (e.g., Jung 1994; de Ceuninck 1998; Ziolkowski 1998, 2007; Bednarik and Khan 2002; Khan 2007, 2013; Bednarik 2005, 2017; Fossati 2017). However, the finding discussed here represents a so far unique discovery within the regional archaeological context and deserves a specific geoarchaeological investigation. In fact, this rock art panel comprises a series of engravings that differs in pecking technique from the most widespread ones, and notably from the large majority of the rock art present in the same oasis. Although being generally strongly abraded by environmental processes, where they are sheltered by a dark, biomineralized rock crust, they are better preserved. The same rock crust allowed dating engravings and elucidating their palaeoenvironmental history.

3.2. The study area

3.2.1. Geographic, climatic and geologic background

The rock art panel here investigated consists of engravings on a limestone boulder outcropping on the southern slope of a hill belonging to Jabal Hammah, in the vicinity of the archaeological site of Salut (Fig. 3.1) in the northern Sultanate of Oman (Degli Esposti et al. 2018b). The Geologic Map of Oman, 1.250,000, sheet NF4007 – NIZWA, reports the toponym Jabal Hammah, although locals currently refer to the same hill as Jabal Salut, which is the toponym used in most of the archaeological literature cited here. The area is located between the modern villages of Bisya (2 km to the South) and Bahla (20 km to the North), and stands at the margin of the palaeo-oasis of Salut, which developed in the Early and Middle Holocene along Wadi Sayfam (Cremaschi et al. 2018a). A variety of sedimentary, metamorphic, and igneous rocks outcrop in this region (Geologic Map of Oman, 1.250,000, sheet NF4007 – NIZWA); the area is delimited to the North by the massif being part of the Mid-Late Cretaceous Samail Ophiolite, and to the West and to the South by Permian to Cretaceous sedimentary formations (limestone and radiolarite). The bedrock of the hill hosting the engraved boulder consists of the Late Jurassic to Cretaceous Wahrah Formation (Glennie et al. 1974), which includes lithoclastic, oolithic, marly limestone, chert, and silicified limestone (Béchennec 1986). Pleistocene and Holocene deposits cover part of the region, including large alluvial fans and fine sediments covering most of the bottom of the alluvial plain along the main wadis (see details in Cremaschi et al. 2018a).

The present climate of Northern Oman is arid to semi-arid and controlled by the seasonal development of the monsoon winds. Rainfall originates from Mediterranean frontal systems in winter and spring (Weyhenmeyer et al. 2000, 2002), and varies between 150 and 250 mm/yr (Alsharhan et al. 2001; Fleitmann et al. 2007). On the contrary, palaeoclimate studies based on speleothems from the Hoti Cave (at ca. 45 km from the study site; Neff et al. 2001; Fleitmann et al. 2007) suggest that in the early Holocene (between ca. 10.5 and 9.5 ka BP) precipitations increased, triggered by the northward shift of the mean latitudinal position of the summer ITCZ and the associated Indian Summer Monsoon (ISM) rainfall belt. After ~7.8 ka BP to present, the mean summer ITCZ continuously migrated southward triggering the gradual decrease in intensity and duration of the ISM season. Other records from southern Oman and Socotra suggest a marked decrease in precipitation at ca. 2 ka BP, thus confirming a general decrease in intensity of the Indian Ocean monsoon in the late Holocene (Fleitmann et al. 2007; Cremaschi et al. 2015)

3.2.2. Archaeological background

The archaeological record of the region of the Sultanate of Oman roughly comprised between the cities of Nizwa and Bahla was initially surveyed in the 1970ies (Hastings et al. 1975; De Cardi et al. 1976), but systematic archaeological investigation started later (e.g. Orchard and Orchard 2002). A project of extensive archaeological research which entailed the excavation of the major archaeological sites in the oasis (Husn Salut; Salut-ST1; Jabal Salut necropolis; Qaryat Salut) was established in 2004, when the work of the Italian Mission to Oman started tracing the outlines of the regional evolution of human occupation (Phillips et al. 2015; Degli Esposti 2015a; Avanzini and Degli Esposti 2018; Tagliamonte and Avanzini 2018). Extensive stratigraphic excavations were combined with a programme of surveys conducted over the plain surrounding the sites, as well as the surrounding hillocks (e.g. Phillips et al. 2012; Condoluci et al. 2014), which led to the identification of several open-air sites, including some rock art panels and isolated representations (Fig. 3.1).

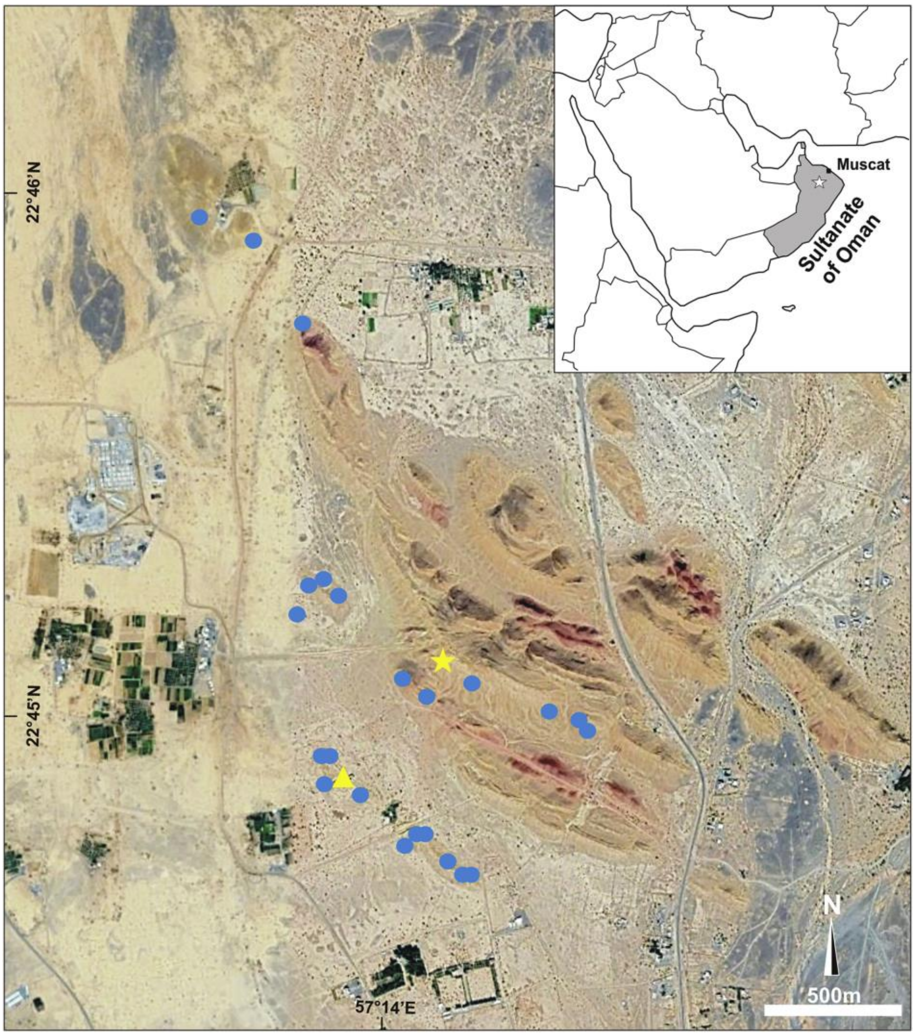

Figure 3.1. The study area on GoogleEarth™ satellite imagery; the star is the position of the boulder, dots are other rock art stations, the triangle is the position of the citadel of Salut. Note the monocline pattern of the Jabal Hammah. The inset indicates the position of the area in the Sultanate of Oman.

Locally, the earliest human traces in the area can be dated back to the Palaeolithic, whereas scatters of lithics suggest the Neolithic exploitation of the region (Fig. 3.2). However, the most evident and systematic occupation of the area and exploitation of natural resources corresponds to the Early Bronze Age (second half of the 3rd millennium BCE). At that time, three monumental stone tower sites were erected along the eastern side of Wadi Sayfam, being an expression of a specific culture that displays homogeneous characters throughout the Oman peninsula, from Musandam in the north to the Masirah Island in the south (Orchard and Stanger 1994; Degli Esposti 2016). A demographic decrease apparently marked the first half of the 2nd millennium BCE, while a reverse trend started around the end of the 15th century BCE, as indicated by the establishment of a dense network of Iron Age sites, clustering around the citadel of Salut (Avanzini and Phillips 2010; Phillips et al. 2010; Condoluci et al. 2014). Archaeological excavation suggests that this settlement, somehow precocious compared to other areas of southeast Arabia, lasted beyond the end of the Early Iron Age period, that is, beyond c. 300 BCE and likely until the 1st century CE (Degli Esposti et al. 2018a, 2019). During this long phase, the palaeo-oasis of Salut reached its greatest spatial extent and apogee of agricultural land use. Around the end of the 1st century CE, the palaeo-oasis of Salut was abandoned for several centuries. After that, the citadel of Salut witnessed three distinct periods of Islamic occupation: (i) Early Islamic (ca. 9th/10th century CE); (ii) Middle Islamic (ca. 12th/13th century CE); and (iii) Late Islamic (after the 16th century CE) (Whitcomb 1975; Avanzini et al. 2005; Avanzini and Degli Esposti 2018). Geoarchaeological evidence indicates that the major phases of expansion of settlements in the area were related to the establishment of efficient systems for water management sustaining intensive cultivation (Cremaschi et al. 2018a).

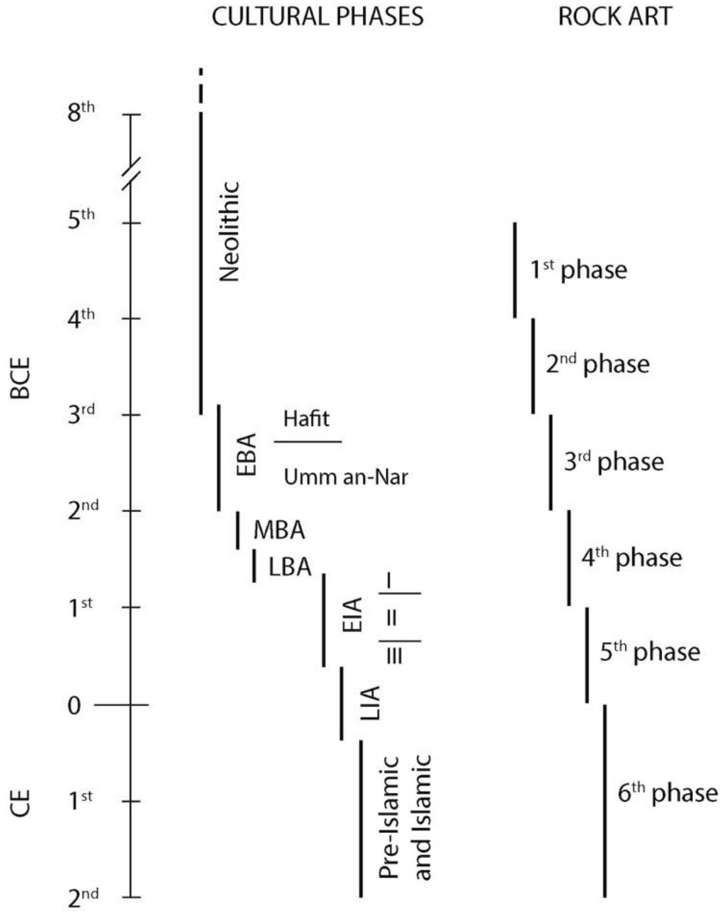

Figure 3.2. Regional cultural periods on the basis of the current archaeological literature, compared with the periodization of rock art in Oman (Jabal Akhdar) as suggested by Fossati (2017).

3.3. Materials and methods

3.3.1. Description of the rock art panel

The petroglyphs discussed in this paper were engraved on the vertical flank of a large boulder on the southwestern slope of Jabal Hammah (Figs. 3.3 and 3.4), northeast of Husn Salut. The Jabal Hammah is a rocky monocline relief dotted by several prehistoric tombs (Condoluci and Degli Esposti 2015; Degli Esposti et al. in press). On the southwestern slope of the hill, several blocks and boulders belonging to the Wahrah Formation are present, corresponding to the remains of an ancient rockfall triggered by undersapping processes that eroded the underlying marls. In the central part of the rockfall, the engraved boulder stands up. Several smaller blocks stand at its foot, possibly adjusted to increase its stability or to allow carving the engravings (Fig. 3.4). Other large boulders found along the slope of the hill were artificially stabilised, but they do not display engravings. On the contrary, in the same area several small, isolated engraved blocks were found. The large boulders and the small ones possibly correspond to a sort of landmark and are part of the same archaeological landscape.

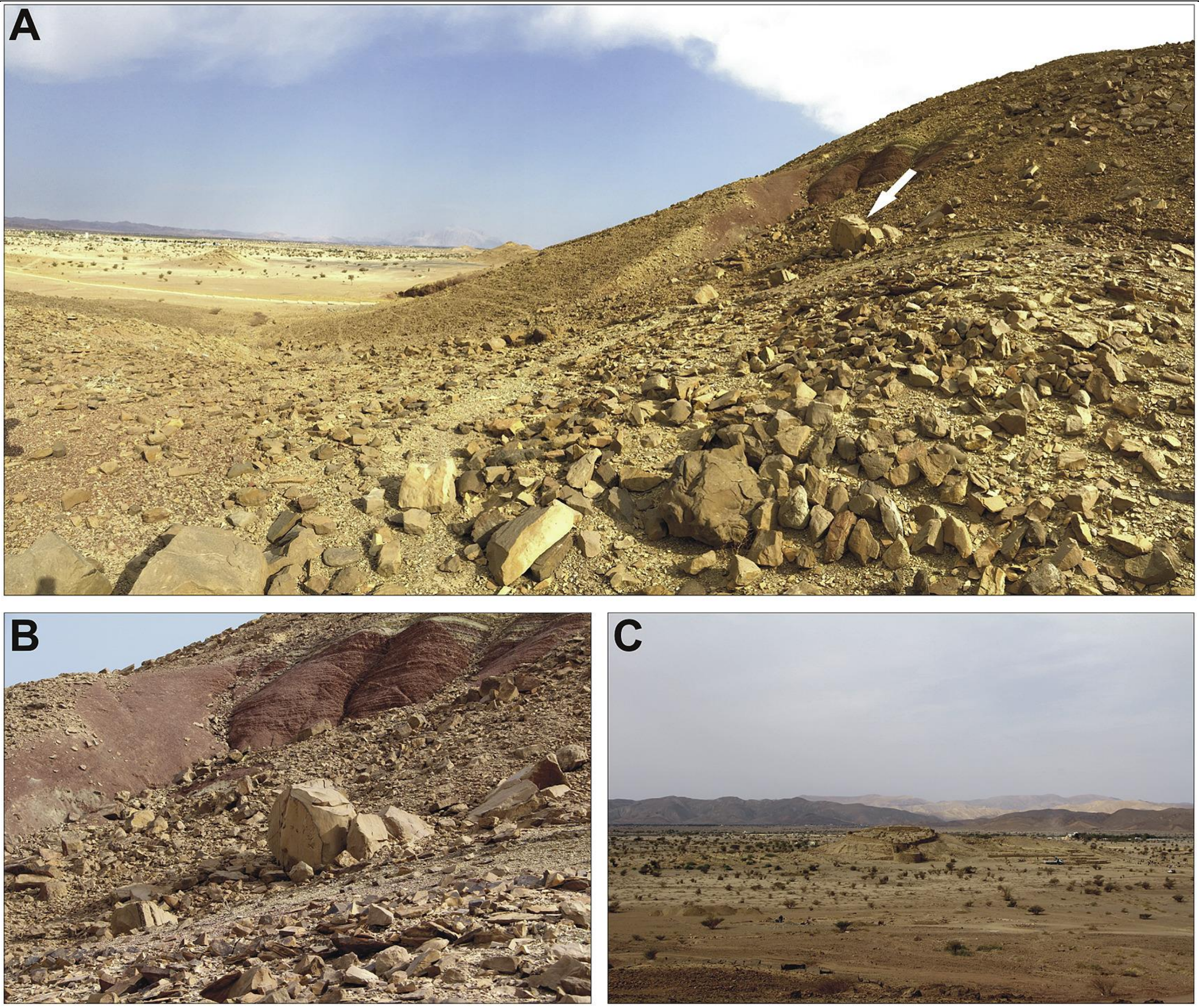

Figure 3.3. (A) General view of the western slope of the Jabal Hammah (the arrow indicates the position of the boulder). (B) A detail of the slope with the engraved boulder in its present-day landscape. (C) A view of the citadel of Salut from the Jabal Hammah.

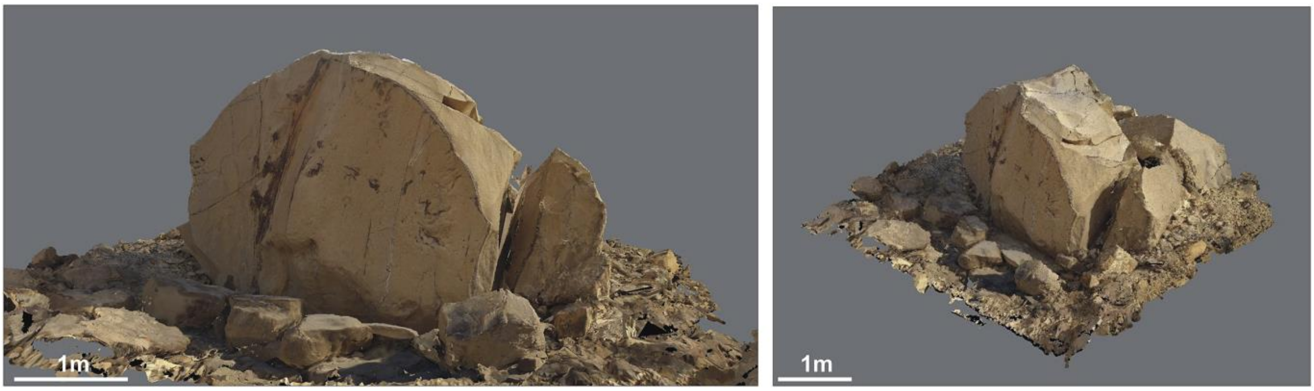

Figure 3.4. Different views of the engraved boulder after 3D reconstruction (see the text for details).

We distinguished three engraved figures carved on the boulder surface (Fig. 3.5). Despite two of them being remarkably less well preserved, all can be recognised as representing the same motif: a standing man holding in his right hand a long shaft ending in a hemi-circular item, possibly the representation of a halberd (henceforth we refer to this item as halberd). The three figures are sketchily made with simple traits ("stick men"), with outstretched arms and open legs. On the best preserved figure, the uppermost, a faintly visible vertical stroke may indicate the penis, which is also vaguely visible in the central figure. The engravings were made with a continuous pecking, lowering the engravings' groves of a few millimetres under the original surface.

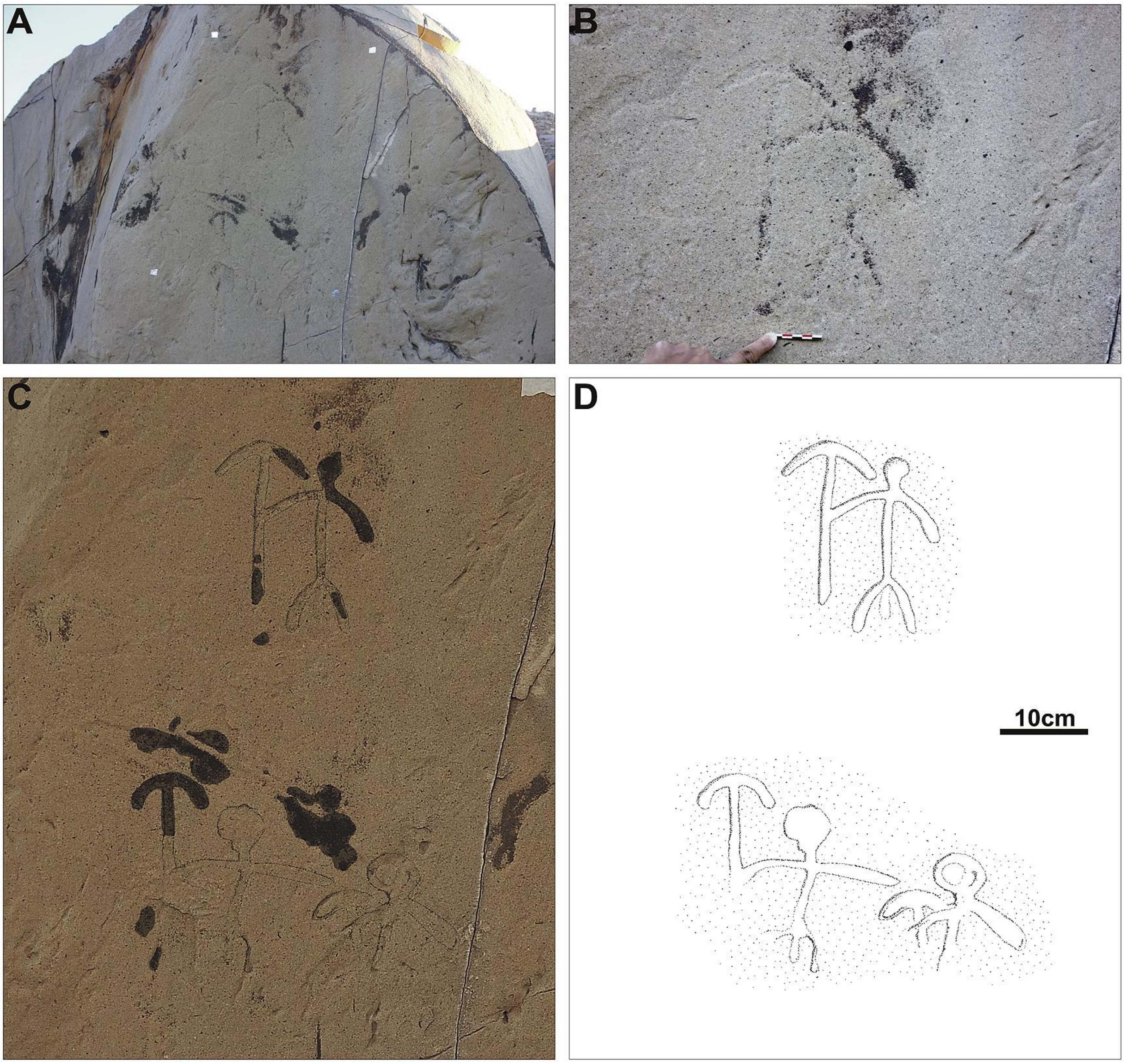

Figure 3.5. (A) Pictures of the main panel of engravings representing men with halberd, and (B) a detail of the best preserved figure. (C, D) Interpretative drawing of the panel of men with halberd.

Other petroglyphs are scattered in the surroundings of Jabal Hammah and they include horse riders, various animals, and several signs. The most of the latter are common in the region, as suggested by several comparisons (e.g., Jung 1994; de Ceuninck 1998; Haerinck 1998; Ziolkowski 1998, 2007; Khan 2007, 2013; Fossati 2017). Although other examples of complete motifs of the standing man with halberd have not been found so far in the area of Salut, we spotted a few representations of the single halberd (Fig. 3.6). Interestingly, these engravings were made using the continuous pecking technique instead of the dotted technique that distinguishes the great majority of the rock art in the area; this is a further similarity to our case study. Three isolated halberds are on the southern face of a boulder employed in the building of one of the structures that is part of an Early Bronze Age tower (T2) standing some 600 m north of the Iron Age complex of Salut (Degli Esposti 2015b). The engraving was manufactured after the block was inserted in the masonry structure; however, it is hard to define the real chronological correlation between the engravings and the age of the tower T2. A few stone seals found during archaeological excavations provide additional, indeed scarce, parallels. A cylinder seal found at the Bronze Age tower ST1 in the area of Salut, dated to the Iron Age re-occupation of the site, bears a representation closely resembling the halberds shown on the discussed boulder (Degli Esposti 2012, 2015b). Moreover, another cylinder seal from Ra’a al Jinz RJ-2, dated by its excavators between 2500 and 2250, is carved with an identical figure (Cleuziou and Tosi 2000).

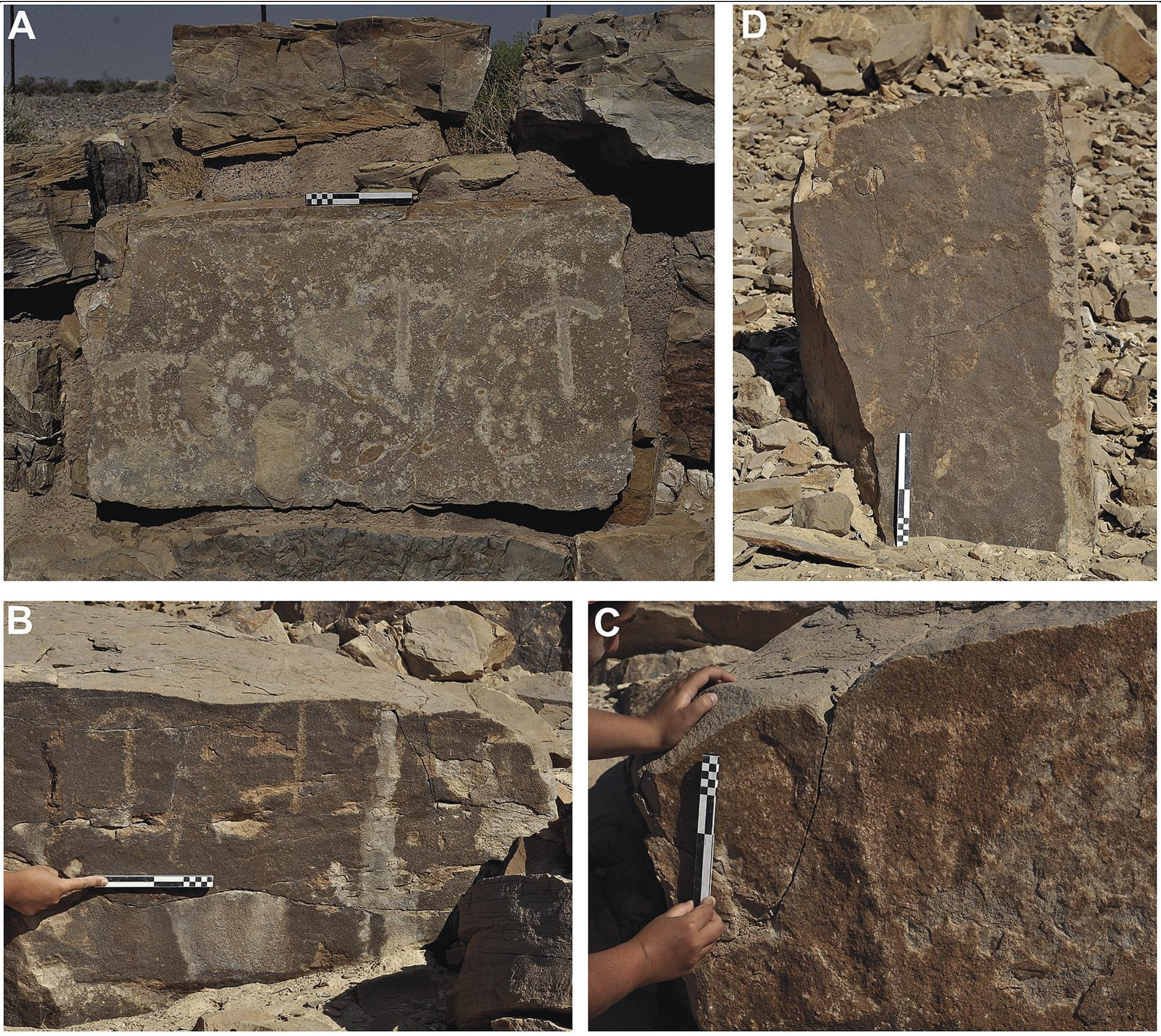

Figure 3.6. (A-C) Local comparisons for the representation of men with halberd; (A) represents the engravings found on the masonry of the Bronze Age tower T2. (D) An example of a different iconography in the local rock art.

3.3.2. Field survey, photographic recording, and analyses

The engraved boulder discussed here was located by one of the authors (MC) during a survey of the rock art panels over the hills surrounding the citadel of Salut. The boulder and the surrounding area were carefully inspected and engravings digitally recorded and drawn. Moreover, a 3D model of the boulder was elaborated with Structure-from-Motion (SfM) photogrammetry. This technique uses an automated process to obtain point clouds, triangular meshes and full 3D textured models from images (Pena-Villasenín et al., in press) and has been applied with relevant results in field archaeology (De Reu et al. 2013; Galeazzi 2016; Waagen 2019) as well as in rock art studies (Sanz et al. 2010; Plisson and Zotkina 2015; Robert et al. 2016; Bea and Angas 2017), being the most appropriate solution for macroscopic 3D recording in the field in terms of time and cost of execution. Moreover, for the majority of rock art sites, SfM 3D-modelling has proved to be a valuable, cost-effective alternative to Terrestrial Laser Scanner (TLS) (Johnson and Solis 2016; Jalandoni et al. 2018). In this paper, we applied a low-cost and high flexible SfM approach (Jalandoni et al. 2018) to elaborate a 3D model for metric analysis and digital tracing of rock art motifs (Supplementary Material). In our case study, we took 156 pictures with a Canon EOS 100D from different perspectives and elevations with at least 60% overlap and markers with a known distance were placed near the boulder; Table 3.1 presents camera location parameters used in the elaboration of the 3D model. The 3D model was generated and scaled using Agisoft Metashape photogrammetric software (Agisoft 2018).

Table 3.1. Camera location parameters used to generate the 3D model of the boulder with rock art.

Number of pictures	**156**	**Camera stations**	**156**
Altitude	2.67 m	**Tie points**	128,354
Ground resolution	0.647 mm/pix	**Projections**	400,483
Coverage area	13 m^2	**Reprojection error**	1.48 pix

As described in the following parts (sections 3.4.1 and 3.4.2), the best preserved part of the rock art panel is covered by a dark, hard mineral coating. The same coating covers or fills small natural depressions along the boulder and along vertical discontinuities (fractures). To establish the nature and age of the weathering crust, we collected it in correspondence of several linear fractures on the sides of the boulder not related to engravings. Several small crust and bedrock samples were removed with a small chisel.

Rock samples were cut perpendicular to the crust surface, polished, and thin section prepared according to the procedure described in Murphy (1986). Micromorphological observation under plane-polarized light (PPL) and cross-polarized light (XPL) of thin sections were conducted with an optical petrographic microscope Olympus BX41 equipped with a digital camera (Olympus E420); during interpretation, we followed the concepts discussed in Cremaschi et al. (2018b). Micromorphological studies of thin sections also employed a Cambridge 360 scanning electron microscope (SEM) imaging both secondary and back-scattered electrons. Energy dispersive X-ray analysis (EDS Link Isis 300) required to carbon coat the thin sections. Energy dispersive X-ray spectroscopy worked with an accelerating voltage of 20 kV, filament intensity 1.70 A, and probe intensity of 280 pA. Every analysed element was previously standardised by using several single element standards (Micro-Analysis Consultants Ltd); elemental concentrations measured by EDS are reported as oxide weights normalized to 100%.

Several fragments of the crust were collected and glued on glass fibers mounted on a goniometer head. An Oxford Diffraction Xcalibur 4- circle diffractometer, equipped with a Mo-Kα X-ray tube (λ = 0.7107 Å) and a CCD area detector, operating at 50 kV and 30 mA, was used for X-ray diffraction investigations. The diffraction patterns were collected by exposing each sample for 45 or 120 s during a full rotation of the goniometer head around the vertical axis. The collected images were integrated into a 2θ-Intensity diffraction pattern adopting the routines implemented in the software Crysalis (Rigaku Oxford Diffraction 2018). The phase assignment was done based on the collected powder diffraction patterns, using the ‘Xpert HighScore suite (Degen et al. 2014).

Several samples of rock crust were observed with a Confocal Laser Scanning Microscope (CLSM) after fluorescent staining. Confocal images were collected using a Nikon A1 laser scanning confocal microscope and a 20x/0.75NA (WD 1 mm) Plan Apo λ objective. Fluorescence was excited and collected using different combinations of the following laser lines and emission parameters: (i) autofluorescence from photosynthetic pigments was viewed in the red channel using the 633 nm line of an Ar/HeNe laser in the emission range of 650–750 nm; (ii) The total biofilm biomass was visualized by DAPI (4′ ,6-diamidino-2-phenylindole, Invitrogen) staining according to manufacturer instructions. This dye was excited with a 405 nm laser with emission collected in the range of 430–480; (iii) metabolically active cells of the biofilms were viewed in the green channel by staining the samples with Calcein AM (Film-Tracer™ calcein green biofilm stain, Invitrogen) according to manufacturer instructions. This dye was excited with a 488 nm laser with emission collected in the range of 500–550 nm; (iv) extracellular polysaccharides were detected by Con A staining (Concanavalin A, Alexa Fluor 488 conjugate, Invitrogen) according to manufacturer instructions. This dye was excited with a 488 nm laser with emission collected in the range of 500–550 nm; (v) extracellular proteins were detected by Bodipy staining (BODIPY® 630/650-X, Invitrogen) according to manufacturer instructions. This dye was excited with a 633 nm laser with emission collected in the range of 650–700 nm. CLSM was used in reflectance mode with the 488 nm argon line for relief imaging of specimens. Captured images were analysed with the software NIS-Elements (Nikon) for 3D reconstructions of biofilms.

Based on both SEM and optical microscopes observations, one sample of weathering surface not experiencing mechanical or biogeochemical weathering (Sowers 2000) was selected for accelerator mass spectrometry radiocarbon dating (AMS-^{14}C), as organic carbon generally occurs trapped under the Mn-rich rock varnish at the rock interface (Dorn et al. 1989). The sample for dating was prepared according to the protocol described in Zerboni (2008). A portion of the same sample was analysed for total organic carbon (TOC) by titration using chromic acid to measure the oxidizable organic carbon

(Walkley and Black 1934). The sample was dated at the facility of the University of Georgia (USA). AMS-^{14}C age result was calibrated using the INTCAL13 calibration curve (Reimer et al. 2013) and results reported with 95.4% and 68.2% accuracy (Table 3.2).

3.4. Results

3.4.1. Description of the rock surface of the boulder

The surface of the boulder, as much as many other rock surfaces in the area, displays evidence of weathering processes. Physical and chemical deterioration are the most evident effects of weathering. In the area, blocks are often disaggregated by thermal stress, and the surface of limestone rocks dotted by small-scale solution pits, karren, and micro-karren (*sensu* Ginés et al. 2009) (Fig. 3.7). One further karst-related feature is evident at the micro-scale (Fig. 8); under the microscope, boulders and blocks display dissolved surfaces with etch pits (Fiol et al. 1992). In this case, where the dominant lithotype is a siliceous limestone, the formation of alveolar micro-depressions at the rock surface is controlled by the dissolution and removal of micritic mud among silicified grains (Fig. 3.8). Moreover, on the considered boulder evidence of calcium carbonate-bearing case hardening (Smith 1988; Dorn 1998) is present. In fact, along the margins of the boulder, we occasionally observe the occurrence of a few mm thick coating of sparitic calcite combined with iron oxides and occasionally clay minerals (Fig. 3.8).

Figure 3.7. Some examples of limestone surfaces displaying evidence of karst dissolution. Arrows in A and B indicate the deepest micro-karren (linear features); arrows in B and C indicate dissolution surfaces marked by small and irregular depressions C.

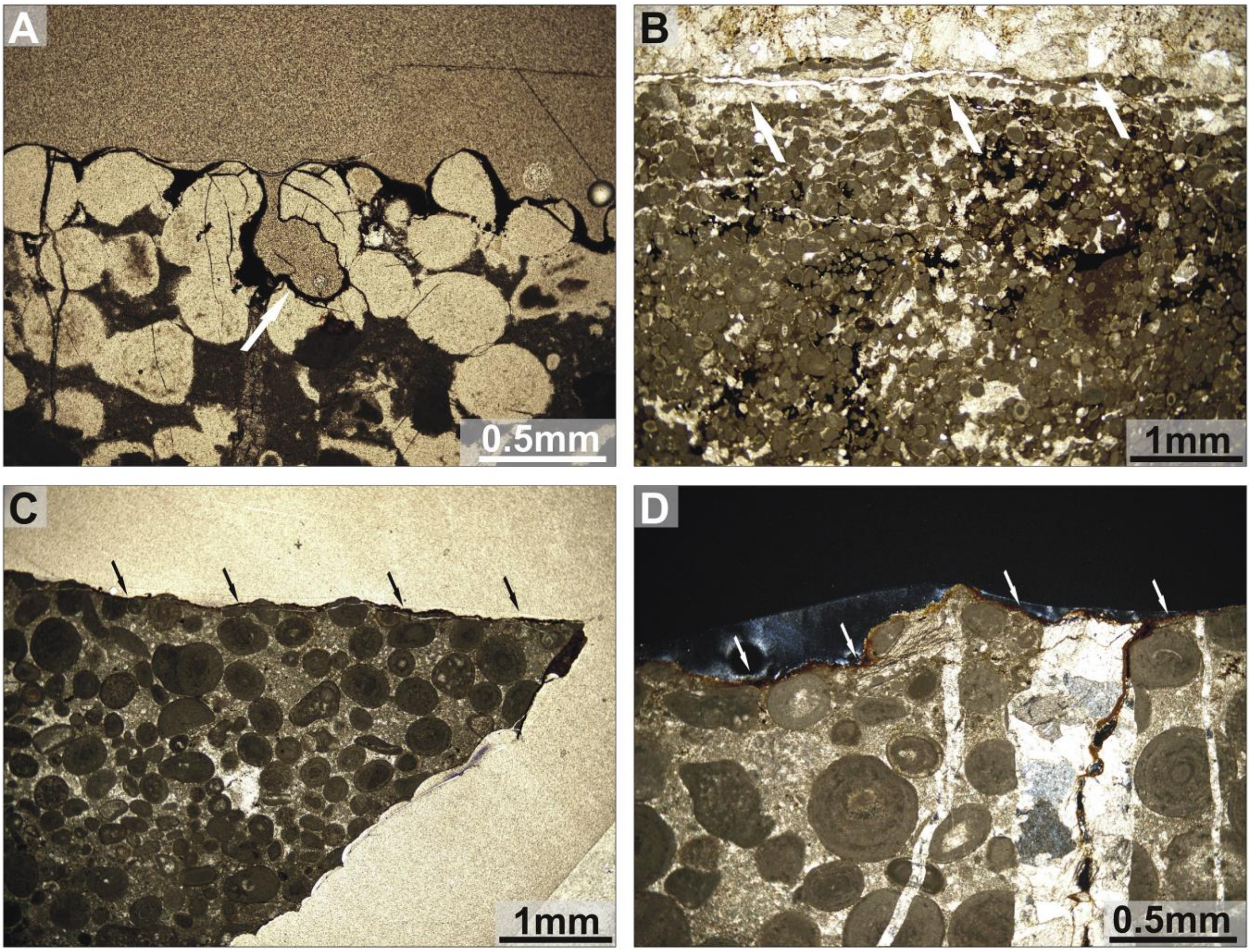

Figure 3.8. Optical images of a cross section of samples from the engraved boulder: (A) Alveolar micro-depressions at the rock surface, note the dissolution of micritic mud (PPL). (B) Sparitic calcite of the case hardening (XPL). (C) Cross section of a limestone surface covered by the thin dust film (PPL) and (D) a detail of the dust film (XPL).

As a consequence of the abovementioned processes, the surface of the boulder is rough and limestone dissolution levelled the micro-relief of engravings, leading to their removal. The groves of the engravings are best preserved in few places, where dark, few mm-thick crusts are present (Fig. 3.9). The same crust is evident also in correspondence of natural depressions, cracks, and fractures along the same boulder, and on many other rocks outcropping along Jabal Hammah (Fig. 3.9). At the macro-scale, the crust is metallic, smooth, massive, and hard; its Munsell® (1994) color varies between 10R 2.5/1 and 10 YR 2/2 (reddish black–very dark brown). Moreover, a very thin layer of a darkish coating covers the whole rock surfaces of subaerially exposed rocks (Fig. 3.7). Generally, the color of this coating is 10 YR 4/1 (dark gray) and contrast with the yellowish color of the fresh Wahrah Formation limestone (10 YR 7/6, yellow). The thin rock coating is widespread in the area, but at some places dismantled by recent wind erosion.

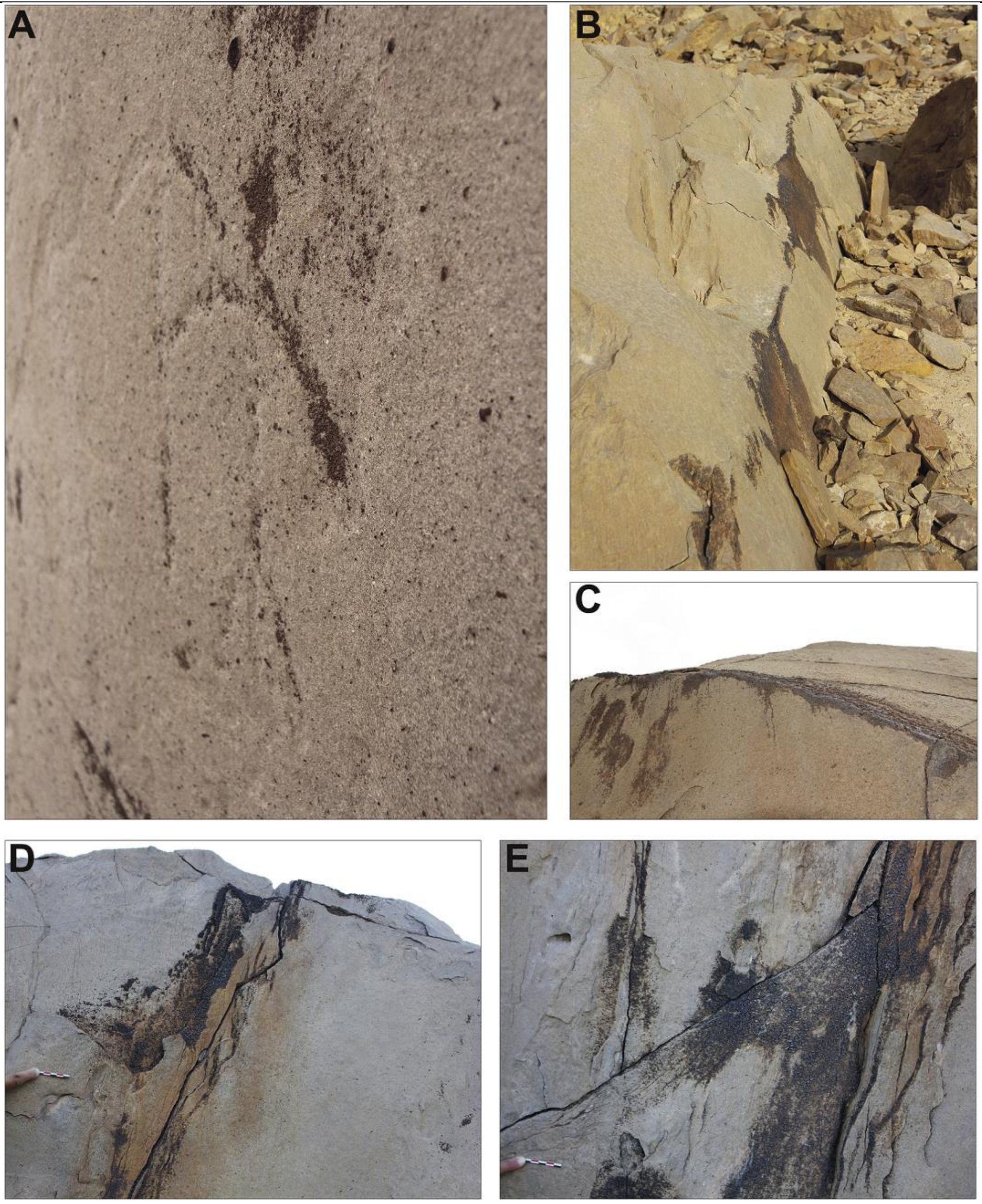

Figure 3.9. Field pictures of the rock crust developed in the groves of one of the engraved figures (A); note the incoming inversion of the relief due to karst dissolution and the crust sheltering the engraving from complete levelling. (B–E) Examples of the same crust developed elsewhere on the same boulder.

Under the petrographic microscope, the dark crust consists of a continuous layer draping the discontinuities between the residual siliceous grains and filling the alveolar depressions at the rock surface (Fig. 3.10). This results in an irregular thickness of the crust along the rock surface, higher in correspondence of alveolar voids and thinner on grains. At PPL and XPL, the dark coating is massive and opaque, with a few reddish reflections along some silicified ooids; reddish parts of the coating show a moderate birefringence under XPL. Conversely, the thin rock coating widespread on all exposed rock surfaces displays almost the same thickness of a few tens of microns (rarely thicker) and a dark brownish color under PPL to a light to dark brown color and very weak birefringence under

XPL (Fig. 3.8). This coating covers also the dark crust and has analogies with dust films (Rivard et al. 1992; Dorn 1998) enriched with local constituents and others originating from the aerosol.

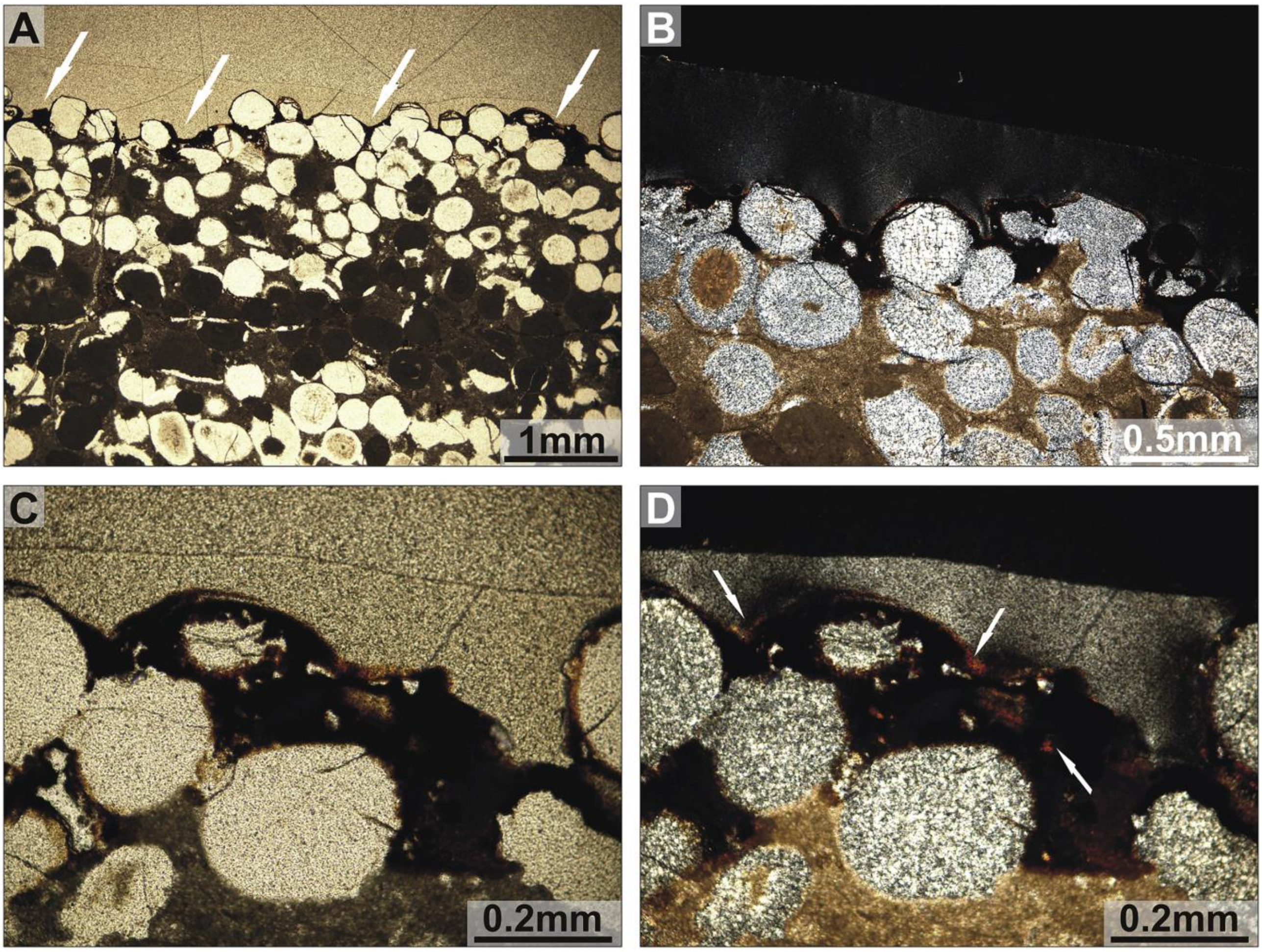

Figure 3.10. Optical images of a cross section of samples of rock crust. (A) General view illustrating the distribution of the crust draping silicified ooids and infilling voids among them (PPL). (B) A detail of the crust in XPL illustrating its optical properties; note the occurrence of completely and partially silicified ooids and the removal of part of the micritic mud due to dissolution. (C) The crust at high magnification illustrating intergranular spaces filled by varnish material (PPL). (D) The same in XPL, note the presence of reddish and birefringent material (indicated by the arrows).

The higher magnification of the SEM better illustrates the occurrence of alveolar voids due to dissolution on top of rock surfaces (Fig. 3.11), the continuity of the thin dust film (Fig. 3.11), and the occurrence of coating infilling and draping discontinuities (Fig. 3.12). The thickness of the dust film never exceeds 100 μm and is dark brown under the SEM; the EDS analysis suggests a composition dominated by Si (~50-60%), Al (~16-20%), Fe (~9%), and Mg (~8-11%), and enrichments of such alkalis as K (~2.5-5%) and Na (~2-2.5%). This layer adheres to the rock surface, but it did not tightly grow on it (Fig. 11); chemical data suggest that this layer consists mostly of clay minerals, possibly Fe-oxides stained, and enriched in alkalis form the aerosol (Zerboni 2008), thus confirming the attribution to dust film. The thickness of the dark crust found in the engravings' furrows and sampled along discontinuities of the block varies from a few up to 500 μm. SEM images in Fig. 3.12 illustrate the different properties of the samples and help in discriminating between the micritic matrix, siliceous ooids, and many components of the crust. The outer part of the crust consists of a convolute to botryoidal mixture of crystals; EDS analysis detected major concentrations of the following elements: Si (~36-44%), Al (~17-20%), Fe (~10-13%), Mg (~6.5-10%), K (~3%), Na (~2.5%), and Ca (~2.5-1.5%). This layer is up to 100 μm thick and dotted by whitish features enriched in Mn (~7-16%); it can be interpreted as a layer of clay minerals enriched in Mn and Fe oxides – a rock varnish (Dorn 1998, 2007). The subsequent part under the SEM appears white and bright; in fact, it is enriched in Fe (~85-90%), with a lower content of Si (~8%), Na (~1.5%), Mg (~1%), and Al (<1%). Towards the bottom of the crust, the Fe content decreases to ~65%, but we noticed an enrichment of Ca (~7.5%), and P (~5.5%). In rock coatings, concentrations of Ca and P showing parallel trends generally indicate the presence of calcium phosphates minerals, ultimately related to biologic processes (Reneau et al. 1992; Dorn 1998; Zerboni 2008).

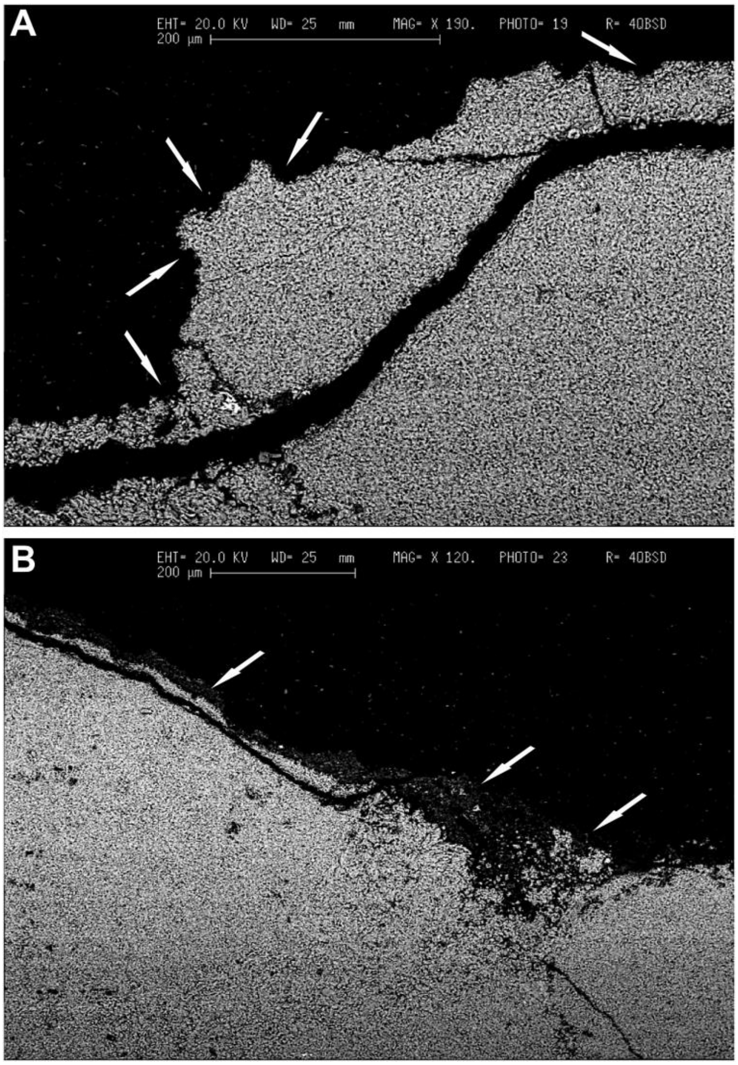

Figure 3.11. Scanning Electron Microscope images of a cross section of rock surface: (A) alveolar voids due to dissolution (etching pits are indicated by the arrows); (B) thin dust film indicated by the arrows.

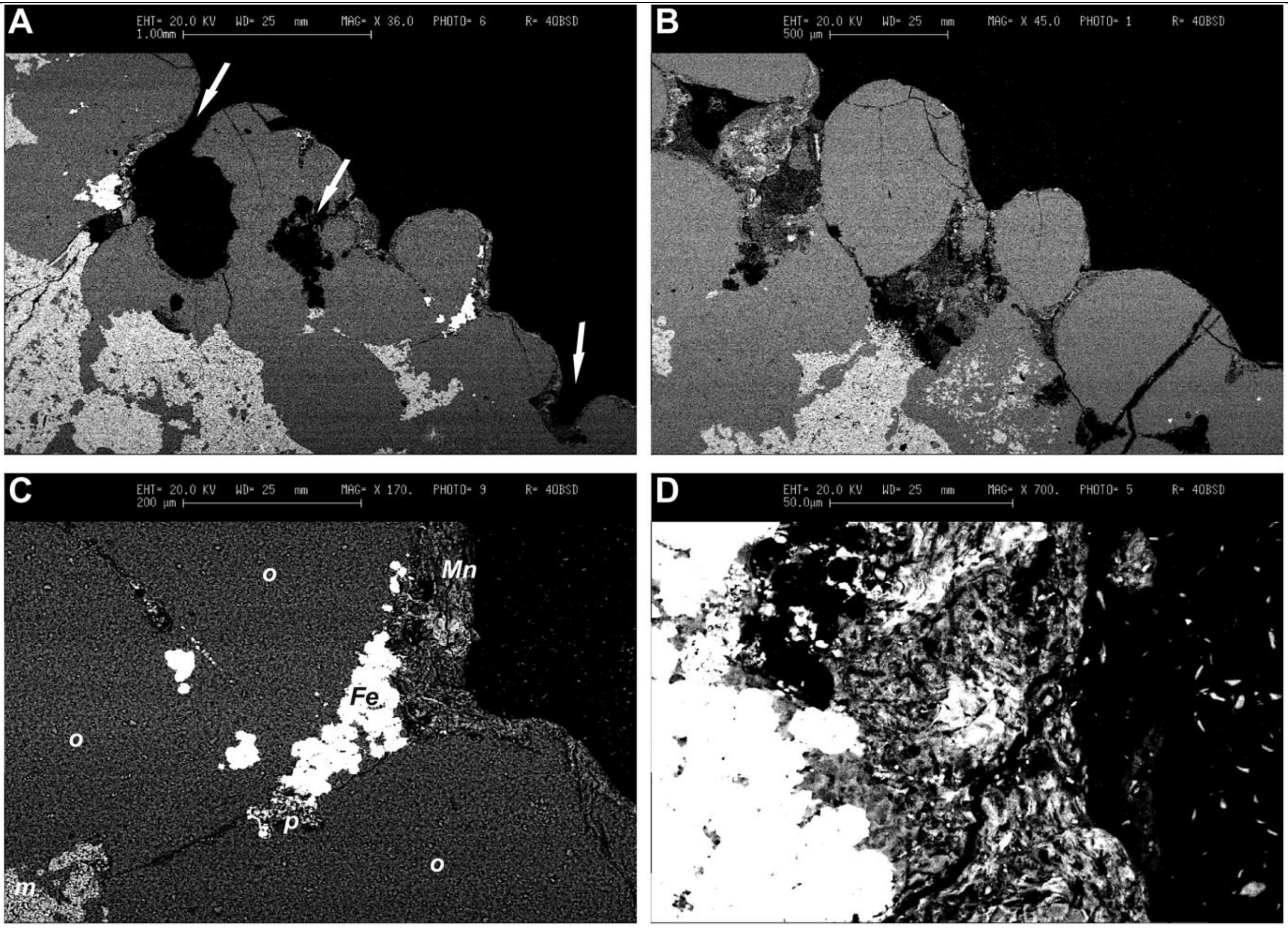

Figure 3.12. Some examples of Scanning Electron Microscope images of the rock varnish. (A) General view of the varnish infilling dissolution pit and draping grains (indicated by the arrows); notice the presence of silicified ooids and micritic mud . (B) Accumulation of rock varnish inside intergranular spaces. (C) The multi-layered rock varnish descried in the text; (o) silicified ooids, (m) micritic muds, (Mn) layer of clay mineral with Mn and Fe oxides, (Fe) Fe-rich layer, (p) accumulation of phosphates. (D) a detail of the transition between the Fe-bearing layer and the Mn-rich one.

The analysis of the X-ray powder diffraction patterns reveals a heterogeneous spatial distribution of the mineral phases making up the weathering crust. Overall, in addition to the ubiquitous contribution of quartz, which can be assigned to the underlying siliceous ooids, the observed diffraction peaks from the collected patterns were assigned to birnessite – ideally $(Na,Ca,K)_{0.5}(Mn^{4+},Mn^{3+})O_4 \cdot 1.5H_2O$ – and, locally, hydroxyapatite and halite, whereas two samples clearly show also the occurrence of an amorphous phase. The identification of amorphous phases is compatible with the occurrence of mixtures of Mn and Fe oxides and hydroxides that are common in rock coatings, whereas birnessite is one of the most common crystalline phases in Mn-bearing rock varnish (Potter and Rossman 1979; Dorn 1998). The detection of hydroxyapatite is compatible with the occurrence of Ca and P, and has been described in rock varnish (Nriagu 1984; Dorn 1998).

3.4.2. CLSM observations

Representative images of the biofilm biomass and the extracellular organic matter (EOM) retrieved on the rock surfaces are presented in Fig. 3.13a and Fig. 3.13b respectively. The images correspond to the

Maximum Intensity Projection (MIP) 3D reconstructions obtained from confocal images series with the dedicated NIS-Elements software.

The fluorescent signals in Fig. 3.13a visualise cells in the biofilms. It is possible to observe a patchy distribution of small cell aggregates following the topography of the rock surfaces. Phototrophs (red signal) appear as few isolated coccoid cells on the rock surfaces, while chemotrophs (green + blue signals) dominate the biofilm community. The fraction of metabolically active cells in the biofilms is distinguished by coupling the results from DAPI staining (blue signal) with those obtained from Calcein AM staining (green signal) (Fig. 3.13a). In fact, while the DNA-binding dye DAPI shows the total biofilm biomass (active and non-active cells), the green fluorescent viability dye Calcein AM can measure intracellular esterase activity, which is an indicator of both cellular metabolic activity and cell membrane integrity. Thus, the green signal in Fig. 3.13a represents the fraction of active cells in the biofilm, which is about 30% of the total biomass. The fluorescent signals in Fig. 3.13b indicate the EOM retrieved on the rock surfaces. The green signal, derived from the lectin-binding dye Con A, reveals the presence of extracellular glycoconjugates (i.e. polysaccharides, including those ones covalently linked to proteins and/or lipids) covering the lithic surfaces. BODIPY® 630/650-X is an amine-reactive dye used to label the primary amines of proteins, amine-modified oligonucleotides, and other amine-containing molecules. The resulting red signal shows the presence of extracellular proteinaceous materials accumulating on the rock surfaces and partially overlapping the green signal of glycoconjugates.

Overall, the microbiological investigations reveal that: i) the lithic substrates are colonised by a monolayer of cells growing in small clusters that follow rock fissures and cracks; ii) chemotrophs comprise the largest proportion of the biofilm community in all the specimens analysed, and iii) only a small fraction (about 30%) of the biofilm community is metabolically active; moreover, iv) a thin layer of extracellular organic materials rich in glycoconjugates cover most of the rock surfaces.

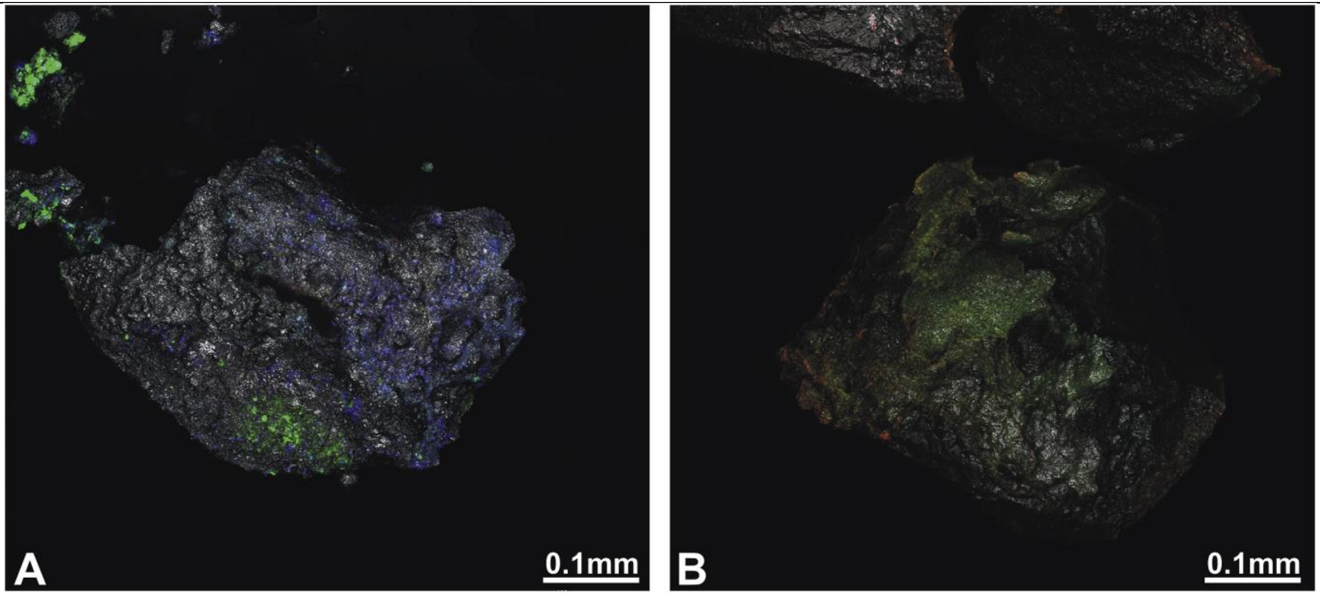

Figure 3.13. (A) Confocal laser scanning imaging of the biofilm; colours key: phototrophs, red (auto-fluorescence); metabolically active chemotrophs, green (Calcein AM); total chemotrophs, blue (DAPI); stone, grey (reflection). (B) Confocal laser scanning imaging of the extracellular organic matter retrieved on the rock surfaces; colours key: extracellular glycoconjugates, green (Con A); extracellular amine-containing molecules, red (BODIPY® 630/650-X); stone, grey (reflection).

3.4.3. Radiocarbon dating

Rock varnishes and rock crusts can be radiocarbon dated if they are biomineralized and if they still preserve organics trapped within weathering products (Dorn et al. 1989, 1992; Zerboni 2008). The latter generally act as a sealing, allowing the isolation of the carbon systems from external contaminations. A sample of crust collected on the boulder – although not in correspondence of engravings – displaying a TOC content of ca. 1.5 g/kg was submitted to AMS-^{14}C dating. The analyses rendered the age of 2600 ± 60 uncal. years BP (see calibration in Table 3.2). This age refers to the growth of the rock crust; thus it is a limit *ante quem* for the making of engravings.

Table 3.2. AMS-^{14}C dating results on rock crust; calibration according to Reimer et al. (2013).

Sample	Latitude	Longitude	TOC (g/Kg)	Laboratory code	δ^{13}C	pMC
Salut 1	N25°54′38.8″	E 12°14′48.9″	1.45–1.5	UGAMS 32708	−25.34	72.39 ± 0.52

AMS^{14}C age years uncal BP	AMS14C age cal. 95.4%		AMS14C age cal. 68.2%	
	years cal BC	years cal BP	years cal BC	years cal BP
2600 ± 60	901–541	2850–2490	836–590	2785–2539

3.5. Discussion

3.5.1. Engravings in the regional context

In recent years, a number of studies highlighted the rich rock art record of the Arabian Peninsula (e.g. Anati 1968-1974; Ash Shari 1994; Nayeem 2000; Crassard 2013; Jennings et al. 2013; Bednarik 2017; Guagnin et al. 2017), and in particular of the Sultanate of Oman (e.g. Clarke 1975a, 1975b; Preston 1976; Jackli 1980; Insall 1999; Yule 2001; Khan 2007; Al-Jahwari and ElMahi 2013; Fossati 2017; Tokunaga et al. 2019). Nevertheless, the engravings of human figures with halberd are almost unique in the region; at least, a few engraved items that can be interpreted as isolate halberds exist. In fact, no fitting comparison is known from South East or South Arabia. One similar engraving was found north of Timna in the Arabah Valley (Negev Desert), among the representations of a densely decorated panel with petroglyphs belonging to different ages (Rothenberg 2001). At this site, a “stick man” in the same outstretched position as those at Salut and holding a double-pointed-head item is present. This figure has some similarities with the one represented at Salut. However, the head of this item (tool or weapon) is less curved, and the man holds it from one end of the head and not from the shaft. Given the archaeological context of the famous mining area of the Arabah (e.g., Rothenberg 1972; Rothenberg et al. 1978), this tool could be reasonably interpreted as a pickaxe.

3.5.2. Formation of rock coatings

The microscopic, chemical, and mineralogical investigation on the rock surface of the boulder with engravings and the identification of an equivalent of the rock crust preserving engravings suggests the occurrence, along time, of many and contrasting weathering processes. Different climatic conditions triggered each weathering process and led to the formation of specific weathering products.

The dissolution of the rock surfaces and the formation – at the microscale – of alveolar etching pits are related to karst process. This requires moderate water availability, higher than the present day one. In fact, the effects of karst processes along Jabal Hammah seem quite significant and not compatible with the present hydroclimate. In fact, the so-called arid karst is controlled by intense evapotranspiration at the surface, low rate of CO_2 enrichment of solutions, and fast percolation of water, resulting in less intense surface karstification and more intense underground dissolution (Kranjc 2010). The greyish layer covering all subaerially exposed rock surfaces in the area of Jabal Hammah is a dust film (Dorn 1998), consisting of fine mineral particles originating from the aerosol and the deflation of surrounding soils aggraded on rock by the wind. Its accretion is related to arid environmental conditions and today its formation could be still active.

Finally, the crust grown along the engravings' furrows can be defined as a complex and multi-layered Fe- and Mn-bearing rock varnish. Its formation required several processes including microorganism-promoted biomineralization. The distribution of this weathering surface is more limited than the dust film, and it seems to be more common on the sheltered portions of siliceous limestone. This is compatible with other data suggesting that rock varnish growth occurs preferentially on silica-bearing rocks (Dorn 1998, 2007). At Salut, rock varnish consists, like many varnishes developed in warm deserts, of a thick accumulation of poorly layered and oxides-impregnated clay minerals. In our case, a large part of the rock varnish is impregnated by Fe-oxides, which represents the central part of the varnish, whereas its outer part mostly consists of the classical Mn-rich rock varnish. The association of iron films with manganese-rich accretions is common in arid and semiarid regions (e.g., Engel and Sharp 1958; Hooke et al. 1969; Hein and Koski 1987; Cremaschi 1996). Both iron and manganese oxides are likely the result of intense biomineralization occurred in presence of clays under environmental settings wetter than today, and interrupted by the onset of severe arid climate (Kiersch 1950; Dorn 1998, 2007). This is the same environmental context of the Mid-Late Holocene rock varnish formed in the Sahara (Cremaschi 1996; Zerboni 2008). Moreover, Krumbrein (1969, 1971) observed rock varnish on silicified limestone from the Negev Desert and, similarly to our case, dissolution created surface pores that were occupied by iron and manganese deposits.

The agent promoting the accumulation of rock varnish could not be unquestionably identified, but we think that a biological mediation occurred. For instance, the surrounded features in the Fe-rich layer are similar to the product of biomineralization (Dorn 1998). Moreover, a number of studies suggest that a plethora of microbial communities including bacteria, algae, fungi, and endolithic and epilithic lichens play a role in iron and manganese fixation on both limestone and siliceous bedrocks (Scheffer et al. 1963; Krumbein 1969, 1971; Ascaso et al. 1976; Friedmann 1982; Dorn 1998). Confocal microscopy images confirmed the presence of a metabolically active microbial community on rock surfaces. These findings also suggest the attribution of rock varnish to biomineralization through the activity of chemotrophs.

3.5.3. Age of rock art and weathering processes, and palaeoenvironmental implications

Once defined the main processes implied in the chemical weathering of rock surfaces and in the decay of engravings, we can try to delineate a tentative relative chronology of events, which can be improved considering the radiocarbon dating and the comparison with regional palaeoclimatic data.

The single radiocarbon age available refers to the formation of rock varnish. The attribution of the result of radiocarbon dating to rock varnish is suggested by the occurrence of phosphates, whose formation is related to biological processes, trapped within the Fe- and Mn-rich layers. As a consequence, the age of rock varnish formation represents a *terminus ante quem* for the age of engravings. The AMS-^{14}C result is also useful in reconstructing the palaeoenvironmental context of rock art.

The oldest process that occurred in the region is the dissolution of limestone, promoted by wet environmental conditions. In the area, most of the Holocene was wetter than today, and arid environmental conditions started in the Late Holocene (e.g., Fleitmann et al. 2007; Cremaschi et al. 2015) also contributing to the abandonment of Early Bronze Age sites and to the transition to a different settlement pattern during the Middle Bronze Age, around c. 2000 BCE (e.g., Magee 2014). The larger water availability recorded in the Early Bronze Age phase prompted the onset of intensive agriculture and the creation of complex water-management systems (Cremaschi et al. 2018a), and probably allowed the biomineralization of rock varnish in the alveolar voids produced by pristine dissolutions. Likely, the rock surface studied here was engraved during this period. In fact, the available radiocarbon date indicates this happened before 2600 ± 60 uncal. years BP, which means at least before the central part of the local Early Iron Age (overall commonly dated between 1300 and 300 BCE). This is compatible with the identification of a similar representation on an architectonic element of the Early Bronze Age tower T2 at Salut, although it only includes isolated halberds and no human figure (Degli Esposti 2015b).

On the contrary, the subsequent Middle and Late Bronze Age, as well as the Early Iron Age, are marked by a progressive reduction of rainfall (Fleitmann et al. 2007). During this arid period, marked by short-timed wet episodes, biological processes triggering biomineralization were interrupted. Archaeological evidence related to water management and excavation of deep wells confirms the reduction of environmental humidity at the beginning of this long period (Cremaschi et al. 2018a). Likely, short-timed resumption of rainfall occurring in the last three millennia of the Holocene (for instance, in Medieval times) conveyed sufficient water to occasionally reactivate karst dissolution and promote the levelling of the engravings' furrow. Fortunately, the presence of the weathering crust had a case-hardening effect on the underlying rock (*sensu* Kiersch 1950; Twidale 1982; Dorn 1998) preserving engravings up to today. Finally, in the last centuries of severe arid conditions, the wind accreted the thin dust film covering all rock surfaces.

Other means of dating the engravings are admittedly of little help. Comparative dating cannot be used as precise complete parallels are unknown, while the most common isolated halberds also lack a safe date. Stylistic issues seem to be a poor tool, given the overall naïve appearance of the wide majority of the rock art in the region (e.g., Ziolkowski 2007). Representations of weapons/tools or animals for which dated archaeological counterparts or the age of first domestication are known would be common benchmarks for dating: suffice it to mention the ubiquitous representations of camel-riders and the currently accepted date for the domestication of this animal in ancient Near East around 1000 BCE (Magee 2015). Unfortunately, there is no evidence in the archaeological record of the area of tools or weapons with a similar shape to the "halberds" depicted in the discussed engravings. The only possible candidate, a tool that actually recalls a small pick-axe, was first known from Mleiha in the Sharjah Emirate (Mouton 2008), and most recently several examples have been excavated in the Salut plain necropolis (Degli Esposti et al. 2019). The deriving chronology, however, fixed between mid-2nd and 1st century BCE, is strongly incoherent with the radiocarbon determination.

Fossati (2017) has recently proposed a tentative, broad phasing for the rock art of Oman (see also Fig. 3.2). There, "Dagger in T shape" are generally linked with 3rd and 2nd millennium BCE engravings. One anchoring point of this hypothesis is the affinity with Yemenite warrior stele, where similar signs actually represent daggers (e.g., Newton and Zarins 2000). It is clear from the case discussed here, as well as from the Arabah Valley comparison mentioned above, that these T signs cannot be univocally interpreted when found in isolation, and in the present case they surely do not represent daggers. A broad date into the 2nd millennium BCE would, nevertheless, be consistent with the other evidence discussed here.

Fossati's (2017) 6th phase, comprising the long period from the 1st millennium BCE to the present, is distinguished by the widespread representation of warriors (often mounted). If the halberds depicted in the Salut boulder indeed are weapons (nothing precludes different interpretations, such as ceremonial items), their date could thus fit in at the very beginning of this long phase, and possibly suggest an earlier start for it.

3.6. Conclusion

In this paper, we describe a previously unknown iconography in the rock art of the Sultanate of Oman – the man with the halberd –, which does not fit any comparison with the engravings and paintings known in the region, although representations of isolated halberds (also called "Capital T" signs) are, conversely, rather well known (e.g., Ziolkowski 2007; Fossati 2017), and are also found scattered over the same oasis of Salut.

We suggest that lithological and environmental reasons are in charge of the scarcity of coeval engravings. The formation of rock varnish is the factor that triggered the preservation of engravings on

Jabal Hammah. This process was promoted by the lithology of the boulder – limestone with silicified ooids – as Si-bearing rocks favour the growth of rock varnish. At Jabal Hammah rock varnish was not found on non-silicified limestone surfaces, which are, therefore, more prone to karst dissolution. In such a case, engravings may have been erased. In the area, the few other representations of isolated halberds were found in more protected contexts, as in the case of the engravings on the architectonic element of the tower T2. There, the block in the wall was buried after the abandonment of the site and, therefore, the rock surface and engravings did not undergo weathering and dissolution. At Jabal Hammah, the formation of a biomineralized crust preserved engravings from complete erosion triggered by karst dissolution. Many of the blocks and boulders of the area of Salut, however, display strong evidence of this process that might have erased other engravings.

In the case of the studied boulder, the interplay of contrasting chemical and biochemical processes (conservative vs. destructive processes) permitted the preservation of engravings, gave the opportunity to radiocarbon date them, and offered fresh data to reconstruct the complex archaeological landscape of the palaeo-oasis of Salut. Our work also confirms (i) the importance of weathering surfaces as proxy data of past environmental conditions (Dorn 2007; Cremaschi et al. 2018b), and (ii) the great potential that combined geoarchaeological investigation on rock art and its rock support (Cremaschi 1996; Cremaschi et al. 2008; Zerboni 2012) has in paleoenvironmental reconstruction.

In a broader perspective, our results suggest that in some cases the absence of rock art evidence is not evidence of rock art absence. Destructive natural processes may have acted dismantling or rejuvenating rock surfaces, thus erasing engravings or paintings. Other case studies confirm this general assumption. For instance, in the hyperarid central Sahara, where prehistoric rock art panels and galleries dating to wetter periods of the Holocene are common, some rock shelters settled in prehistoric times do not present any rock art representations, as in the case of the Takarkori Cave (Cremaschi et al. 2014). This is the consequence of climate-driven processes that contributed to the continuous abrasion and dismantling of the friable sandstone surface of the vault of the rock shelter and to the loss of rock art, whose existence is suggested by archaeological evidence discovered in the anthropogenic infilling of the cave (di Lernia et al. 2016). Future regional rock art studies shall seriously take into account this conclusion.

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Chapter 4

Geomicrobial Investigations of Colored Outer Coatings from an Ethiopian Rock Art Gallery[3]

Abstract

The open rock shelter of Yabelo in Ethiopia hosts diverse Holocene paintings of great cultural importance. The paintings are characterized by the presence of different mineral coatings, whose features have not been studied yet. Our goal was to understand whether different rock samples from the Yabelo paintings collected in close proximity may reveal coatings with different minerology and biology. Thus, elemental analyses combined with microscopic and molecular investigations were performed on two coatings, one whitish (sample 1) and one reddish (sample 2). Although both samples were dominated by heterotrophic bacteria, the two coatings showed distinct mineralogical and microbiological characteristics. Sample 1 contained higher amounts of Ca and P than sample 2, which was likely related to the presence of organic matter. Sample 1 hosted bacterial genera that are potentially involved in biomineralization processes, metal redox cycles and metal resistance. In contrast, sample 2 showed mainly pathogenic and commensal bacteria that are characteristic of animal and human microbiota, and other microorganisms that are involved in nitrogen and metal biogeochemical cycles. Overall, our results indicated that the bacterial communities were particular to the coating mineralogy, suggesting a potential role of the biological components in the crust genesis.

Keywords: bacterial community; rock art; rock coating; SEM-EDS; subaerial biofilms; CLSM; 16S rRNA gene sequencing

4.1. Introduction

Rock art is a fragile cultural heritage that requires conservation and proper methodological approaches to understand its state of preservation (Whitley 2011; Darvill and Batarda Fernandes 2014). In fact, rock art—both in forms of paintings and petroglyphs—is subject to physical, biological and geochemical changes in the natural environment (Dorn 2013). Mineral coatings covering large portions of the paintings and engravings are all too familiar to anyone who has looked closely at a rock art. Examples of these coatings are natural formations of calcium oxalate crusts (Hernanz et al. 2014; Roberts et al. 2015), calcium carbonate layers (Rousaki et al. 2018; Mauran et al. 2019), manganese-iron oxide deposits (Šebela et al. 2015) and other mineral accretions (Green et al. 2017) that may overlap and hide motifs and symbols of ancient rock paintings. Although the study of rock coatings has important implications in several fields, mineral depositions on rock art have been scarcely investigated, and their mechanisms of formation are still under investigation. Furthermore, limited information is available regarding the composition of the epilithic bacterial communities inhabiting the mineral coatings associated with prehistoric paintings, although the geoactive role of microorganisms has been well established (Gadd 2010; Villa and Cappitelli 2019). Complex microbial communities are

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3. Wu, Y.-L., Villa, F., Mugnai, G., Gallinaro, M., Spinapolice, E. E., Zerboni, A., 2020. Geomicrobial Investigations of Colored Outer Coatings from an Ethiopian Rock Art Gallery. *Coatings 10* (6). https://doi.org/10.3390/coatings10060536.

embedded in a self-produced extracellular polymeric matrix (EPM) growth in the form of biofilms (Villa and Cappitelli 2019). Biofilms can participate in rock varnish genesis (Esposito et al. 2015; Kuhlman et al. 2006), mineral formations in calcitic cave speleothems (Dhami et al. 2018), sulfate deposit formation (Bostick 2019) and acid-mine drainage deposits (Meier et al. 2012).

Dorn (Hernanz et al. 2014) suggested that to understand the weathering and stabilization of rock art, it is important to study rock coatings; however, their contribution to the biodeterioration or bioprotection of rock art is still controversial. For instance, rock varnish in arid lands is a type of rock weathering that contributes to surface stabilization, thus hampering the effects of physical disaggregation (Dorn 2009; Dorn 1998; Zerboni 2008).

The Yabelo area (Borana Zone, Oromia, southern Ethiopia) hosts diverse Holocene paintings of outstanding cultural importance. For many years these prehistoric paintings have been largely ignored by scholars, local communities and local authorities in charge of cultural heritage management. The paintings and rock surface are severely affected by deterioration processes causing loss of pigments, exfoliation, and the formation of different coatings as well as severe cracks (Gallinaro et al. 2018). Paintings and engravings are widespread, both in rock shelters and not-sheltered rock walls (open-air sites), often located in remote places that are currently inhabited by communities of different ethnic groups. The rock art in East Africa has been mainly studied focusing on two major aspects, namely chronology and stylistic interpretations. Only recently did scholars propose to study this rock art in a multidisciplinary approach involving archaeology and ethnography to help reconstruct the past, especially the emergence of pastoralism in this region (Gallinaro et al. 2018).

Besides the archaeological context of African rock art, the characterization of the mineral coatings associated with the prehistoric paintings is needed to assess their putative role in the preservation or deterioration of the raw materials and pigments used. Furthermore, there are many open questions regarding the biological and mineralogical features of different colored outer coatings from the same bulk rock.

Our hypothesis is that coating types associated with the Yabelo paintings may show different chemical and biological compositions, despite being exposed to the same environment. In fact, the host rock provides the same moisture sources with the same chemical content to the rock coatings. Since the parental rock and the environmental exposure are the same, the contribution of microbial activities to the coatings’ genesis should be considered. Thus, our goal was to understand whether different rock samples from the Yabelo paintings collected in close proximity may reveal coatings with different minerology and biology.

To this end, an integrated approach involving high-throughput sequencing and rock surface analyses was applied to investigate the bacterial diversity of two different pigmented areas close to the prehistoric paintings belonging to the same rock shelter. By sampling two different colored coatings from the same bulk rock, we were able to investigate how different patinas in close proximity may host different microbiota and, consequently, offer different biogeochemical processes. This research provides a novel combination of detailed microscopic images of the biofilm on the mineral surface coupled with analysis of chemical elements and DNA sequencing of microbial communities, which will be a new way of comprehending rock art preservation. This research is, to our knowledge, the first microbial investigation on African rock art.

4.2. Materials and Methods

4.2.1. Site Description and Sampling

The regional focus of this study is in East Africa, where recent archaeological investigation in southern Ethiopia confirmed that the rock art of the Yabelo region is of great cultural significance and requires further research as well as preservation (Gallinaro et al. 2018; Spinapolice et al. 2017). The rock shelter YAB6 named by Hundie (Hundie 2001) opens along the cliff of a hill consisting of poorly to moderately metamorphosed granite. The hill is approximately 2 km northwest of the town Yabelo and referred to locally as Dhaka Kura (Crow’s Rock) (Fig. 4.1a). Paintings were identified, including cattle, wild animals and geometric shapes (Fig. 4.1b). The pigments are vanishing, and the rock surface suffers exfoliation, as well as the formation of red and white crusts or patinas. Direct anthropic damage was not detected, but local herders used and still use the rock shelter for grazing (Gallinaro et al. 2018).

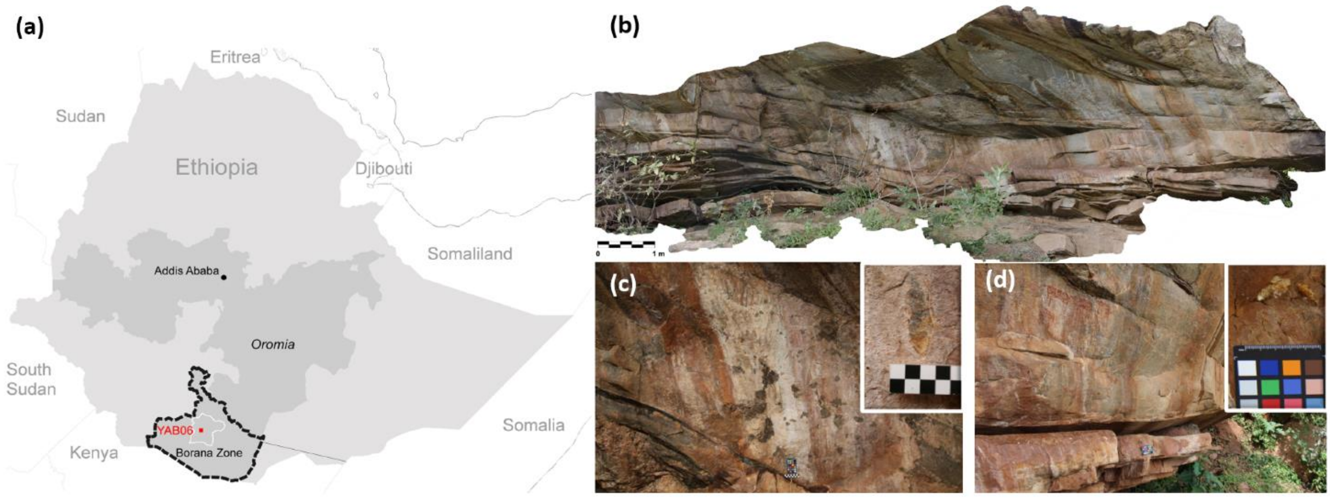

Figure 4.1. (a) Position of the Yabelo site; (b) overview of the rock art gallery of YAB6; (c) sample 1 was taken from white colored coatings close to the middle of the gallery; (d) sample 2 was taken from red colored coatings at the lower right.

Several samples were taken from the shelter with chisels to investigate the many categories of rock weathering, crusts/patinas formation and pigments. Among them, we selected two samples representative of the two most common coatings present on the rock wall at YAB6, and from the most problematic panels in terms of conservation. These panels also present a high concentration of paintings; for the sake of heritage conservation, we collected samples at the margin of panels, far from paintings. The results of multiple analyses on two of these samples are reported here: sample 1 was a piece of exfoliating rock close to the bottom of the rock art panel with whitish patina overrunning the surface (Fig. 4.1c); sample 2 was taken from the lower right with red patina on the surface (Fig. 4.1d). Samples were collected by using sterile tools (mostly small chisels and lancets) and stored in sterile containers. Samples for DNA investigations were stored in sterile falcon tubes containing the stabilization solution DNA/RNA Shield (Zymo Research) to preserves the genetic integrity at ambient temperatures.

4.2.2. Microscopic Characterization of the Rock Substrate and Mineral Coating

Samples were cut perpendicularly to the surface and polished into thin sections mounted on glass slides according to the protocol described in (Zerboni 2008), in order to investigate the effects of weathering. Micromorphology of weathering of thin sections (Cremaschi et al. 2018) was examined with an optical petrographic microscope (OPTIKA B-1000 POL, Ponteranica, Bergamo, Italy) under plane-polarized light (PPL) and cross-polarized light (XPL). Afterwards the thin sections were carbon coated and observed with a Scanning Electron Microscope (SEM, JEOL JSM-IT500) (JEOL Ltd., Tokyo, Japan) coupled with energy dispersive X-ray spectrometry (EDS) for chemical characterization with accelerating voltage 20 kV. All measurement data normalized to 100 weight % oxide.

4.2.3. Visualization of Biofilm Structure

Surface crusts from the rock samples were taken by using adhesive tapes (Fungi-tape, Fisher scientific) and observed with a Confocal Laser Scanning Microscope (CLSM) (Nikon, Amsterdam, Netherlands) in both fluorescence and reflection modes. Confocal images were collected using a Nikon A1 laser scanning confocal microscope and a 20×/0.75 numerical aperture Plan Apo λ (Nikon, Amsterdam, Netherlands) objective with a working distance of 1 mm. Captured images were analyzed with the software NIS-Elements by Nikon (Nikon, Amsterdam, Netherlands) for the 3D reconstructions of the biofilms. The reflection mode (excitation 488 nm) records the mineral surface of the lithic sample. Autofluorescence from phototrophic pigments was assigned to the blue channel and collected using the 633 nm laser line in the emission range of 663–738 nm. Biofilm components were visualized after fluorescent staining. SYTO® 9 (excitation 485 nm/498 nm, BacLight™ ThermoFisher, Milan, Italy) labelled nucleic acids and emitted green fluorescence. For its acquisition the 488 nm laser line and the 500–550 nm emission filter were used. Polysaccharide components of the EPM were labelled with Concanavalin-A-Texas red (excitation 561 nm/emission 570-620 nm).

4.2.4. DNA Extraction and Sequencing

Rock samples, conserved in the stabilization solution DNA/RNA Shield (Zymo Research), were processed a few days after their collection. Total genomic DNA were extracted according to the manufacturer's instructions of PowerBiofilm DNA Isolation Kit (MoBio Laboratories Inc., Carlsbad, CA, USA). High-throughput sequencing analysis of the V3–V4 region of the bacterial 16S rRNA gene (primers CS1_341F/CS2_806R) was performed by using a MiSeq platform (Illumina) with v3 chemistry providing 2 x 300 paired-end reads (Rapin et al. 2017). Raw data were pre-processed, quality filtered, trimmed, de-noised, paired, and modelled via QIIME2 (Bolyen et al. 2019) and DADA2. Chimeras were detected using DADA2 according to the "consensus" method (Callahan et al. 2016). Sequences were clustered into Amplicon Sequences Variants (ASV). ASVs were assigned using a Naïve-Bayes classifier trained on the SILVA database (Quast et al. 2013). Multi packages of R software (R Core Team, Version 3.3.0, Vienna, Austria) (R Core Team 2013) were used to calculate the alpha diversity, to generate the rarefaction curves and the Venn diagram. Samples were rarefied at the minimum library size of 92,380. The rarefaction curves were drawn using the function "rarefaction" (Jacobs 2012) from the vegan R-package (Oksanen et al. 2007).

4.3. Results

4.3.1. Optical Microscopy and SEM-EDS Analysis

Thin section of sample 1 under PPL showed a dark coating growing along the lithic surface and extending into cracks of the rock substrate with reddish organic substances (Fig. 4.2a). Under higher magnification (in PPL and XPL) the coatings appeared brownish, and in XPL there were some small bright (birefringent) crystals (Fig. 4.2c,d). SEM images in back scattering electron mode from sample 1 revealed a continuous layer of mineral coating that was 10-20 μm thick and exceeded 30 μm in certain locations. The coating grew on the silicate-bearing surface of the substrate and it was more developed for instance in correspondence with an iron oxides inclusion (Fig. 4.3a). SEM-EDS analysis suggested the coating is mainly quartz and clay minerals, being enriched in Si, Al, Mg, and K (Table 4.1). In the thicker coating that grew inside the cavity of the iron-rich inclusion (Figure 4.3a), the amount of P_2O_5 (25%-33%) and CaO (33%-41%) increased from the bottom of the cavity towards the opening, but at the surface they were reduced (P_2O_5 7.73%, CaO 13.43%) and replaced by higher SiO_2. The amount of FeO in this cavity remains in the range of 1%-6% (Table 4.1). At another location (Fig. 4.3b), the surface coatings contained higher amounts of P_2O_5 (6%-16%), CaO (9%-19%) and FeO (4%-13%) than the underlying mineral that is mostly SiO_2 (52%), Al_2O_3 (15%) and K_2O (11%). Other examples of thick coatings (Fig. 4.3c) that contain high amounts of P_2O_5 (25.11%, 14.64%) and CaO (31.97%, 13.75%) are shown in Table 4.1. Such parts of the coatings have more complex layers including a layer of clay minerals and a layer of iron and calcium phosphates.

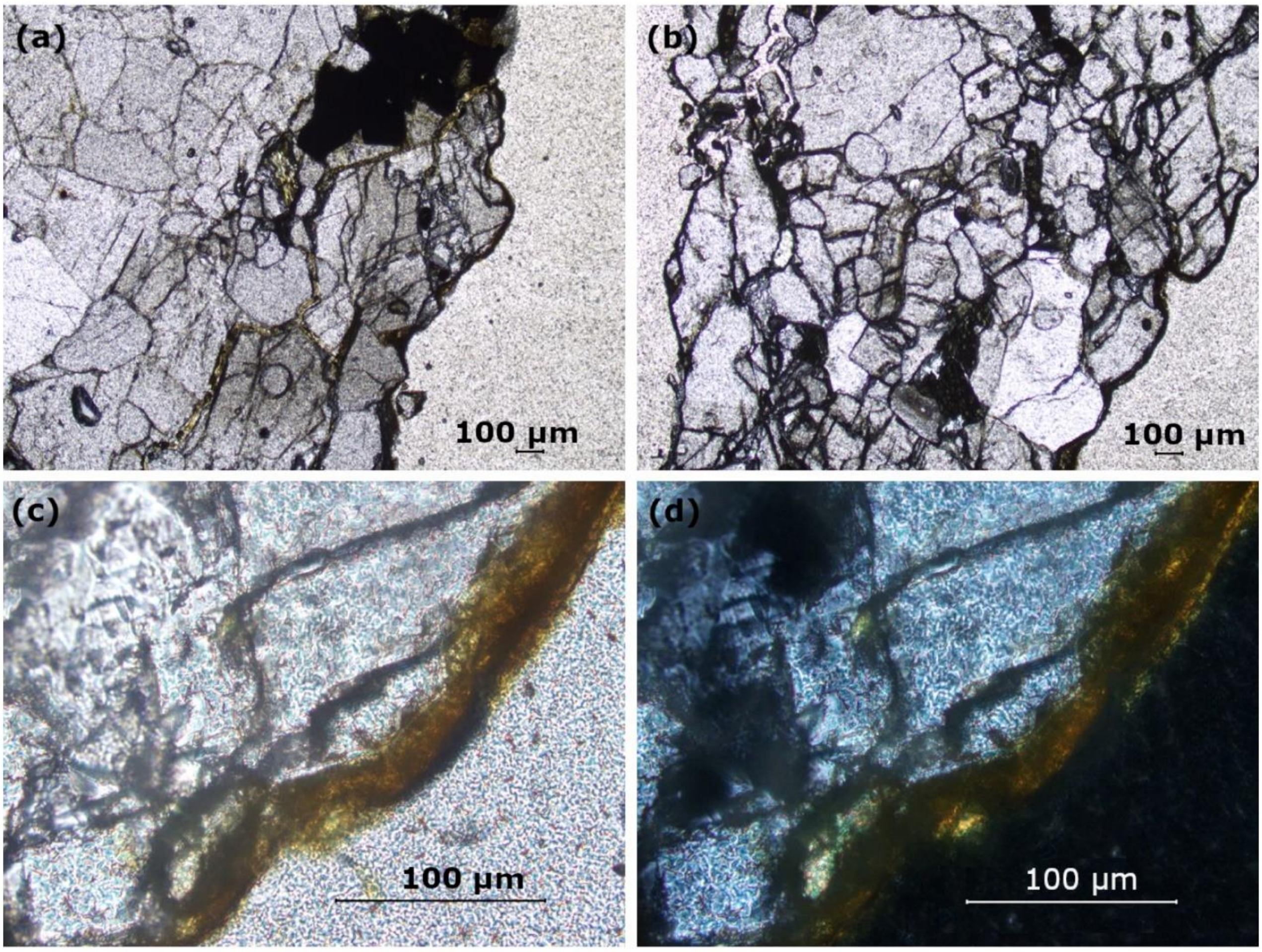

Figure 4.2. (a) Plane-polarized light (PPL) microscopic image showing black coating growing on the lithic surface of sample 1 and extending into cracks, the iron inclusion is black; (b) PPL microscopic image showing black coating growing on the lithic surface of sample 2; (c) higher magnification of the coating from sample 1 in PPL and (d) in cross-polarized light (XPL).

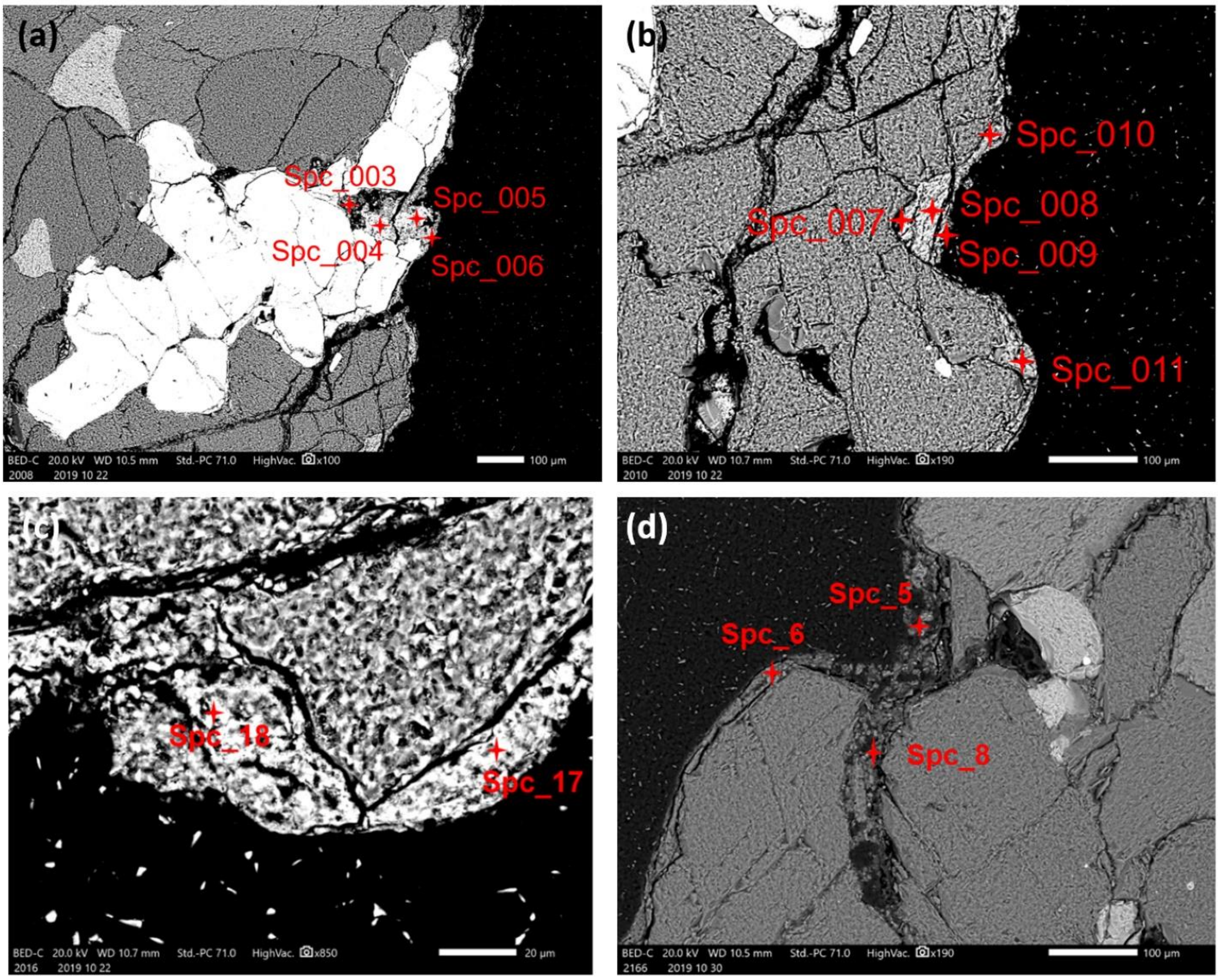

Figure 4.3. (a) Backscattered electron images in SEM showing the iron inclusion in rock sample 1 as white reveal a continuous inorganic coating; (b) site indications of EDS analyses of coatings on sample 1; (c) site indications of EDS analyses of coatings on sample 1; (d) backscattered electron images in SEM of sample 2 with inorganic coating and sites of EDS analyses.

Table 4.1. EDS analyses of coatings from sample 1 and 2. All measurement data normalized to 100 weight % oxides, not all elements are shown. n.d. = not detected.

		SiO_2	**Al_2O_3**	**FeO**	**CaO**	**MgO**	**P_2O_5**	**SO_3**	**K_2O**
Sample 1	Spc_003	3.45	1.01	1.21	33.93	5.03	25.47	1.53	0.84
Sample 1	Spc_004	5.22	0.77	6.60	36.99	2.72	29.57	0.76	0.69
Sample 1	Spc_005	3.44	0.55	1.97	41.13	2.00	33.62	0.75	n.d.
Sample 1	Spc_006	23.44	9.58	5.22	13.43	4.26	7.73	0.46	1.52
Sample 1	Spc_007	52.20	15.08	n.d.	n.d.	n.d.	n.d.	n.d.	11.45
Sample 1	Spc_008	52.64	15.28	n.d.	n.d.	n.d.	n.d.	n.d.	11.51
Sample 1	Spc_009	14.37	6.56	4.37	19.99	4.57	16.00	1.05	1.08
Sample 1	Spc_010	28.35	5.76	13.18	9.33	4.22	7.64	0.53	2.33
Sample 1	Spc_011	28.95	12.66	6.25	10.04	7.13	6.26	0.59	2.86
Sample 1	Spc_17	13.19	5.68	2.11	31.97	3.92	25.11	0.56	0.89
Sample 1	Spc_18	27.01	14.85	4.44	13.75	n.d.	14.64	n.d.	2.05
Sample 2	Spc_5	38.77	14.05	7.42	2.39	2.34	0.98	0.32	2.31
Sample 2	Spc_6	21.45	9.94	5.44	15.97	2.61	5.54	0.51	1.92
Sample 2	Spc_8	19.55	16.64	2.00	10.78	n.d.	2.29	n.d.	0.92

4.3.2. Biofilm Characterization

Fig. 4.4 showed the reconstructed 3D biofilm images from confocal images with the software NIS-Elements by Nikon. Sample 1 had coccoid aggregates of photosynthetic bacteria dispersed sporadically on the higher part of the sample, and smaller clusters of chemotrophs colonizing the whole rock surface evenly. Spaces between the phototrophs and chemotrophs appeared to be covered in a monolayer of extracellular polysaccharides. Together these components constituted a dynamic biofilm that covered the whole surface of sample 1, with phototrophic and chemotrophic communities embedded in a continuous layer of polysaccharide matrix.

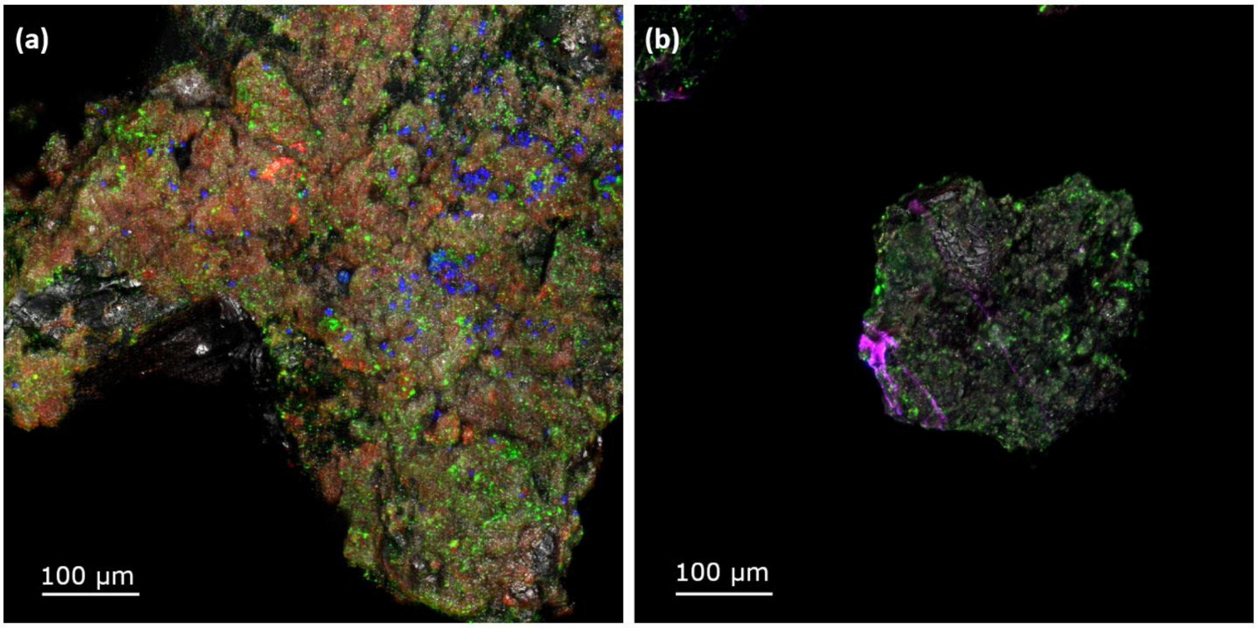

Figure 4.4. Confocal microscope images of biofilms on (a) Sample 1 and (b) Sample 2. Color key: phototrophs, blue (autofluorescence); chemotrophs, green (SYTO 9); polysaccharides, red (Con A); stone, gray (reflection).

In comparison, sample 2 had a less dense biofilm coverage. Congregates of chemotrophs differing in size grew on most of the lithic surface, concentrating on the ridges and exempting voids or smooth mineral surfaces. Faint traces of polysaccharides could be seen through some of the chemotrophs. In general, neither phototrophs nor fungal structures were observed on sample 2. As a result, sample 1 presented a more diverse biofilm with higher phototrophs, chemotrophs and the EPM than sample 2.

4.3.3. Bacterial Communities

The 16S rRNA data set consisted of 186,517 reads clustered in 173 ASVs. The rarefaction curves showed that sequencing depth was sufficient for a thorough description of the bacterial communities (Fig. 4.5a). According to the Venn diagram only 2 ASVs were shared between the two samples, where 139 species were unique to sample 1 and 32 species were unique to sample 2, showing differences between the bacterial communities (Fig. 4.5b). Two species that were common for both samples are: *Curvibacter* and *Undibacterium*, belonged to the *Burkholderiaceae* family. Alpha-diversity indices indicated that the diversity level of bacterial community was higher in sample 1 than in sample 2 (Table 2).

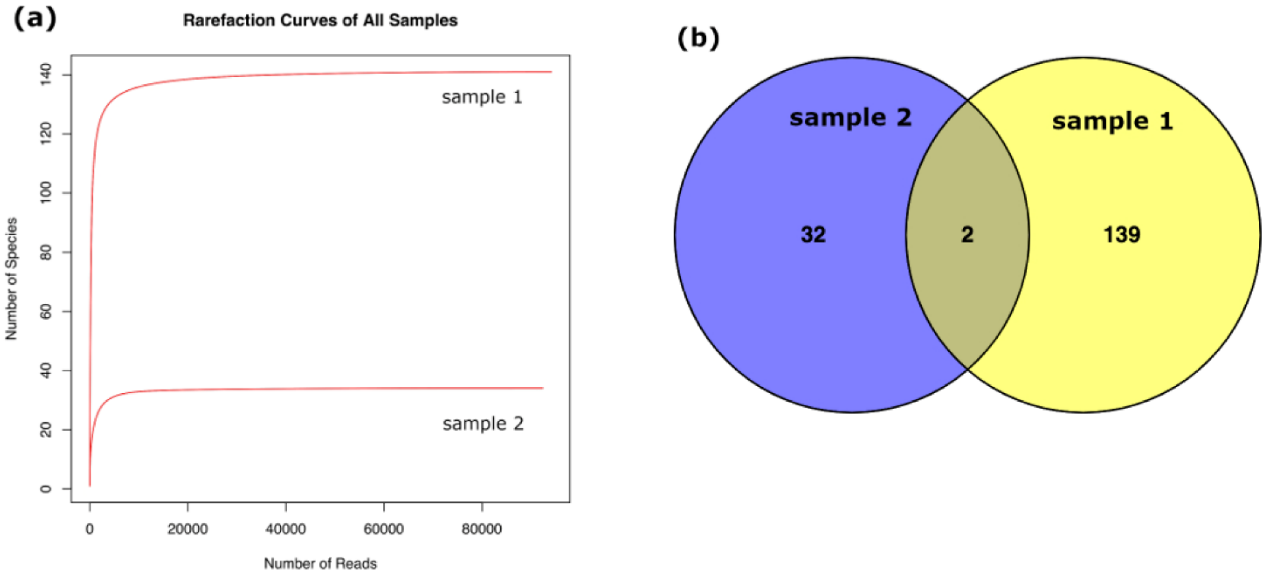

Figure 4.5. (a) Rarefaction curves for bacterial communities in sample 1 and sample 2; (b) Venn diagram drawn from number of amplicon sequences variants (ASVs) representative of sample 1 and sample 2.

Table 4.2. Bacterial community richness and alpha-diversity indices.

Samples	**Reads**	**Richness**	**Eveness**	**Shannon**	**Simpson**
Sample 1	94137	141	0.60	4.26	0.02
Sample 2	92380	34	0.33	1.70	0.27

Ten bacterial phyla, 18 classes, 36 orders, 56 families and 59 genera were detected overall (Fig. 4.6). Sample 1 was dominated by Firmicutes (77.17%) and Proteobacteria (8.75%), with notable percentages of Actinobacteria (8.06%), Cyanobacteria (2.94%) and Acidobacteria (1.76%). At genus level, the core microbiome of sample 1 was mainly composed of *Bacillus* (Firmicutes, Bacilli, Bacillales, Bacillaceae, 21.58%), *Streptococcus* (Firmicutes, Bacilli, Lactobacillales, Streptococcaceae, 20.81%), *Staphylococcus* (Firmicutes, Bacilli, Bacillales, Staphylococcaceae, 17.34%) and *Paenibacillus* (Firmicutes, Bacilli, Bacillales, Paenibacillaceae, 6.48%). Sample 2 consisted mainly of Firmicutes (66.77%) and Proteobacteria (33.07%), with the genera *Facklamia* (Firmicutes, Bacilli, Lactobacillales, Aerococcaceae, 48.05%), *Comamonas* (Proteobacteria, Betaproteobacteria, Burkholderiales, Comamonadaceae, 29.06%) and *Atopostipes* (Firmicutes, Bacilli, Lactobacillales, Carnobacteriaceae, 11.43%) the most abundant taxa.

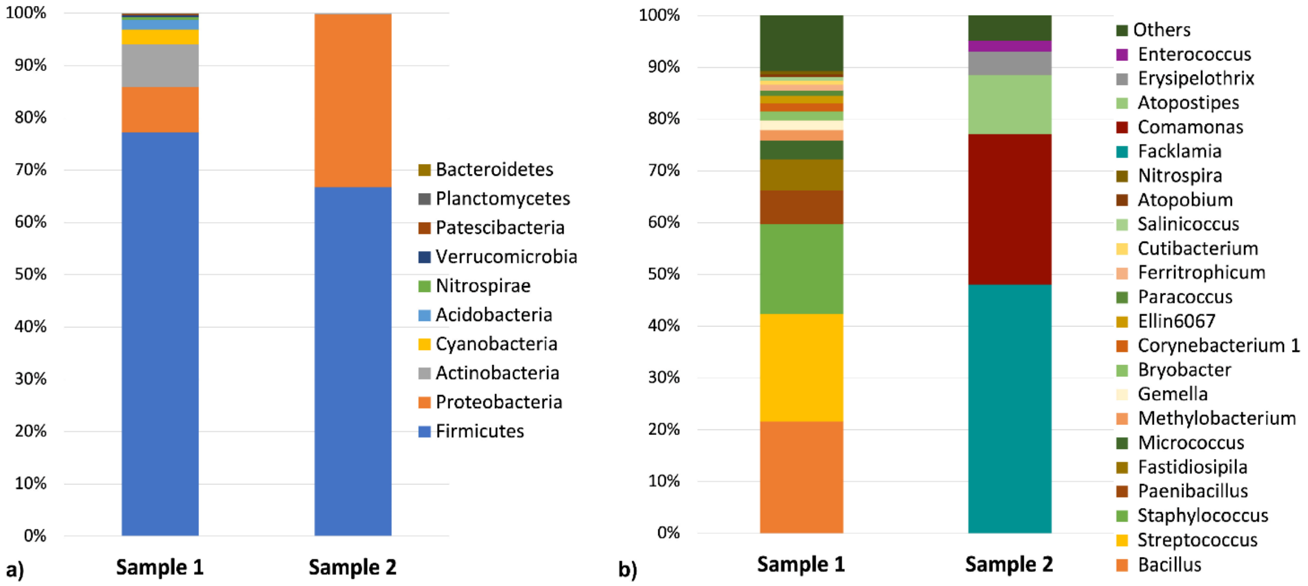

Figure 4.6. Metagenomic data of rock samples to (a) Phylum level; (b) Genus level with a relative abundance of higher than 1% in the samples.

After bibliographic research, the ASVs were classified according to eight main categories reported in Fig. 4.7. The reads allocated to each category were presented as a heatmap to show the main features of the biofilm communities in samples 1 and 2. Inferred physiology showed the predominance of bacteria involved in mineral precipitation, metal redox cycle and metal resistance in sample 1, while microorganisms derived from animal and human sources and those involved in the biochemical cycle of elements, such as metal and nitrogen, were the signature of sample 2.

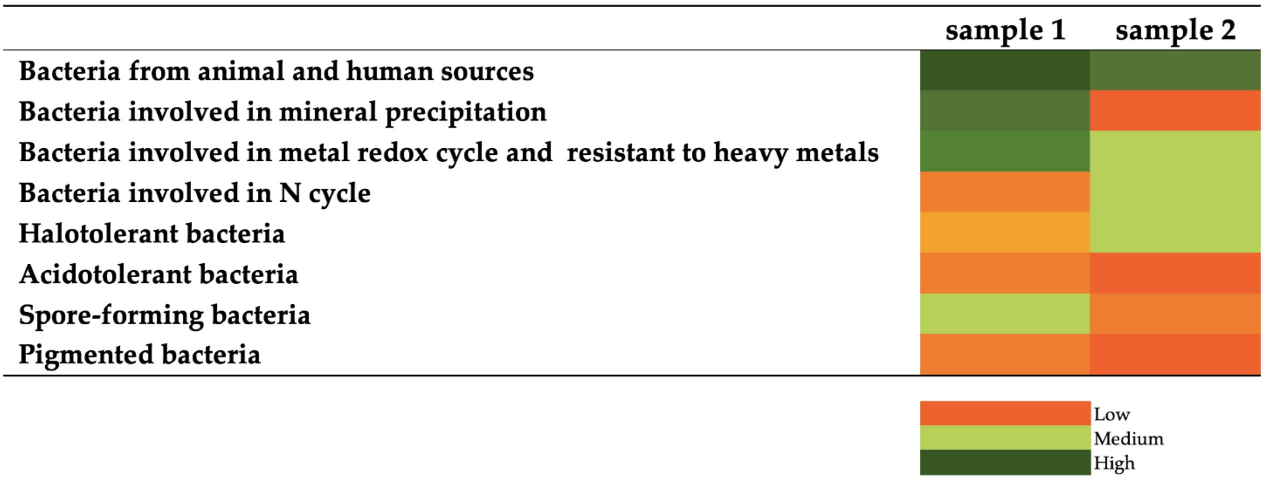

Figure 4.7. Heatmap representing the main features of the biofilm communities inhabiting the coating samples.

4.4. Discussion

There is an abundance of literature that highlights the cultural significance of rock art as well as the attempts to date paintings and petroglyphs in many parts of the world (David and McNiven 2018;

Bonneau et al. 2017). However, rock art is one of the most fragile forms of ancient heritage and its preservation is understudied. In particular, the nature and the role of mineral coatings covering rock paintings are poorly investigated. For instance, Zerboni et al. (2019), discussed the preservation and dating of petroglyphs in the Sultanate of Oman, where a dark crust that preserved the grooves could be related to biological deposition. In Australia, Chalmin et al. (2017) performed a geochemical analysis on microlayers of rock art surface, finding that the weathered rock layer contained Si, Al, K, P and Fe. Green et al. (2017) observed four types of mineral depositions in Australian rock art sites, and identified sulfate, phosphate and calcium minerals. Krinsley et al. (2012; 2017) used scanning electron microscope (SEM) and chemical characterization on Mn-rich biologically formed rock varnish in fast accreting moist environments. Lang-Yona et al. (2018) performed a metagenomic analysis on desert varnish and discussed the indirect contribution of microorganisms to the varnish formation. However, outside the domain of rock varnish, there is a substantial lack of knowledge concerning the role of microorganisms in rock coatings development. Furthermore, little is known about whether different rock samples collected in close proximity on the same bulk rock may accommodate coatings with different minerology and biology. To answer this question, comprehensive research that combines the geochemical characterization and the biofilm community investigation of these colored coatings retrieved from the rock art is necessary.

The SEM investigation revealed that rock coatings consists of layers of clay minerals, calcium-bearing minerals, phosphates- and iron-rich oxides-hydroxides. The formation of some of these mineral coatings can be related to some biological processes. Calcium-rich minerals in the form of oxalates are common secondary minerals formed by microbial mobilization, while iron-rich minerals have also been reported as a biological precipitation (Gorbushina 2007). Calcium- and phosphate-rich coatings were reported on desert pavement cobbles (Dorn 1998; Zerboni 2008) as a product of microbial activity or endolithobionts decay, and iron phosphate coatings have been found in stone coatings, where the iron is possibly from a bacterial precipitation (Dorn 1998; Konhauser et al. 1994). Both scanning electron and optical microscopies showed that the coatings in sample 1 were thicker than sample 2. Moreover, chemical analyses of the coatings showed that sample 1 had higher amounts of Ca, P, and Fe than sample 2. The analysis demonstrated that coating types with different chemical compositions can still occur despite their close proximity to each other. Iron films and sulfate crusts have been found within a couple of meters from one another, a well-delineated phenomenon in the Yosemite Valley and in Kärkevagge, Swedish Lapland (Larson and Dorn 2012; Marnocha and Dixon 2014b). Since the different coatings are presumably exposed to the same physical and chemical macro environment, a possible biological origin of the colored coatings is considered.

To this end, the bacterial communities associated with two colored coatings from the Yabelo painting collected in close proximity were studied. From the confocal images, the biofilms of sample 1 were shown to be more developed and complex than those of sample 2. Overall, the taxonomic profiles detected in both samples were dominated by Firmicutes. In contrast, previous studies investigating the microbial communities associated with Palaeolithic art in caves reported an abundance of Proteobacteria, Actinobacteria, Acidobacteria and Cyanobacteria in the patinas (Schabereiter-Gurtner et al. 2004; Nir et al. 2019a, 2019b). At the phylum level, our findings are in line with those of Roldán et al. (2018), proving the similarity of the communities of our samples to the bacterial communities found in the prehistoric Spanish Levantine rock art and Atlanterra and La Graja shelters in Southern Spain (Gonzalez et al. 1999; Laiz et al. 2000).

The 16S rRNA gene sequencing analysis clearly indicated that the bacterial communities are particular to the coating mineralogy at genus level. The only two species shared by the samples are *Curvibacter*, which is involved in the precipitation of calcium carbonate (Zhang et al. 2017), and *Undibacterium*, which is responsible for carbonate dissolution and Fe-oxyhydroxides (Kakoti et al. 2020). A common feature between the two coating samples is the predominance of heterotrophic bacteria, which suggests that the most common energy-acquiring mechanism in the rock art system under investigation is relying on the organic compounds for nourishment. The dominance of heterotrophs may be derived from the organic materials generated by the human activities at the rock shelter and animal herding (Gallinaro et al. 2018). Despite the dominance of heterotrophs, the bacterial community in sample 1 also includes phototrophs and chemolithoautotrophs that use light and inorganic compounds to grow, respectively. The cyanobacteria identified in the sample 1 belongs to the class of Melainabacteria that is prevalent in the gut of herbivorous mammals and humans with a predominantly vegetarian diet (Di Rienzi et al. 2013). The microaerobic and chemolithoautotrophic bacterium *Ferritrophicum* plays an important role in Fe cycling in many terrestrial and freshwater environments (Weiss et al. 2007).

From the taxonomic assignment, the main futures of the community can be inferred, and the associations between the mineralizing metabolisms and the rock coating mineralogy can be obtained. Although the dominance of biomineralizing bacteria does not indicate whether these microorganisms are actively involved in the coating's origin, there is a clear relationship between the coating mineralogy and the bacterial community structures. In fact, the biofilm inhabiting the coating of sample 1 is mainly characterized by bacteria involved in mineral precipitation, metal redox cycle and metal resistance. Among the ASVs in sample 1, the dominant genera are *Bacillus*, *Streptococcus*, and *Staphylococcus*. *Bacillus* was associated with carbonate precipitation of speleothem formations in

Tjuv-Ante's Cave, Northern Sweden (Mendoza et al. 2016). *Bacillus* strains were also involved in the biofilm formation and calcite binding because of their EPM production capability (Perry et al. 2005). *Bacillus* is also the most important calcium-carbonate biomineralization bacteria that has an industrial application ranging from the CO_2 sequestration to reduce atmospheric CO_2, to the rescue of historical buildings. Bacterial biomineralization has been reported by Konhauser and Riding (Konhauser and Riding 2012) and the ability of *Bacillus* to precipitate calcium carbonates was studied for its impact on built heritage, for instance on rock monument surfaces (Urzí et al. 1999), and even tested as a potential bioconsolidating agent for monumental stones (Jroundi et al. 2017; Perito et al. 2014; Soffritti et al. 2019). Furthermore, Gonzalez et al. (Gonzalez et al. 1999) isolated a number of iron reducing *Bacillus* strains from rock paintings based on the red iron oxides in a sandstone shelter in Spain. Fishman et al. (Fishman et al. 2018) reported that the calcium-phosphate-based mineral apatite is created via the biomineralization by a large number of bacteria including Streptococcus and Corynebacterium, which are the strains identified in sample 1. Although Adetutu et al. (Adetutu et al. 2012) considered *Staphylococcus* as an indicator of the human interaction in the open shelters, the bacterium can also precipitate carbonates (Han et al. 2018) and can be involved in Hg, Fe(III) reduction (Marnocha and Dixon 2014a). Thus, the biomineralization processes in sample 1 are primarily driven by the heterotrophic communities that alter the local condition to promote the mineral precipitation (Rusznyák et al. 2012; Maciejewska et al. 2017). *Acinetobacter* and *Arthrobacter* are both involved in Cr(VI) and Mn(IV) reduction (Marnocha and Dixon 2014a). At the genus level, a large number of metal-resistance bacteria in sample 1 were observed, including the species of *Corynebacterium*, *Bryobacter*, *Gemella* and *Methylobacterium* (Marnocha and Dixon 2014a; Viti et al. 2003; Barns et al. 2007; Marzan et al. 2017).

Bacteria from animals and humans and those involved in the biochemical cycle of elements such as metal and nitrogen, are the signature of a biofilm inhabiting the coating of sample 2. In fact, species of the genus *Facklamia* are associated with warm-blooded animals, including the gut system of pigs (Crespo-Piazuelo et al. 2018) and the urinogenital tract of female cows, where the bacterium may cause an opportunistic infection (Takamatsu et al. 2006). *Erysipelothrix*, facultative spore-forming Gram-positive bacilli, can cause a wide range of diseases in a variety of species such as sheep, poultry, cattle and humans (Atienzar et al. 2019). *Comamonas* is a halotolerant, saprotrophic bacterium routinely found in oligotrophic environments (like rock surfaces and cave environments), capable of degrading a number of nitrogen-containing aromatic compounds while releasing usable nitrate and ammonia (Barton et al. 2007). *Atopostipes*, previously isolated from swine manure (Cotta et al. 2004), was the predominant bacterium in chicken litters that is responsible for odor production (Wadud et al.

2012). *Enterococcus* is a lactic acid bacterium comprising both pathogenic and commensal species ubiquitous in various environments including the gut system. Due to its tolerance to salts and acids, strains of *Enterococcus* spp. are highly competitive and adaptable to several ecosystems including lithic environments (Roldán et al. 2018; Hanchi et al. 2018). In addition, several members of *Enterococcus* genus have been reported to produce bacteriocins, which are antimicrobial compounds that contribute to either the stability or the dynamics of the microbial communities (Franz et al. 2007).

4.5. Conclusions

For the first time, a mineralogical characterization is combined with a microbial characterization to study the different coatings on an African rock art site. The results demonstrated the following conclusions:

- The two coatings show distinct features, where the coating in sample 1 contains higher amounts of Ca and P than that of sample 2, which is likely related to the presence of organic matter.
- Heterotrophism is the most common energy-acquiring mechanism shared between the two coating communities. The widespread distribution of heterotrophic bacteria is likely to be derived from animal and human sources, which also provide important sources of organic materials generated by the herding activities around the rock art shelter.
- The core bacterial community of sample 1 is substantially different from sample 2, indicating that the microbiota is unique to the coating minerals. In fact, sample 1—the coating with the highest Fe, Ca and P content—hosts bacterial genera that are potentially involved in biomineralization processes, metal redox cycles and metal resistance. In contrast, sample 2 shows mainly pathogenic and commensal bacteria that are characteristic of the animal and human microbiota, and other microorganisms that are involved in nitrogen and metal biogeochemical cycles.

Unveiling the composition of the microbial communities in the coatings of prehistoric paintings has important implications for the conservation strategy. In future work, the coating minerals will be precisely identified using X-ray powder diffraction (XRD) analysis, while the role of the active bacterial community in coating genesis and rock art protection or biodeterioration will be deciphered using the RNA-based analysis.

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Chapter 5

In Living Color: Pigments-Based Microbial Ecology at the Mineral-Air Interface[4]

Abstract

Pigment-based color is one of the most important phenotypic traits of biofilms at the mineral-air interface (subaerial biofilms, SABs), which reflects the physiology of the microbial community. Since color is the hallmark of all SABs, we argue that pigment-based color could be used to convey information about the mechanisms driving microbial adaptation and coexistence across different terrestrial environments, possibly linking phenotypic traits to community fitness and ecological dynamics. Within this framework, we present the most relevant microbial pigments at the mineral-air interface and discuss some of the evolutionary landscapes that make pigments adaptive strategies for resource allocation and survivability. We also report the multifaceted features of pigments that reflect the structure and function of SAB communities and we present the pigment ecology in the context of microbial life-history strategies and coexistence theory. Finally, we set out key questions for future pigment-based ecology research and we picture promising applications.

Keywords: pigments; color; subaerial biofilm; mineral-air interface; pigment ecology

5.1. Introduction

The mineral-air interface is a large and ancient ecological niche that exists on a wide range of scales from microscopic to planetary. Despite the stark environment, the mineral-air interface hosts a micron to a millimeter thick veneer of densely packed microorganisms that operate within self-organized structures called subaerial biofilms (SABs) (Villa et al. 2016). Examples of SAB communities are those retrieved from mineral grain in the topsoil of hot and cold deserts (e.g., biocrusts), air-exposed rocks, and outdoor stone monuments. Given the oligotrophic nature of mineral substrates, the SABs are mainly supported by photosynthetic interactions, where chemoorganotrophic assemblages in this ecosystem are driven by phototrophic carbon fixation. Chemoorganotrophs, as it turns out, promote cyanobacterial growth by consuming oxygen, supplying key metabolites, and scavenging waste products. According to an estimate, the global photoautotrophic communities on terrestrial mineral surfaces contribute to approximately 7% of the net primary production by terrestrial vegetation and half of the biological nitrogen fixation on land (Elbert et al. 2012). This highlights the fundamental role of the mineral-air interface in the global biogeochemical cycles of carbon and nitrogen, which is to serve as a junction for shaping phototroph–chemoorganotroph partnerships (Villa and Cappitelli 2019).

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4. Villa, F., Wu, Y.-L., Zerboni, A., and Cappitelli, F. “In Living Color: Pigments-Based Microbial Ecology at the Mineral/Air Interface.” *BioScience*. (submitted revised version)

Besides being a nutrient-poor habitat, the mineral-air interface often undergoes rapid changes in moisture, temperature, and irradiation level, all of which promote the development of specialized microorganisms with efficient metabolic stress responses (Gorbushina 2007). Thus, to adapt to fluctuating conditions, SAB-dwelling cells must engage and coevolve with their neighbors and their polymeric extracellular matrix (EPM) (Gorbushina 2007, Villa and Cappitelli 2019). Furthermore, SABs are excellent ecosystem models not only for studying multiple survival strategies and ecological succession but also for providing clues to extraterrestrial life.

An omnipresent phenotypic feature of the SABs is color. The green, yellow-brown, or black patinas are familiar colors on a rock in the backyard, a historic stone building, and a sculpture exposed to the outdoor environment (Fig. 5.1). To generate colors, most SAB-dwelling cells rely on light-absorbing compounds called pigments. Pigmented microorganisms for industrial and medical applications are under intense research (*inter alia* Ramesh et al. 2019, Kalra et al. 2020, Sajjad et al. 2020, Venil et al. 2020, Celedón and Díaz 2021, Chatragadda and Dufossé 2021). However, the implications of pigmentation have been overlooked in microbial ecology, despite its recognizable importance in our lives. Most studies on pigmented biofilms focus on aquatic environments instead of terrestrial ecosystems, which host the SABs. One should keep in mind that the mineral-SAB-air interactive system is a fundamental component of the Earth Critical Zone—a thin veneer where critical processes (physical, chemical, and biological) interact to sustain the life (Brantley et al. 2007, Richter and Mobley 2009).

Pigment-based color is one of the most important phenotypic traits of SABs, that reflects the physiology of the microbial community. Since color is the hallmark of all SABs, we argue that pigment-based color could be used to convey information about the mechanisms driving microbial adaptation and coexistence across different environments, possibly linking phenotypic traits to community fitness and ecological dynamics. In other words, we would apply the concept of pigment ecology at the mineral-air interface to describe SAB's requirement, environmental interaction, and effects on ecosystems.

Within this framework, we review pigment-based colors of SAB-dwelling cells and their ecological significance by integrating research in microbiology, biogeochemistry, biogeomorphology, and microbial ecology. Evidence is drawn from various mineral-air ecosystems where microorganisms play crucial roles in primary productivity and global element cycles, such as biological soil crust (biocrust), topsoil, subaerially-exposed rocks, and stone monuments. This work provides: (1) an overview of the most relevant microbial pigments at the air-mineral interface, including photoreceptors in SAB color vision (Box 1); 2) an explanation of some of the evolutionary landscapes that make pigments adaptive strategies for resource allocation and survivability; 3) a discussion of the multifaceted features of pigments that reflect the physiology and activities of SAB communities in different ecosystems, with a focus on the pigmentation of stone heritage (Box 2) and on paleobiology and astrobiology (Box 3); 4) a presentation of pigment ecology in microbial life-history strategies and coexistence theory, and 5) a summary of key questions for future pigment-based ecology research and promising applications.

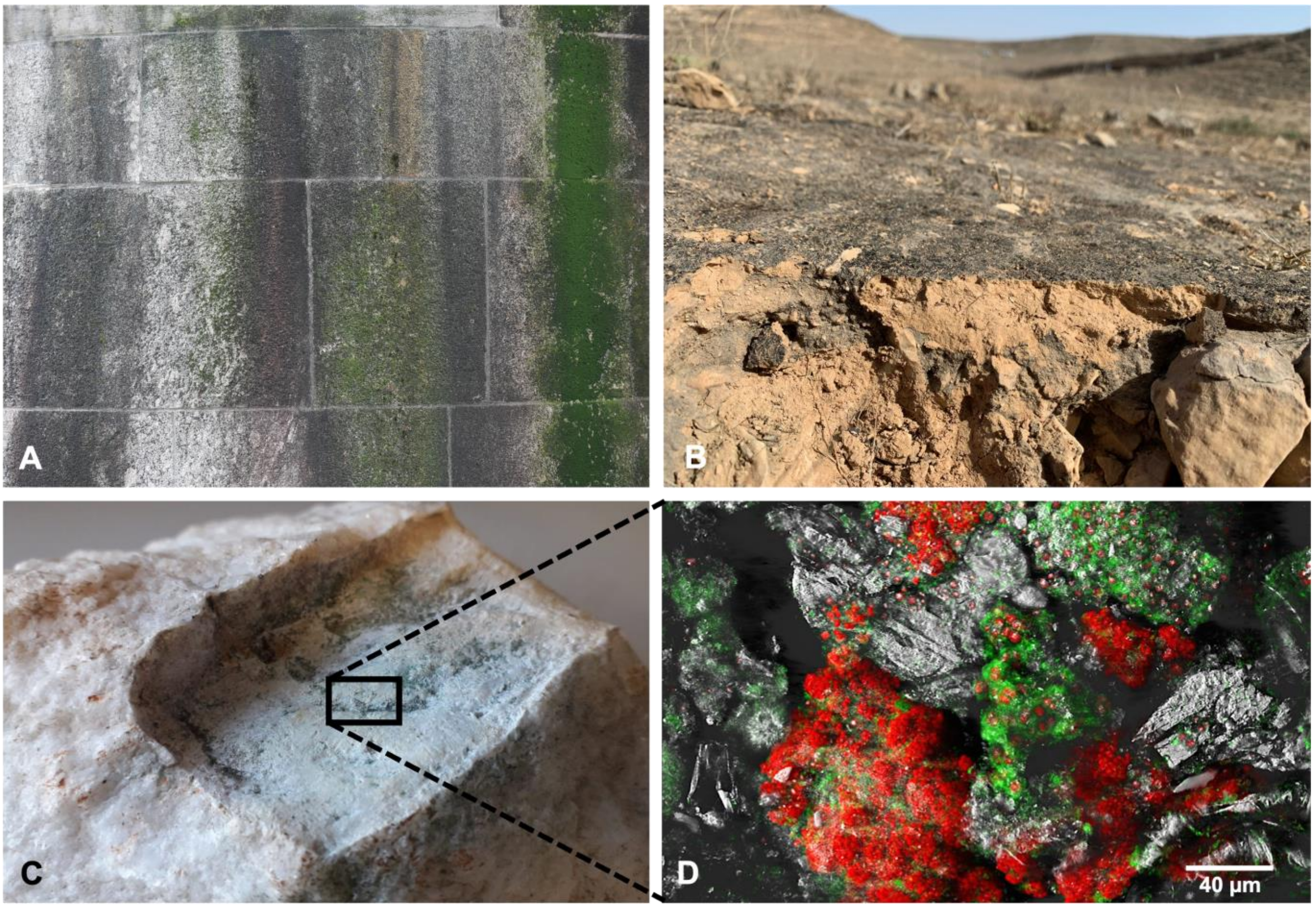


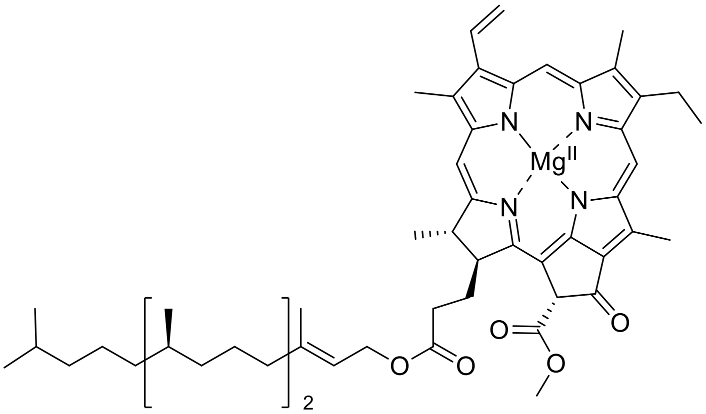
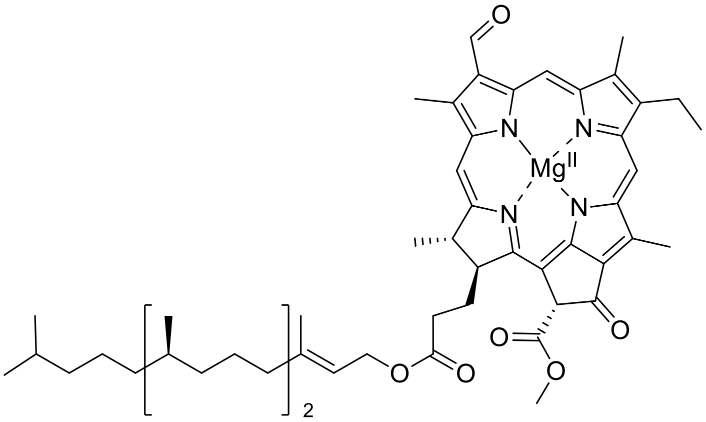
Figure 5.1: Colored SABs on mineral substrates. A) green and purple SABs growing on a vertical wall of a stone monument in Milan, Italy. B) a dark biocrust in the Jebel Qara, Sultanate of Oman. C) endolithic growth (green SAB) in a block of white marble used for construction. D) Confocal laser scanning imaging of the endolithic biofilm. Red are microcolonies of photoautotrophic microbes, green are chemoheterotrophic microbes.

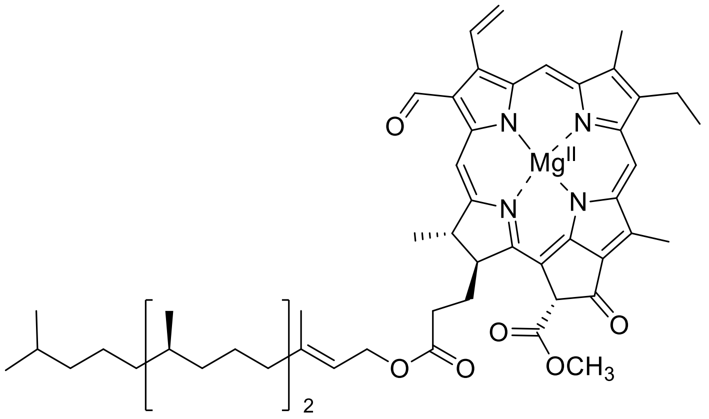
5.2. Microbial pigments and their role in the mineral-air interface

Pigments are chemical compounds that absorb light at a certain wavelength and reflect the remaining (Ramesh et al. 2019). All pigments contain conjugated moieties that allow electronic resonances and mediate energy transfer reactions in the cells. The pigments also serve multiple biological functions, such as energy harvest, photoprotection, free radical quenching, thermal stress protection, metal chelation, cell strength, desiccation, resistance, and cell development (Cordero and Casadevall 2017, Orlandi et al. 2021). Furthermore, the pigments are also involved in processes that underlie photomorphogenesis, photoperiodism, photomovement, and color vision through photoreceptor pigments (Box 1). Thus, microorganisms exploit light signals as a source of information regarding their location and surroundings, as well as a cue for acclimation and behavior.

The main pigments retrieved at the mineral-air interface are energy-related pigments such as chlorophylls (Chls), bacteriochlorophylls (BChls), rhodopsin, and phycobiliproteins, and protection-related pigments like carotenoids, scytonemin, and melanin. Chemical structures and maximum absorbance wavelengths of the principal SABs' pigments are reported in Table 5.1. Pigment biosynthesis responds to multiple environmental and physiological cues, such as temperature, oxygen supply, pH, light, ionic strength, and carbon and nitrogen sources.

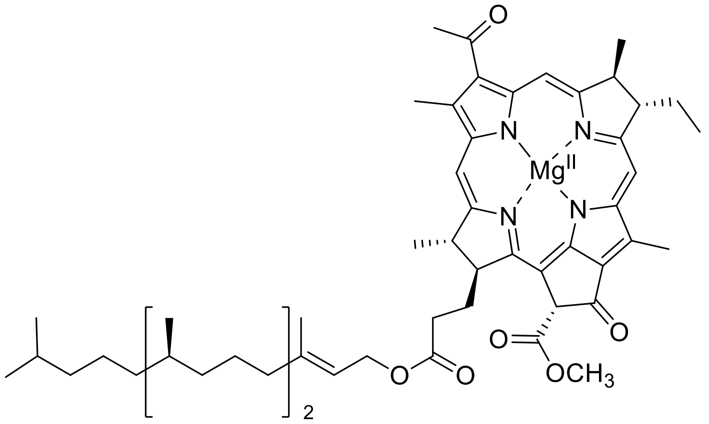
Table 5.1: Chemical structures, maximum absorbance wavelengths, functions, and microorganisms of the principal SABs' pigments.

Chemical structure	Details
	**Chlorophyll *a*** - Absorption peak: 642 nm and 372 nm. - Role: energy harvest. - Microorganisms: Cyanobacteria and algae.
	**Chlorophyll *b*** - Absorption peak: 626 nm and 392 nm. - Role: energy harvest. - Microorganisms: Cyanobacteria and algae.



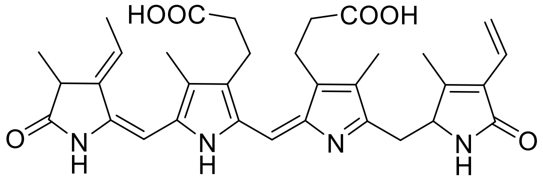
Chlorophyll *f*

- **Absorption peak: 706 nm.**
- **Role: energy harvest.**
- **Microorganisms: cyanobacteria, red algae and other oxygenic microorganisms.**



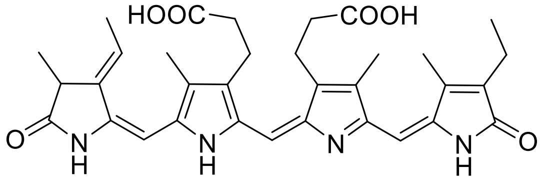
Bacteriochlorophyll *a*

- Absorption peak: 805 nm and 870 nm.
- Role: energy harvest.
- Microorganisms: purple and green sulfur bacteria and some groups of aerobic bacteria, such as members of the *Acidiphilium* genus.



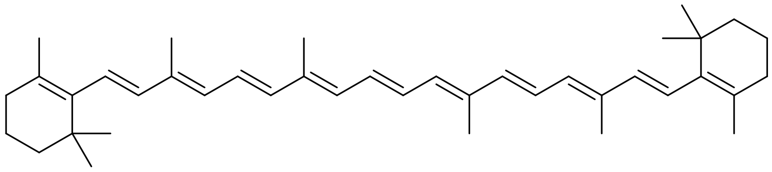
Phycoerythrobilin

- Absorption peak: 530-570 nm.
- Role: energy harvest, antioxidative and radical-scavenging activity.
- Microorganisms: cyanobacteria and algae.



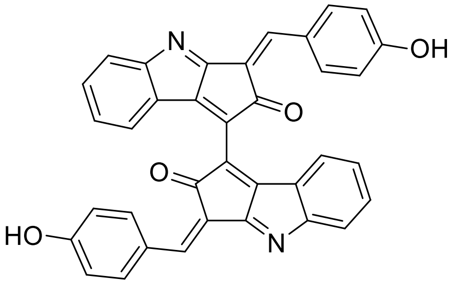
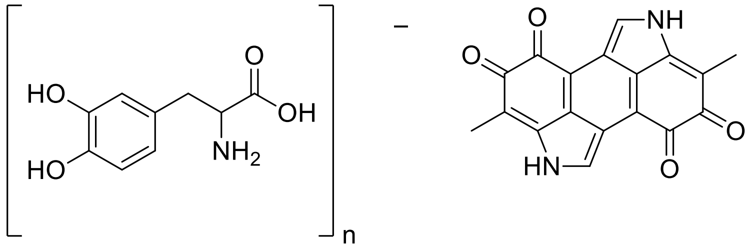
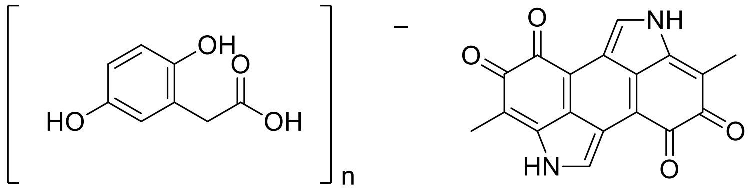
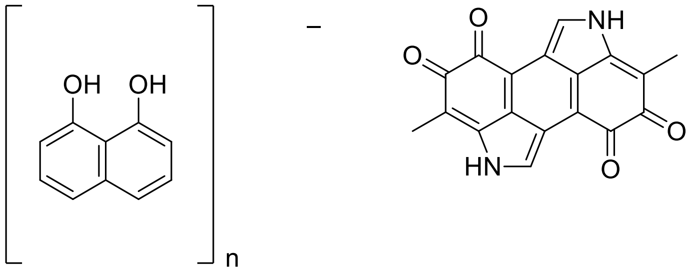
Phycocyanobilin

- Absorption peak: 640-660 nm.
- Role: energy harvest, antioxidative and radical-scavenging activity.
- Microorganisms: cyanobacteria and algae



β-carotene

- Absorption peak: 448 nm.
- Role: energy harvest, antioxidative and radical-scavenging activity.
- Microorganisms: bacteria, algae and fungi.

	**Scytonemin** - **Absorption peak: 252 nm, 278 nm, 300 nm, 384 nm.** - **Role: photoprotection, free radical quenching, thermal stress protection, desiccation.** - **Microorganisms: cyanobacteria and algal symbionts of some lichens.**
	**DOPA-Melanin** - Absorption peak: 300-600 nm. - Role: photoprotection, free radical quenching, thermal stress protection, metal chelation, cell strength, desiccation, resistance. - Microorganisms: bacteria and fungi.
	**Pyomelanin** - Absorption peak: 200-300 nm - Role: photoprotection, free radical quenching, thermal stress protection, metal chelation, cell strength, desiccation, resistance. - Microorganisms: bacteria and fungi.
	**DHN-Melanin** - Absorption peak: 300-600 nm. - Role: photoprotection, free radical quenching, thermal stress protection, metal chelation, cell strength, desiccation, resistance, and cell development. - Microorganisms: bacteria and fungi.

5.2.1. Energy-related pigments.

The variety of energy-related pigments reflects the need for microorganisms to evolve special photosystems to exploit any available spectral region with sufficient energy. Among the best-known energy-related pigments are the Chls from oxygenic phototrophs, and the BChls and rhodopsin from the anoxygenic phototrophic prokaryotes (Chew and Bryant 2007). In addition, cyanobacteria and red algae also synthesize accessorial light-harvesting pigment molecules called phycobiliproteins (Adir et al. 2020, Puzorjov and McCormick 2020). There are four types of phycobiliproteins based on their maxima light absorption peak: phycoerythrin (red pigment), phycocyanin (blue pigment), phycoerythrocyanin (orange pigment), and allophycocyanin (bluish green pigment) (Pagels et al. 2019). Phycocyanin and allophycocyanin are present in all cyanobacteria worldwide, while phycoerythrin and phycoerythrocyanin have a limited distribution in cyanobacteria (Bryant 1982). Depending on cyanobacteria habitats the ratio of these pigments can be changed (Murton et al. 2017). Phycobiliproteins have been shown to display antioxidative and radical-scavenging activity. It has been observed that the intracellular content of each specific phycobiliprotein depends on the incident light intensity, suggesting different roles of phycobiliproteins in light dissipation in a photon-excessive condition (Montero-Lobato et al. 2020). The resources dedicated to phycobiliproteins synthesis are as much as 60% of the total soluble cellular protein, proving the substantial fitness benefits of producing these pigments (Wiltbank and Kehoe 2019).

While Chls in cyanobacteria and other oxygenic photoautotrophs absorb light primarily at 514–700 nm, BChl*a* in aerobic anoxygenic phototrophic bacteria can absorb near-infrared light at 760–1130 nm with minimum absorption of the visible red (Tang et al. 2018). BChl*a*-containing bacteria have been found in rock varnish communities (Kuhlman et al. 2006), biocrust (Csotonyi et al. 2010), oligotrophic soils from the Sør Rondane Mountains (Tahon et al. 2016b, 2016a, Tahon and Willems 2017) and the Himalaya plateau (Rehakova et al. 2019). These microorganisms can use both organic and inorganic electron donors for light-dependent fixation of carbon dioxide without generating oxygen (George et al. 2020).

Chls pigments display a very low degree of light absorption in the range of 500-600 nm, an energy-rich region of the solar spectrum (Kume et al. 2018). By contrast, microbial rhodopsins show a strong well-defined peak of absorbance centered at a 568 nm (DasSarma and Schwieterman 2021). Microbial rhodopsins are a family of transmembrane proteins that contain opsin and a covalently bound retinal cofactor as a chromophore. Microbial rhodopsins have two main biological functions: i) as light-driven proton pumps, which respond to light by transporting ions across the cell membrane and generating a chemiosmotic potential for ATP synthesis; ii) as photosensory receptors that mediate light-induced behavioral responses (Govorunova et al. 2017). The dissemination and diversification of microbial rhodopsins across Archaea and Bacteria can be explained by considering the minimal genetic determinants of rhodopsins synthesis (a single opsin gene and another gene for retinal synthesis from carotenoid) subjected to horizontal gene transfer. A recent metagenomic study of soil crusts showed that half of the microbial genomes encode for rhodopsins (Finkel et al. 2013), while 20% of bacterial taxa of hypolithic communities (the assemblages found below translucent rocks) in the Antarctic Dry Valley harbored rhodopsin genes (Guerrero et al. 2017). The presence of actino-opsin (*actR*) genes in the genome of Actinobacteria—one of the most abundant phyla in the SABs—indicates the microorganisms' ability to boost heterotrophic growth via phototrophy (Chuon et al. 2021). This new insight reveals the microorganisms' coping mechanism in the harsh and energy-deficient mineral-air interface, which is by maximally exploiting the solar energy. BChl- and rhodopsins-based phototrophy ensure heterotrophs meet the energy demand during starvation conditions by using sunlight as an alternative energy source, which is an important energetic mechanism underpinning the microbial function in the desert ecosystems (Leung et al. 2020). In addition, the widespread distribution of prokaryotic rhodopsins on Earth, and their capacity to generate chemical energy using an energy-rich portion of the electromagnetic spectrum, suggest the early appearance of these biopigments on our planet (DasSarma and Schwieterman 2021). Recently, BChl- and rhodopsin-based dual phototrophy has been observed in Alphaproteobacteria isolated from the "Lille Firn" glacier and the nearby exposed soil in northeast Greenland (Zeng et al. 2020). This process would reduce the consumption of organic matter for energy production in bacteria by increasing the flexibility and efficiency in conserving light energy. Such diversity of photosynthetic pigments is instrumental for efficiently capturing the available light spectrum and, therefore, for determining the distribution and composition of SAB communities. The pigment set increases the survival and fitness of phototrophs by allowing microorganisms to capture light that is unused by others in their local environment. This is a widely utilized practice in a natural environment where microorganisms must compete with their neighbors for space and resources.

5.2.2. Protection-related pigments.

Carotenoids are organic liposoluble pigments produced by bacteria, archaea, algae, and fungi, whose colors range from yellow to purple. Carotenoids include over 1100 substances belonging to isoprenoids, which are characterized by the presence of a conjugated tetraterpene (C40). Carotenoid production in some non-phototropic bacteria occurs in a light-dependent manner to protect cells from photo-oxidants (Sumi et al. 2019). The conjugated double bonds are responsible for the photoprotective and antioxidative mechanisms of the carotenoids. Carotenoids scavenge oxygen radicals by releasing energy through polyene chain vibration (physical reaction), but recent research revealed their ability to inhibit these radicals through chemical reactions (Maoka 2020). For instance, astaxanthin can react with peroxynitrite and nitrogen dioxide radicals to form nitro-astaxanthin to prevent the nitration of tyrosine. Similar results were obtained in cases of β-carotene, lutein, zeaxanthin, capsanthin, and fucoxanthin (Maoka 2020).

Other protective pigments include potent sunscreens, namely scytonemin and melanin. The alkaloid biomolecule scytonemin is a yellow-to-brown extracellular pigment produced only by certain strains of sheathed or encapsulated cyanobacteria (Garcia-Pichel and Castenholz 1991, Rastogi et al. 2015). Certain environmental stresses such as UVA radiation, temperature, periodic desiccation, and fixed-nitrogen deficiency can modulate the synthesis and levels of scytonemin (Fleming and Castenholz 2007, Sorrels et al. 2009, Orellana et al. 2020). In Atacama halites under natural solar radiation, epilithic (rock surface) cyanobacteria had a scytonemin content 12-fold higher than their endolithic (rock pores) counterparts (Vítek et al. 2014). Gao et al. (2021) reported that this pigment can prevent up to 90 % of the incident solar UV radiation from entering the cell, by absorbing short wavelength UV radiation and dissipating energy through harmless thermal de-excitation. However, the localized warming caused by the accumulation of scytonemin, and the thermal de-excitation are not without consequences. Couradeau and colleagues (2016) showed that scytonemin in mature biocrusts can increase the soil surface temperature by as much as 10 °C, which replaces thermosensitive bacterial species with thermotolerant microorganisms. Thus, scytonemin shapes the composition of the SAB communities indirectly. Scytonemin also serves as a potent antioxidant that prevents cellular damage caused by reactive oxygen species (ROS) (Matsui et al. 2012).

Melanin is a general term for a group of heterogeneous polyphenols produced by a variety of organisms including bacteria and fungi (Tran-Ly et al. 2020). Microbial melanin is classified into two types: allomelanin and eumelanin. Allomelanin encompasses a variety of non-nitrogenous subgroups of melanin derived from different catecholic and dihydroxynaphtalene precursors, which are known as 1,8-dihydroxynaphthalene (DHN)-melanin and pyomelanin. Eumelanin includes 3,4-dihydroxyphenylalanine (DOPA)-melanin, which is synthesized via a similar pathway as mammalian melanin synthesis (Singh et al. 2021). These dark pigments are characterized by their unique physicochemical properties such as broadband optical absorption, paramagnetism, charge transport, and remarkable structural stability, which confer diverse biological functions. Microbial melanization is used for photoprotection, thermoregulation, energy harvest, free radical quenching, metal chelation, cell strength, and resistance to the desiccation (Pavan et al. 2020). For instance, the presence of a thicker melanized cell wall increases the resistance of fungi to acute and chronic ionizing radiation (Pacelli et al. 2020, Liu et al. 2021) and bolsters fungal cell-wall strength that enhances penetration into mineral substrates (Liu et al. 2020b, Prenafeta-Boldú et al. 2022). Similar to scytonemin, the role of melanin in thermoregulation stems from its ability to effectively absorb solar radiation and dissipate it non-radiatively in the form of heat. Cordero et al. (2018) studied the distribution of 358 isolated fungal species worldwide and found the first empirical evidence of a geographical pattern in color lightness. They reported that color lightness, as it decreased from the equator to the poles, is relevant for the thermoregulation of fungi. Recently, untargeted metabolomics revealed an increase in melanin expression in southern communities on exposed rocks, which confers high tolerance for low temperature and drought (Coleine et al. 2020). These findings suggest the significance of thermal melanism for the evolution of eukaryotic life as it represents another important mechanism that enabled the earliest terrestrial organisms to survive and grow alongside photosynthesis. Besides thermoregulation, melanin is also involved in energy transduction in a process called radiosynthesis, where it harvests energy from electromagnetic radiation for metabolic use using the electrical properties (Casadevall et al. 2017).

While the sunscreen pigments chemically and physically protect the producing microorganisms, the protection is extended to other cells in proximity. Mature biocrusts display pigments stratification with the highest concentrations of mycosporin-like amino acids, scytonemin, and carotenoids in the upper layers, while Chl*a* and phycobiliproteins are in the lower portions. In line with the pigments' stratification, biocrusts from the Needles District of the Canyonlands National Park presented dark-pigmented *Nostoc* spp. and *Scytonema myochrous* restricted to the soil surface, and motile filamentous *Microcoleus vaginatus* extending underneath (Bowker et al. 2002). *Microcoleus vaginatus* showed significant mortality without *Nostoc* spp. and *Scytonema myochrous*, suggesting that the sunscreen pigments produced by *Nostoc* and *Scytonema* in the crust surface protected the less-pigmented *Microcoleus vaginatus* from the photobleaching (Bowker et al. 2002). In Antarctic endolithic ecosystems, melanized fungi form a black "sunscreen" above the photobiont stratification to protect the whole community (Selbmann et al. 2013). Similarly, the relative enrichment of scytonemin in the 'black' layer on the halite crust surface was documented by Vítek et al. (2014). Scytonemin is a very stable pigment that remains intact within the EPM, conferring passive UV protection to both metabolically active and inactive cells. Moreover, the scytonemin-rich layer acts as a screen for the Chl-rich layer located below. Interestingly, Vítek and colleagues (2014) noted that the Chl-rich layer underneath was composed of the same cyanobacterial taxa as the black layer but lacking in scytonemin. They concluded that, since the biosynthesis of scytonemin is an energy-consuming process, the protective black layer allowed the underlying cells to minimize energy expenditure for scytonemin biosynthesis.

Various analytical techniques developed over the years are still in use to detect and analyze microbial pigments. Pigments extraction from SABs followed by separation with chromatography (e.g., high-performance liquid chromatographic, HPLC) and identification/quantification with mass spectrometry (MS), and nuclear magnetic resonance (NMR) are techniques largely used for studying pigments, even at trace levels (Cappitelli et al. 2012, Keshari and Adhikary 2013, Montero-Lobato et al. 2020). Flow cytometry has been occasionally applied to microbial colonization on heritage samples to study fluorescence produced by photosynthetic pigments (Cappitelli et al. 2009). In recent years, Raman spectroscopy has become a popular analytical tool for the qualitative and quantitative assessment of microbial pigments in the SABs (Vítek et al. 2017, 2020). Such studies have used high-resolution laboratory Raman spectrometers and more recently, portable handheld instruments that can be used in the field. Raman microspectrometry has allowed the direct study of pigment type and distribution in endolithic zones without sample treatment or extractions. The investigations revealed differences in pigment distribution in SABs colonizing differing lithotypes such as dolomite, gypsum, or altered orthoquartzite (Edwards et al. 2005, 2007). Raman microspectrometry was able to detect SAB pigments such as melanin, scytonemin, chlorophyll, and carotenoids in samples of halite endoliths from the Atacama Desert in Chile (Vítek et al. 2010, Culka et al. 2017). Importantly, Raman spectroscopy reliably identifies pigments but cannot identify the producing species, because the same pigment may be produced by more than one species. It is the combination of molecular investigations and Raman spectroscopy that provides such an association (Imperi et al. 2007).

5.3. SAB’s pigmentation as an adaptive strategy for resource allocation and survivability

The color change is a form of phenotypic plasticity, which confers fitness advantages to the whole microbial community by optimizing resource allocation. From an adaptation standpoint, pigments can serve several functions, and the resulting change in color patterns frequently represent a trade-off between the different evolutionary drivers, such as light quantity (irradiance) and quality (wavelength). These trade-offs can vary even within the population, and the color can be altered strategically according to the condition and the purpose. At the mineral-air interface, differences in light quality are not only related to the daylight/annual cycles but also to the system condition that constrains part of the spectrum (e.g., translucence and lithology) to be available to the microorganisms. A Monte Carlo model of light propagation in translucent rocks described the light levels at the bottom and the subsurface edges of desert quartz. The model showed the variation in light levels, with the available light resulting higher at the sunward subsurface edge than at the basal surface (Cappitelli et al. 2009). Meslier and colleagues (2018) observed that different lithologies (limestone, gypsum, ignimbrite, and granite) collected in the Atacama Desert have different light transmission properties. The solar irradiance transmission spectra measured at the ventral surfaces of quartz stones in the Namib Desert showed complete comminution of the short wavelengths (UV and blue) and higher transmission in the longer wavelength (Gwizdala et al. 2021). Thus, the hypolithic communities of mineral substrates experience reduced illumination and a relatively red-enriched light. Overall, this light-filtering process yields a series of distinct spectral niches for phototrophic microorganisms, leading to the development of different spectrum-response mechanisms. To this end, pigments contribute to the acclimation (short-term) and adaptation (long-term) of microorganisms to light variability.

Fast spectrum-response strategies include the mechanisms for dissipating the excess excitation energy from the light-harvesting complexes in the form of heat. It is well known that cyanobacteria use sophisticated molecular machinery composed of light-harvesting complexes, energy-converting photosystems, electron carriers, and regulatory complexes, to convert photosynthetically active radiation (PAR) into chemical energy in the thylakoid membranes (Stirbet et al. 2020). The complexity of this molecular machinery makes photosynthetic organisms sensitive to fluctuating light intensities. Indeed, light becomes dangerous when the entire photosynthetic electron transport chain becomes excessively reduced, forming ROS that leads to severe cell damage. Thus, the survival and growth of photosynthetic microorganisms strongly depend on the balance between the collection of light energy for photosynthesis and protection against photo-oxidation. As an example of a fast acclimation process, the orange carotenoid protein (OCP) of cyanobacteria senses light intensity and induces thermal dissipation of excess excitation energy by interacting with the phycobilisome (Muzzopappa and Kirilovsky 2020). By studying the pigments of an endolithic cyanobacterial community along an ignimbrite cross-section profile, Vítek et al. (2017) reported the Raman shifts of the $\nu 1(C=C)$ carotenoid band positions with respect to the depth. The findings suggested that red-shifted carotenoids (OCP-OCP^R transition) in endolithic *Chroococcidiopsis* sp. in proximity to the surface is a light-dependent phenomenon. This was interpreted as *Chroococcidiopsis* sp.'s photoprotection to cope with the excess light near the rock surface.

One of the best-characterized spectrum-response strategies is chromatic acclimation, in which cyanobacteria modulate the composition of primary pigments in their photosynthetic light-harvesting antennae (phycobilisomes) to compensate for light quality changes (Sanfilippo et al. 2019). Thus, chromatic acclimation involves tuning the cellular metabolism and physiology to external light cues through pigment alteration. During chromatic acclimation, the synthesis of phycoerythrin partially replaces phycocyanin in the phycobilisome complexes, leading cyanobacteria to use a significantly greater amount of green light for the photosynthesis (Hirose et al. 2019). Thus, cyanobacteria can appear in different colors based on the light they endure, with red and green light causing the cells to turn blue-green and brick red, respectively. The regulation of this photoreversible color change involves sophisticated light-color-sensing and signal-transduction systems (Kehoe and Gutu 2006). The chromatic acclimation process is generally slower than OCP because it involves gene expression changes and the synthesis of new protein complexes and pigments. Smith and colleagues (2014) suggested that chromatic acclimation takes place in cyanobacteria that colonize hypolithic substrates from the Mojave Desert. A red shift in the spectral emission profiles of *Chroococcidiopsis* cells inhabiting quartz, carbonate, and talc was observed and correlated to the modification of the phycobilisome composition (Smith et al. 2014).

One of the most recently identified forms of chromatic acclimation is the far-red light photoacclimation (FaRLiP). During this acclimation, cyanobacteria remodel photosystems and phycobilisomes, while synthesizing atypical Chls (Chl*d* or Chl*f*), and far-red-absorbing phycobiliproteins (Gan et al. 2014). Although FaRLiP reduces photosystem efficiency, it allows cyanobacteria to sustain photosynthetic activity under conditions where PAR is limited and energy yield from normal photosynthesis is compromised (i.e., Chl*a*-based). FaRLiP is instrumental to terrestrial cyanobacteria because they frequently occur in niches where visible light is strongly filtered or scattered, leading to enrichment in wavelengths longer than 700 nm. A Chl*f*-containing cyanobacterium with the capability of near infrared-driven oxygenic photosynthesis was found on moist limestone outside Jenolan Caves in Australia (Behrendt et al. 2015). Recently, Antonaru and colleagues (2020) used the marker gene *apcE2*—which encodes a phycobilisome linker associated with FRL-photosynthesis—to detect Chl*f* microorganisms in metagenomic data from environmental samples. The results showed the presence of the gene in cyanobacteria commonly retrieved at the mineral-air interface and producing Chl*f*, such as *Chroococcidiopsis* sp. and *Calothrix* sp. Furthermore, the study highlighted the distribution of Chl*f*-containing cyanobacteria in a variety of habitats at the mineral-air interface across the globe, such as the Frasassi Cave in Italy and soils in Germany and Cuba (Antonaru et al. 2020).

Gwizdala et al. (2021) stated that neither complementary chromatic adaptation nor FaRLiP was adaptation mechanisms used by hypolithic cyanobacteria inhabiting quartz pebbles in the Namib Desert. The researchers did not detect any Chl*d* or Chl*f* in SAB pigment extracts, and they did not identify any open reading frames mapping to phycoerythrin, suggesting that other mechanisms could have been adopted by the hypolithic cyanobacteria to respond to light stress. Similar studies reported the lack of redshifted Chls in hypoliths while observing an increase in phycobiliproteins (Smith et al. 2014, Mehda et al. 2022). These findings were interpreted as the response of cyanobacteria to low-light conditions in the deeper part of the colonized rocks. Thus, an alternative to FaRLiP is the low-light photoacclimation, where cyanobacteria remodel their photosynthetic apparatus to absorb light beyond the wavelength of 700 nm to increase their light-harvesting capacity.

The chromatic tuning ability of SAB-dwelling cells provides enormous flexibility in adjusting cellular response to light and redox conditions. Thus, change in pigmentation can be used to maximize energy collection for photosynthesis and/or to protect the photosynthetic apparatus, guaranteeing the level of organic carbon for the sustainability of the whole community. Since organic carbon availability is the highest during the day when photosynthesis is the most active, if a microorganism can use sunlight as a sensor to initiate carbohydrates transport and metabolism, it could theoretically gain a growth advantage over the other components of the SAB community. Maresca et al. (2019) reported that some actinobacteria use light as a signal for synchronizing their metabolism with their phototrophic neighbor, maximizing organic carbon uptake at the moment photosynthates are released. This fine control was enabled by cryptochromes—flavin pigments that control the circadian rhythm—that sense specific wavelengths of light. Because of actinobacteria’s abundance in SAB, it seems that the regulation of sugar metabolism is a mechanism to efficiently use the nutrients excreted by phototrophs, which are plenty at the mineral-air interface.

5.4. Pigmentation as a reflection of SAB physiology and activity

Microbial pigments are not merely colors, as they possess multifaceted features that reflect the physiology and activities of the SAB communities. Thus, pigments can provide detailed information about the stochastic response of SABs to climatic and environmental conditions. Therefore, SAB’s pigments may be applied as a tool for biodiversity conservation strategies, ecosystem functioning, ecological modeling, and climate change monitoring.

Discrete color phenotypes are often associated with differences in morphological, physiological, and behavioral traits. For instance, chlorosis is the acclimation process pursued by non-diazotrophic cyanobacteria in response to nitrogen shortage. During nitrogen starvation, the cells gradually turn down their metabolism and enter a dormant-like state characterized by minimum photosynthesis and pigmentation, which allows long-term survival with resuscitation under favorable environmental conditions (Spät et al. 2018). A hallmark of chlorosis is the rapid proteolytic degradation of phycobiliproteins—the major light-harvesting pigments in red algae and most cyanobacteria—accompanied by cell color change from blue-green to yellow. It has been shown that the phycobiliproteins degradation favors the release of soluble amino acids and reduces the intracellular ROS level by decreasing the light absorption capability (Forchhammer and Schwarz 2019, Krauspe et al. 2021). The current understanding of the chlorotic cells' regulatory processes depicts the intricate mechanism that modulates ribosomal proteins, RuBisCO components, central regulators, and metabolic enzymes, as well as hyperphosphorylation of nondegraded phycobiliproteins (Spät et al. 2018, Krauspe et al. 2021a). The degradation and re-synthesis of phycobiliproteins were examined using hyperspectral confocal fluorescence microscopy coupled with single-cell analysis based on the pigment content and localization in live cyanobacterial cells (Murton et al. 2017). The findings revealed that phycobiliproteins degradation and re-synthesis are coordinated by highly synchronized cells that undergo pigment modifications. Chl fluorescence was decreasing during nitrogen starvation without any change in the localization of subcellular Chl.

Chl*a* detection and quantification have been used to characterize the biocrust development (Caesar et al. 2018, Román et al. 2021) and recovery after environmental disturbances (Ayuso et al. 2016, Faist et al. 2020). Abed et al. (2014) used chlorophyll analyses to investigate the correlation between cyanobacteria hydrotaxis and the rapid biocrust greening upon wetting. By using HPLC and hyperspectral imaging, the researchers concluded that Chl*a* concentration increased after wetting only at the top layer, while its vertical profile did not change. Furthermore, ^{13}C labeling experiments demonstrated that the greening upon wetting was not due to de novo synthesis of Chl*a*, but rather to the rapid recovery of the pigment already present in the biocrust. Overall, the photosynthetic apparatus remains essentially intact and return to a functional state with remarkable speed. Additionally, cyanobacteria did not exhibit any hydrostatic movement to track water, but instead increased their Chl*a* production and restored their photosynthetic activities within minutes of water addition. Román et al. (2019) demonstrated that biocrust reflectance provides a non-destructive and reliable method to quantify Chl*a* and, therefore, to study the biocrust state and development. Recently, the Chl*a* absorption feature in the red region was recognized as a robust and sensitive index of biocrust greening being minimally affected by the soil moisture content. The index was then applied to the optical images obtained from the satellite Sentinel-2 and proved to be suitable for tracking the spatial and temporal development of biocrust along the rainfall gradient in the Negev Desert (Panigada et al. 2019).

The content of photosynthetic pigments has been widely used to estimate the biomass of phototrophic SABs colonizing the construction materials and the ornamental stones. For instance, Vázquez-Nion et al. (2018) measured Chl fluorescence (as a proxy of growth and physiological state) and color variation (as a proxy of biomass estimation and pigment production) to assess the primary bioreceptivity to phototrophic SABs of eleven varieties of granitic rocks. The color variation was measured by using the CIELAB color system coordinates that represent each color using three scalar parameters: L*, lightness or luminosity of color; a*, associated with changes in redness-greenness; and b*, associated with changes in yellowness-blueness. Vázquez-Nion et al. (2018) reported enhanced granite colonization rates on substrates with high open porosity, capillary water content, and surface roughness. The effects of substrate bioreceptivity and environmental parameters on SAB development were investigated by using the same pigment-based techniques: Chl fluorescence and color variation (Fuentes and Prieto 2021a). The study revealed that granite bioreceptivity affected microorganisms' adhesion to the substrate, water stress hindered biofilm formation, and temperature promoted photosynthetic efficiency. Chl fluorescence measurements were also used to investigate the effects of water-induced stress on phototrophic SABs, where 2 days of drought were found to influence the vitality and recovery of the microorganisms (Fuentes and Prieto 2021b). Photosynthetic pigments content and color measurements revealed that high levels of atmospheric CO_2 favor the development of phototrophic SABs, although the effects of increased CO_2 levels were counteracted by increased water availability (Prieto et al. 2020).

The cyanobacteria-dominated endolithic colonization in halite deposits of the Atacama Desert hyperarid zone was investigated with λ-scan confocal laser scanning microscopy to detect autofluorescence emission spectra of photosynthetic pigments (Roldán et al. 2014). Photosynthetic pigments could be identified according to the shapes of the emission spectra and wavelengths of fluorescence peaks. Red fluorescence produced by Chl*a* and phycobiliproteins was considered an indicator of cell viability. Once faded, a nonspecific green fluorescence appeared, either due to the variety of compounds, e.g. flavonoids and pyridine nucleotides, or the degradation of the photosynthetic pigments and the increasing amount of denatured proteins (Roldán et al. 2014). The same technique was applied by Villa et al. (2020) to study the healthy state of phototrophs colonizing a stone monument before and after a cleaning treatment. The pigment spectral profile of SABs exposed to a chemical treatment showed characteristic emission peaks between 540 and 590 nm, indicating the disorganization of the light-harvesting complex.

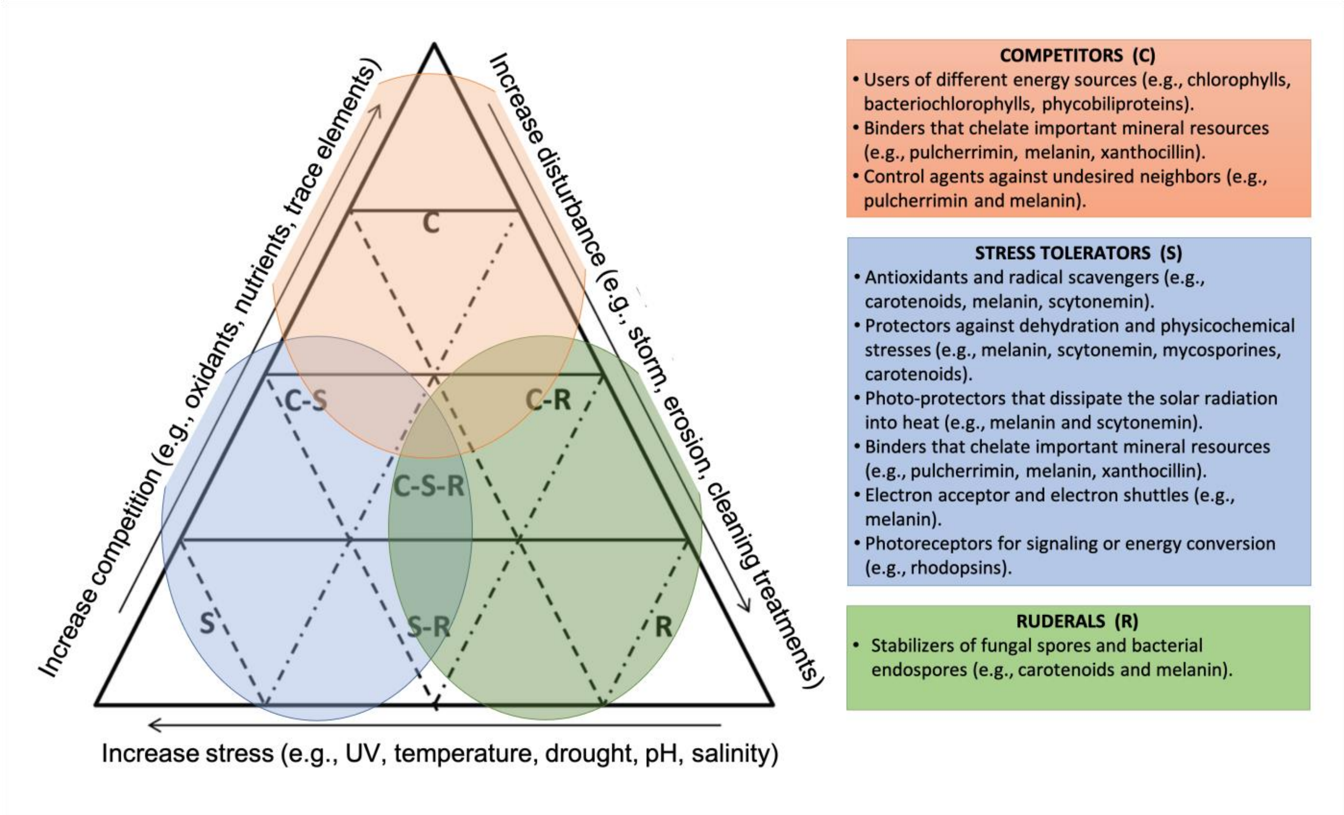
To study the effects of increasing nitrogen (N) inputs on biocrusts structure and function, Dias et al. (2020) used the number of pigments and their relative abundance as a proxy for SAB richness and evenness, respectively. The results revealed that biocrust diversity—as shown by pigment richness and evenness—increased with increased N inputs. Thus, pigment-based data suggested that increased N inputs promoted biocrusts multifunctionality, which may be instrumental in counteracting the negative effects of climate change and desertification in drylands.

5.5. Toward microbial pigment ecology

Since pigment-based color is the hallmark of all SABs, it could be exploited to study the mechanisms driving changes in community structure and function under the influence of perturbations, and microbial coexistence across different environments.

So far, we have presented pigment-based color as a trait that promotes adaptation for resource allocation and survival benefits and that reflects the physiology and activity of the SAB community. These concepts can also be seen in the framework of trait-based life-history strategies. Trait-based life-history strategies simplify the complex trait information into a few ecologically relevant elements and simultaneously offer tools to characterize changes in community structure and function due to stress (persistent adverse climatic and environmental conditions) and disturbance (event leading to significant loss of biomass or rock surfaces rejuvenation, and surface instability).

Grime’s CSR (Competitor, Stress tolerator, Ruderal) framework is a classic trait-based approach that classifies organisms responding to stress and disturbance. Although originally developed to explain plant-community assembly, the CSR theory can be applied to seek general patterns of microbial communities’ organization (Villa et al. 2016, Wood et al. 2018). Thus, it is possible to use the CSR framework to illustrate the interface between pigments and microbial life history (Fig. 5.2). According to the CSR theory, “competitors” thrive in low stress and low disturbance environments, where they gain a competitive advantage by maximizing resource acquisition. Competitive traits associated with pigments are related to the improvement of resource acquisition and the control against undesired neighbors. An interesting example of a competitive trait is provided by the brown pigment pulcherrimin, which is excreted by *Bacillus* and yeast species generally found on mineral substrates. From the chemical point of view, the pulcherrimin is a chelate complex of pulcherriminic acid and ferric ions. Iron is the most important metal in biology, and previous studies showed that external Fe strongly influences biofilm formation in several microorganisms (Rizzi et al. 2018). Pulcherrimin manipulates the microenvironment of the biofilm through the depletion of the iron (Arnaouteli et al. 2019). A partial depletion in high-iron conditions allows *B. subtilis* to colonize a surface and gain access to nutrients. Furthermore, the presence of the iron chelating pulcherrimin can prevent bacteria found in the surrounding environment to invade the biofilm through the generation of an “iron-free” zone (Arnaouteli et al. 2019). In the same way, fungal melanin is an excellent metal-binding compound, and its metal scavenging activity allows the bioabsorption of essential metals from rocks and other mineral niches (Oh et al. 2021), conferring a competitive advantage by enhancing the mineral use. The yellow fungal pigment xanthocillin binds copper, impacts cellular copper content, and possesses significant metal-dependent antimicrobial properties (Raffa et al. 2021). In summary, microorganisms capable of synthesizing certain pigments deprive competing cells of important resources while antagonizing undesired neighbors.



COMPETITORS (C)
- Users of different energy sources (e.g., chlorophylls, bacteriochlorophylls, phycobiliproteins).
- Binders that chelate important mineral resources (e.g., pulcherrimin, melanin, xanthocillin).
- Control agents against undesired neighbors (e.g., pulcherrimin and melanin).

STRESS TOLERATORS (S)
- Antioxidants and radical scavengers (e.g., carotenoids, melanin, scytonemin).
- Protectors against dehydration and physicochemical stresses (e.g., melanin, scytonemin, mycosporines, carotenoids).
- Photo-protectors that dissipate the solar radiation into heat (e.g., melanin and scytonemin).
- Binders that chelate important mineral resources (e.g., pulcherrimin, melanin, xanthocillin).
- Electron acceptor and electron shuttles (e.g., melanin).
- Photoreceptors for signaling or energy conversion (e.g., rhodopsins).

RUDERALS (R)
- Stabilizers of fungal spores and bacterial endospores (e.g., carotenoids and melanin).

Figure 5.2: A reflection of pigmentation traits on the competitor (C)–ruderal (R)–stress tolerator (S) life strategy framework, as was proposed for plants by Grime (1977). The graphical representation combines the CSR context with resource acquisition and survival benefits for the microbial community.

Stress tolerators endure suboptimal environments by maintaining metabolic performance in unproductive niches, which increases the efficient use of resources in the long term. It is suggested that microorganisms can synthesize pigments in response to stressful conditions to improve long-term growth and survival strategies in extreme environments. For example, heavy melanization was associated with the survival of microcolonial fungi under desiccation. Microcolonial fungi are rock specialists that lack sexual reproductive structures and that form compact melanized colonies on bare rock surfaces (Coleine et al. 2021). Gorbushina et al. (2008) observed the maintenance of protective carotenoid precursors and constitutive antioxidants like melanin and mycosporines in dormant microcolonial fungi following fast drying. Fungal melanin increases the cell’s ability to absorb and retain water as a protection against desiccation. Melanin is known for its hygroscopic character and strong association with water, which determines its electroconductive properties (Cordero and Casadevall 2017). In addition, melanization can change the strengths and rigidity of the cell wall by crosslinking with different macromolecules. Melanin can also act as an osmoregulator by reducing the size of pores in the cell wall (Plemenitaš et al. 2008). Thus, melanin affects the osmolyte exchange by reducing water loss and increasing cellular turgor forces (Casadevall Arturo et al. 2017). Desiccation also affects the EPM by changing its structural rigidity/elasticity. Scytonemin, widely distributed in the EPM of cyanobacteria, seems to stabilize the matrix that would give a survival advantage for terrestrial cyanobacterial during drought (Gao 2017). To survive periods of depleting oxygen concentrations, some microorganisms use melanin as terminal electron acceptors for anaerobic respiration and also as electron shuttles during the respiration of Fe(III) minerals (Turick et al. 2009). This unusual utilization of melanin pigments advantages microorganisms by enhancing metabolic versatility in response to changing environmental conditions and by accelerating the biogeochemical cycling of metals. An increase in carotenoid concentration promotes cells' resistance to freeze-thaw stress by regulating membrane fluidity, which promotes cell viability at low temperatures and facilitates the nutrient transport (Seel et al. 2020). Carotenoids also improve bacterial tolerance in cells upon acute xenobiotic shocks (Chia et al. 2021). In addition, the accumulation of the pigment protects the proteins involved in recovery, thereby, reducing the energy costs of the repair (Leung et al. 2020). All SAB-dwelling cells use a variety of mechanisms to allocate limited resources according to their current needs in the environment. Under xeric conditions and starvation, heterotrophs can meet energy demands by using sunlight as an alternative energy source through a BChl-based reaction center or proton-pumping rhodopsin (Zeng et al. 2020). In addition, to survive the fluctuating environment, microorganisms integrate light signals to produce appropriate behavioral responses. For instance, some Haloarchaea utilizes microbial rhodopsins to sense light and guide phototaxis in search of the optimal environmental niches (Chuon et al. 2021). It has been shown that light signals activate specific cellular acclimation programs through photoreceptors, preparing biocrust-inhabiting cyanobacteria for the impending dehydration (Oren et al. 2019). Since hydration/dehydration cycles are likely to affect inorganic carbon (Ci) levels, cyanobacteria acclimate to this fluctuation via their Ci-concentrating mechanism (CCM). The results by Oren et al. (2021) showed that far-red-light-sensing photoreceptors—such as phytochromes—regulate the activity of the cyanobacterial CCM, affecting desiccation tolerance in cyanobacteria. Overall, pigments provide SAB-dwelling cells with special properties that translate into survival and adaptation advantages during environmentally stressful conditions. Pigments can support the stress-tolerant strategy by serving as i) antioxidants and radical scavengers, ii) protectors against dehydration and physicochemical stresses, iii) photo-protectors that dissipate the solar radiation in the form of heat, iv) binders that chelate important mineral resources, v) electron acceptor and electron shuttles, and vi) photoreceptors for signaling or energy conversion.

Ruderals are microorganisms that cope with frequent disturbances by relying on high colonizing ability, rapid production of low-cost biomass, and inefficient resource uptake. The ruderal trait associated with pigments is the dispersal that is related to community re-colonization. Some pigments confer advantages in ecological dispersion. For instance, the red pigment prodigiosin increases cell concentration in the aerosol produced by bursting bubbles (Syzdek 1985). Pigments are also involved in the development and stability of fungal spores and bacterial endospores. Studies demonstrated that pigment synthesis has provided an evolutionary advantage during sporulation, protecting the spores against the harmful radiation (Moeller et al. 2005, Halaouli et al. 2006). We expect that microorganisms following ruderal strategies make less use of pigments than competitors or stress tolerators because the disturbance might increase the risk of death before the pigment synthesis.

Pigments contribute to the differentiation of resource-use strategy with profound implications on the ecosystem structure and function. According to the coexistence theory, species should vary their utilization of resources (e.g., nutrients, water, light) to coexist in the same habitat (Chesson 2000). In other words, the more microorganisms vary in resource utilization, the greater the range of competitive abilities. Thus, the presence of different pigments in a SAB community has consequences on the diversification of resource-use strategies, which enables different microorganisms to stably share a habitat, explaining the dazzling biodiversity of SABs. For instance, photosynthetic pigments cover the different regions of the solar light spectrum. The utilization of the light spectrum offers opportunities for niche differentiation and the coexistence of microorganisms with different light color absorbance (Stomp et al. 2004). A recent study carried out by Spaak and De Laender (2021) revealed that the combination of photosynthetic pigments in phytoplankton—a multispecies biofilm—is necessary for the species coexistence with a 40% increase in productivity compared to the single-pigment biofilm communities. Thus, having such a wide range of pigments provides SAB communities enormous flexibility in tuning cellular responses to cues such as light and redox conditions.

Moreover, microorganisms sharing the same habitat can use pigments to different degrees (e.g., modulating levels of the phycobilisome), and in pursuit of different resource strategies (e.g., promoting melanization that affects osmolytes exchange), leading to a redistribution of resource demand over space and time. In addition, pigments can drive facilitation (any interaction where the action of one microorganism benefits another) by boosting the growth of intrinsically less fit or more vulnerable microorganisms while relieving facilitators from the competitive pressure (Zélé et al. 2018).

Wierzchos and colleagues (2015) observed a succession of organized colored horizons of algae and cyanobacteria within gypsum deposits in the Atacama Desert: orange-colored algae cells close to the surface, green algae and cyanobacteria just below the orange layer, and a dark cyanobacterial layer at the bottom of the gypsum deposits in the hypoendolithic habitat (pore spaces in the undermost layer of the rock). The analyses revealed an enhancement of carotenoids and depletion of Chl in the orange cells in the cryptoendolithic habitat (pore spaces beneath the rock surface), suggesting that Chl-containing microorganisms took advantage of the protective carotenoids layer to escape from intense solar irradiation. The sunscreen pigment scytonemin was detected at the bottom of the gypsum deposits colonized by dark cyanobacteria. The compact and thick bottom layer of the gypcrete—generated by gypsum dissolution-recrystallization and water evaporation—has a high UV transmission rate that promoted the synthesis of scytonemin. Altogether, these results show that the spatial distribution of pigments can be linked to different colonization zones and microorganism types.

The differences in timing and speed of microbial responses to environmental fluctuations, including resource-use patterns, are found to underlie the ecosystem resistance and resilience. Therefore, pigments may stabilize the SAB communities that are exposed to extreme events predicted to occur more frequently in the future. Overall, pigments may promote biodiversity, coexistence, and resilience through increased resource access, increased habitat heterogeneity, stress reduction, service sharing, and facilitation. These processes are expected to provide niche and fitness differences necessary to uncover the ecological mechanisms responsible for diversity-function relationships.

5.6. Outlook and future directions

Pigmentation is a ubiquitous trait among SAB-dwelling cells as it increases their fitness and plays a key role in the cells' life-history strategies. Although pigments are instrumental in supporting microbial life, there are clear gaps in our understanding of pigments' physiological function and SAB structure and activity that need to be filled. Many questions arise: the correlation between SAB features and pigment-based color, the physiological responses that are mediated by pigments; the change in pigmentation over time and space; the genetics of pigment-based coloration; potential crosstalk and networks between pigment patterns and cell-to-cell communication; and the interplay between pigmentation and biogeochemical processes such as nutrient cycling.

A major hurdle in the field of pigments ecology is the experimental determination of the pigments' physiological and ecological functions and their relationship with the SAB community. Unfortunately, most research on this topic investigated pigment functions by comparing non-pigmented and pigmented isolates or by studying the properties of purified pigments. Therefore, other factors that are different between the strains, or the use of non-physiological concentrations of the pigment, might have influenced the phenotypes that were ascribed to the pigment. Furthermore, despite the knowledge of SAB community composition and SAB-relevant pigments, the role of different pigments in the ecology of biofilms at the mineral-air interface is still hard to understand. The major issue relies on the different methods used until now to characterize SABs (microscopy, cultivation, omics), which make comparisons between data of different articles quite challenging to interpret. Thus, standardized investigations are needed to untangle complex pigment-SAB relationships observed in the field. Recently, Lee and colleagues (2021) developed an automated sorting platform that separates the individual cell from the microbial communities based on their Raman spectra. Sorted cells can be further analyzed by single-cell omics, coupling the phenotypic observation of the pigments to the molecular characterization of SAB-dwelling cells.

Multi-omics approaches are revolutionizing the way we study the structure and function of SAB communities. However, these molecular investigations required expensive equipment and qualified workers to execute the analyses and process the data. By contrast, color measurements (e.g., portable spectrophotometers) are simple, cheap, non-invasive, and not destructive. The groundbreaking idea would be to create a database where to collect multidisciplinary datasets including molecular, pigments, and color data. Then, data-analysis techniques and predictive algorithms can be applied to establish the correlation patterns between color profiles and SAB features. In this way, researchers and stakeholders can simply measure the SAB's color, and from this analysis infer the composition and activity of the microbial community at the mineral-air interface (Fig. 5.3). If we can use pigment-based color to predict SAB composition and/or behavior under different scenarios, we can better manage the microbial adaptation to anticipate climate change, habitat loss, invasive species, or emerging infectious diseases. For instance, the contribution of microbial pigmentation in heat and water absorption is relevant not only for studying geographical patterns of SAB communities but also for estimating heat flow and physiological performance after a perturbation. Again, pigmentation may also help in answering important questions surrounding SAB ecology such as the shaping of ecosystems by microbial communities via color-mediated processes. Color can also indicate the effect of ongoing climate changes on microbial communities. For instance, the production of dark pigments is an ancient adaptation mechanism for obtaining heat from solar radiation and could be an important variable in modeling climate change. SABs in colder climates darken themselves to capture more heat from the sun and improve their ability to survive (Cordero et al. 2018). These results suggest that dark pigmentation could help to predict whether a microorganism can survive at a given latitude as temperatures increase. However, dark pigmentation not only responds to climate change but also enhances it. As these dark pigmented SABs developed, they may set up a positive reinforcing loop in which the colonized area becomes warmer, speeding the glacial melt. There is already evidence that as glaciers melt, dark microbial colonies bloom in the meltwater (Azzoni et al. 2016).

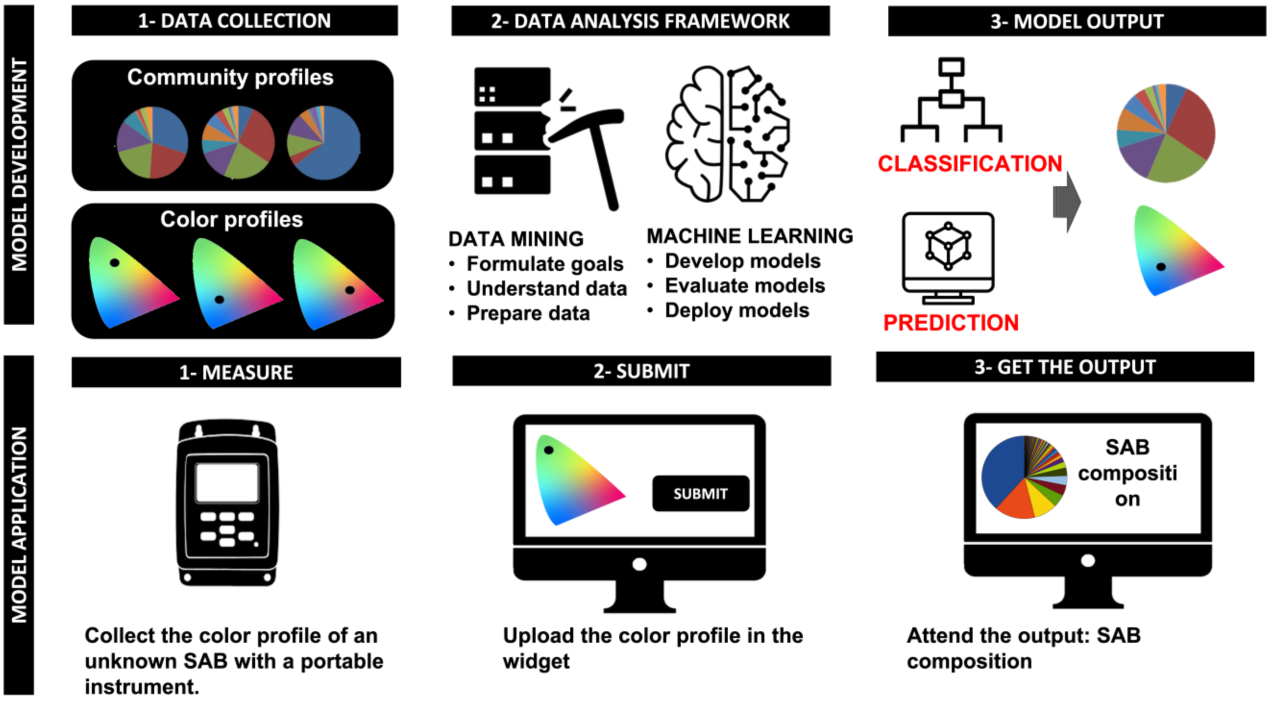


Figure 5.3: Use of color profiles to predict SAB community structure. Data-analysis techniques and predictive algorithms can be applied to establish the correlation patterns between color profiles and SAB composition. Researchers and stakeholders can simply measure the SAB's color, and from this analysis infer the composition of the SAB community.

The diverse structures and multifaceted roles of pigments make these molecules attractive targets for synthetic biology. The current trend is to use light to control biological circuits at the transcription level and to exploit wavelength-sensitive proteins. Bacterial behavior programming using light has shown great promise because of minimal toxicity and rapid activation/deactivation kinetics compared to chemical inducers. Thus, it will be possible to regulate biosynthetic pathways or modify the structure and function of a SAB using a spectrum of individual light colors. Furthermore, there is a growing interest in engineering photosynthetic proteins as a component of biohybrid devices for solar energy conversion that is environmentally sustainable, efficient, and versatile. Recently Liu et al. (2020a) reported self-assembling chimeric photosystems, which employed a hybrid Chl/BChl solar energy conversion system for polychromatic solar energy harvesting and conversion. This approach was inspired by synthetic biology, where different pigments are exploited to create a programmable polychromatic photosystem. The photosystems are interesting materials for biohybrid devices with applications in photoelectrochemical solar energy conversion, fuel molecule synthesis, energy storage, biosensing, touch sensing, and photodetection.

The pigment-based ecology is a field that exemplifies modern research because it deals with a multifactorial phenomenon—pigmentation—that needs an interdisciplinary, technology-driven, multilevel, and integrative approach. Microbiologists, ecologists, biochemists, biophysics, and evolutionary biologists sharing a similar interest in pigmentation should come together to study the mechanisms of pigment production and perception, their intricacies of function, and their patterns of evolution. Mathematicians should incorporate these multidisciplinary datasets into new predictive models to test hypotheses about the role of SAB pigmentation (or SAB color) in biogeochemical cycles, biodeterioration vs. bioprotection of stone heritage, ecosystem productivity, and climate change. The synergistic interplay among different disciplines will produce novel transformative ideas that will not only enrich our instinctive curiosity about colors but also provide a scientific basis for engineering SAB growth and obtaining colored biomolecules for artistic and biotechnological applications. Interdisciplinary studies of the functional attributes of pigments at the mineral-air interface will promote the development of novel (bio)indicators for land degradation and desertification based on the color profiles of rocks and soil crusts' SABs. We are at the frontier of a new era in color science, and the interdisciplinary nature of this collaborative enterprise holds enormous promise for tackling today's global challenges, such as the management of natural and human-made ecosystems and the mitigation of climate change.

BOX 1: Color vision in microorganisms

Just because we see through our eyes, it does not mean that eyeless microorganisms cannot perceive color. Perceived color depends on how an object absorbs and reflects light of a certain wavelength. As light impinges on a surface, the surface absorbs and reflects some wavelengths. The reflected light arrives in our retina, where it is transformed into electrical signals to be interpreted by our brain. So, we do not really "see" color, but rather signals transmitted to our brain, which are converted by photoreceptors in our retinas.

Similarly, microorganisms can use their photoreceptors to transform the reflected light from a surface into a signal that indirectly informs the microorganisms about the substrate color. Photoreceptors are proteins covalently bonded to light-absorbing pigments called chromophores. Upon receiving the light signal, chromophores either change their conformation or undergo photoreduction to trigger biochemical signals for the transduction cascade (Fraikin et al. 2015, Multamäki et al. 2021). It is well known that microorganisms have evolved a variety of tuned photoreceptors to sense and respond to the intensity, quality, and directionality of light. Putative light-sensing proteins are ubiquitously encoded in the genomes of chemotrophic, non-photosynthetic bacteria and fungi (Herrera-Estrella and Horwitz 2007, van der Horst et al. 2007, Corrochano 2019).

Members of the phylum Cyanobacteria encode a vast array of photosensors, such as cyanobacteriochromes. Remarkably, the cyanobacteriochromes can be activated by different wavelengths that cover almost the entire light spectrum, from UV to near-infrared (Bhaya 2016). For instance, *Nostoc* spp., a typical cyanobacterium of the mineral-air interface, has a red/far-red photosensor containing a phycocyanobilin chromophore that leverages light-triggered photoisomerization of bilin-bound chromophores to regulate gene expression, movement, and cell-to-cell communication (Xu et al. 2019, Moreno et al. 2020). The cyanobacteriochromes Cph1 and Cph2 contributed to the growth of *Synechocystis* in response to distinct wavelengths and intensities of light. Cph1 supports growth in far-red light and Cph2 modulates growth under red light (Fiedler et al. 2004). The cyanobacteriochrome IflA from the cyanobacterium *Fremyella diplosiphon* impacted the growth in response to ratios of red/far-red light in the environment (Bussell and Kehoe 2013).

Non-photosynthetic microorganisms such as fungi can sense near-ultraviolet, blue, green, red, and far-red light using up to 11 photoreceptors and signaling cascades to control gene expression and thereby improve fungal adaptation to environmental conditions (Yu and Fischer 2019). The genomes of black fungi belonging to the Eurotiomycetes, Dothideomycetes, and Lecanoromycetes, including lichen-forming species and rock-inhabiting extremotolerant species, contain multiple photoreceptors, suggesting that photoregulation is an important trait of fungal fitness at the mineral-air interface (Schumacher and Gorbushina 2020).

Although microbial photoreceptors are well known, only a few studies focused their attention on photoreceptors and color vision in microorganisms, especially at the mineral-air interface.

The first attempt to illustrate the response of a microorganism to colors was carried out by Fernandez-Rodriguez et al. (Fernandez-Rodriguez et al. 2017). They designed a genetic circuit that enables *E. coli* to use various wavelengths of light to control the multiple gene expression. The circuit consisted of 18 genes for photosensors, biological circuit breakers, and enzymes to produce red, green, and blue pigments. Once the bacterium was exposed to the colors red, green, and blue, the genetic circuit activated the production of the corresponding pigments. Thus, by exposing the engineered *E. coli* strain to colored images, bacterial "photographs" could be obtained. This research demonstrates that bacteria can recognize a variety of colors by producing pigments that are the same color as the (reflected) light they are exposed to. Engineering bacteria with color vision could be useful in fine-tuning the control of biofilms in different medical and industrial settings.

Gambino et al. (2019) investigated the effects of 4 surface colors (white, red, blue, and black) on the development of a cyanobacterial SAB. They found that the surface color impacts SAB formation, where white and red substrates produced more biomass than blue and black. Differences in SAB growth were correlated to the differences in both quality (spectral composition) and quantity (photon flux) of the reflected light. Indeed, the biofilm growth on the white-bottom plates was supported by the highest intensity and the full spectrum of light. Meanwhile, the biofilms on the blue- and black-bottom plates experienced the lowest intensity and the filtered spectrum of light, which changed the red/far-red ratio received by the cells.

The influence of color on the primary bioreceptivity of granite to the terrestrial green alga *Apatococcus lobatus* (Trebouxiophyceae, Chlorophyta) was recently assessed (Sanmartín et al. 2020a). The results showed that granite with red hues favored the formation of subaerial microalgal communities compared with grey-hued granite. The red light reflected by the reddish surface is a well-known stimulus for photoreceptors in algae. The red/far-red light ratio increases photosynthetic yield stimulating chlorophyll production and cell growth in *Chlorella vulgaris* (Kula et al. 2014).

Altogether, these findings suggest that photosensors provide microorganisms with colored vision, consequently lending enormous capacity to fine-tune cellular responses to colored surfaces. Thus, even microorganisms can “see” colors.

BOX 2: Relevance of SABs' pigmentation traits in stone heritage conservation

Colorful microorganisms in form of SABs colonize the surfaces of outdoor stone monuments, mural paintings, archaeological surface findings, and rock art (Figure 2). Gaylarde and Gaylarde (2005) analyzed 230 SABs on a wide variety of building substrates around the world. They showed that phototrophs were the most abundant biomass in both Latin America and Europe, followed by pigmented fungi. Thus, pigmentation is one of the most important phenotypic traits of SABs on monuments that reflect environmental adaptation to lithic surfaces in extreme conditions.

Thanks to the cyanobacteria predominance in many SABs on monuments, scytonemin is a widespread sunscreen pigment that allows microorganisms to self-protect from harmful UV and to survive desiccation before wetting and reanimation (Keshari and Adhikary 2013). Cappitelli et al. (2012) reported that some areas of the National Museum of the American Indian building (Washington DC, USA) contained dark pigments with high content of scytonemin, a likely product of the cyanobacteria *Gloeocapsa* and *Lyngbya*. Similarly, scytonemin was more abundant than carotene and Chl*a* in all SABs sampled from eight historical monuments of Varanasi (India) (Pathak et al. 2017). Recently, Mondal et al. (2022) found the reduced form of scytonemin in the cyanobacteria *Brasilonema* sp. from the monuments of Santiniketan and Bishnupur (India).

Pigmentation can be affected by materials, locations, environmental conditions, structure, and dynamics of the microbial communities. Gaylarde and Baptista-Neto (2021) stated that dark brown or black stains and orange-yellow patinas can be produced by both bacteria and fungi, while the surface humidity strongly affects the SAB components and colors. For instance, the red color produced by the overgrowth of carotenoid-producing alga *Trentepohlia* was seen on North- and East-facing walls of the Rio Bec style Mayan buildings in Campeche state, Mexico. Grey/black biofilms, a color characteristic of UV-resistant cyanobacteria (mainly *Gloeocapsa* and *Chroococcidiopsis*), colonized the sun-exposed areas of the buildings. There was no evidence that stone degradation was occurring beneath the colored biofilms (Ortega-Morales et al. 2013). Two areas of a fort in Niteroi, Rio de Janeiro showed different colored SABs according to the climatic and positional factors: a dark green-brown biofilm below leaking pipes containing filamentous anoxygenic phototrophs, while a grey-pale-green dry biofilm was mainly non-photosynthetic Proteobacteria (Ogawa et al. 2017).

Within biodeterioration processes, a rosy alteration is a widespread phenomenon on both lithic and painted surfaces. The phenomenon was related mainly to the presence of photosynthetic microorganisms (cyanobacteria and algae) and extremophiles (alkaliphilic, thermophilic, and halophilic microorganisms). The rosy discoloration masking Luca Signorelli’s frescoes in the Orvieto Cathedral (Italy) for many years was due to the cyanobacterial phycoerythrin (Cappitelli et al. 2009). The red-orange SABs are also often associated with the green algal order *Trentepohliales,* an abundant producer of ß-carotene and haematochrome pigments (Ortega-Morales et al. 2013, Bartoli et al. 2019). In the Crypt of the Original Sin (Matera, Italy), bacterioruberins—the carotenoids of the extremophile *Rubrobacter radiotolerans*—were responsible for the chromatic alteration of the medieval frescoes (Imperi et al. 2007). In France, a rosy discoloration was observed on the stone steps in the main spiral staircase of the Sully-sur-Loire castle donjon (Leplat et al. 2019). The pink discoloration was associated with the presence of carotenoids and the genus *Nitriliruptor*, while *Rubrobacter* was the main genus identified in the white area. Since *Nitriliruptor* is not known to produce pink pigment, the authors suggested that bacteria belonging to *Rubrobacter* genus—which was associated with rosy discolorations in the past—may have been the initial producer of the pink pigment, before being replaced by other bacteria. De Felice et al. (2010) found many halophilic bacteria while studying the massive rosy discoloration that affected the most superficial layer of the 18th century Italian 'Palazzo De Francesco' made of tuff. Among these halophilic bacteria, the Bacteroidetes *Salinibacter ruber* that dwells in the salt efflorescence of the monument was likely to incur the rosy discoloration by producing carotenoids. Direct examination revealed pink pigmentation in Humor Monastery, Tismana Monastery, and the refectory of Hurezi monastic complex from Romania on the pictorial layer and mortar caused by halophilic carotenoid-producing *Halobacillus* sp., *Halobacillus naozhouensis*, and *Nesterenkonia* sp. (Gomoiu et al. 2017). Halotolerant and halophilic bacteria with brilliant rosy to purple colors were responsible for the rosy discoloration of other European monuments (Ettenauer et al. 2014, Cojoc et al. 2019). The common link among different monuments colonized by pink biofilms seems to be the crystallization of salts aided by water infiltration, which creates an optimal condition for the growth of the halophilic microorganisms (Ettenauer et al. 2014). Carotenoids are known to act as membrane stabilizers against salt stress (Seel et al. 2020).

Although pigments are instrumental in supporting microbial life (e.g., protecting cells against solar radiation and oxidizing agents), the discoloration of stone surfaces is often disconcerting. Colors can mask the artist's or architect's intent or can be perceived as soiling, indicating poor management and maintenance. However, we should keep in mind that the discoloration is not due to soiling, but rather pigments that are produced by specialized microorganisms in a multifunctional community responding to external stimuli. Furthermore, not all colored biofilms are detrimental to the mineral substrates (Pinna 2014, Gulotta et al. 2018) and their removal may cause irreversible damage. In addition, the chemicals commonly used in cleaning the stone are harmful to humans and the environment, which may lead to the proliferation of antimicrobial-resistant microorganisms (Cappitelli et al. 2020). As an iconic example, the processional cloister of the Monastery of San Martiño Pinario (Santiago de Compostela, Spain) was colonized by a green highly-hydrophobic SAB that acted as a natural waterproofing agent for the building (Sanmartín et al. 2020b). This biofilm was mainly formed by *Apatococcus lobatus* (Chodat) J.B.Petersen (Chlorophyta), and it was proven to only have an aesthetic impact, without damaging or protecting the substrate. The researchers recommended not removing the existing green SABs according to the 'minimal intervention' concept. The 46,000-year-old Bradshaw rock art, often exposed to sun and rain, can be vivid with high contrast, even though it has never been repainted. Pettigrew et al. (2010) reported that the original paint is no longer present in this rock art but has been replaced by a living pigmented SAB, which naturally contributes to the longevity and vividness of these ancient paintings. The nutrients contained in the original paint kick-started a mutual relationship between black fungi and red cyanobacteria. The fungi can provide water to the bacteria, while the bacteria provide carbohydrates to the fungi. The black fungi were identified as *Chaetothyriales*, an extremely conservative rock-adapted taxon that replicate by cannibalizing their predecessors in-situ but remained strictly within the art's boundaries.

Thus, while the presence of SABs on mineral surfaces implies current or past interactions with the substrates, its presence is not necessarily biodegradative as is frequently thought (Favero-Longo and Viles 2020). Since the correlation between SABs and stone decay is a matter of controversy, the proper understanding of the SABs' role is instrumental to the sustainability of stone heritage management. Since bio-pigments offer an indication of a specific condition of the SAB community, we hypothesize that SAB's color can be exploited to categorize the SAB's impact on monuments based on their deteriorative, neutral, or protective roles. To this end, a mathematical correlation between SABs' color fingerprints and multidisciplinary investigations on monuments—used to characterize the deteriorative, neutral, and bioprotective roles of SABs under different conditions—should be carried out. Once this correlation is established, heritage professionals could simply read in-situ the SAB's color and use it as a bioindicator of SABs' impacts on the stone. These same principles have been successfully applied in the food industry where fruit and vegetable colors are used to estimate maturity, sugar content, moisture content, and vitamin levels.

By exploiting the SAB color as a benchmark of stone biodecay, a simple and non-destructive monitoring tool with an easy-to-understand language (the color) will be provided to those who preserve the cultural heritage. In this way, it would be possible to prevent useless attempts of restoring and plan the most appropriate conservation strategies.

BOX 3: Biopigments in palaeobiology and astrobiology

Paleobiology—the investigations about the origin and evolution of life on our planet—has strong connections with astrobiology—the studies on the existence of life in other parts of the cosmos. Paleobiology and astrobiology are bound by their common interest in untangling the link between life and the environment. To this end, both disciplines seek to detect putative extinct or even extant life forms on the Earth and exoplanets by studying biosignatures—universal molecules considered as the fingerprints of the past or the present biological activity.

Microbial pigments can persist and be recognized over vast spans of geological time, resolving facets of palaeobiology and evolution. Gueneli et al. (2018) discovered molecular fossils of Chls, the porphyrins, from 1,1-billion-year-old marine black shales of the Taoudeni Basin (Mauritania). By measuring the N-isotope ratios of porphyrins retrieved in sedimentary rocks, the researchers were able to quantify the abundance of different phototrophs in the ocean, establishing the dominance of cyanobacteria over planktonic algae. This finding suggested that small cells at the base of the food chain had limited the flow of energy to higher trophic levels, potentially retarding the emergence of larger and more complex life in that ancient environment. Sforna et al. (2022) discovered in-situ the first evidence of Chls remnants in a billion-year-old multicellular algal microfossil preserved in shales from the Congo Basin. The result provides new clues about the evolution of eukaryotic phototrophy during the Precambrian and the diversification of primary producers in early ecosystems. Cui and colleagues (2020) reported that carotenoids from green sulfur bacteria dominated in Phanerozoic marine sediments, while cyanobacterial aromatic carotenoids—which have distinct chemical structures and occurrence patterns—dominated in phanerozoic lacustrine settings with low sulfate inventories. Before the investigation by Vinnichenko et al. (2020), the oldest indigenous biosignatures were claimed to be from the 1.64 Ga Barney Creek Formation in the northern Australian McArthur Basin. Vinnichenko et al. (2020) reported the discovery of degradation and isomerization products of carotenoids from carbonaceous shales of the 1.73 Ga Wollogorang Formation in the southern McArthur Basin. Thus, the findings extend the biomarker record of phototrophic bacteria by ~90 million years. Altogether, these results show how fossil microbial pigments, exemplified by carotenoids and Chls, capture the history of Earth’s aquatic primary producers. The succession of primary producers in the oceans is instrumental to understanding the marine ecology throughout the Earth’s history. Moreover, the role of pigments is crucial in the interaction between the host rock and the community, promoting the environmental conditions suitable for life and, at the same time, enhancing the processes related to rock decay. The latter can result alternatively in the stabilization or weakening of the rock support (Krumbein et al. 2003). Many studies also explore the stability of pigments over time, suggesting that pigmentation of rock surfaces can survive after the disappearance of the SAB community as in the case of rock varnish (Dorn, 1998). Within rock varnishes and crusts, organo-mineral interactions occur, resulting in the weathering of minerals of the rock support and neoformations of secondary minerals (Dorn and Oberlander 1981a, 1981b, Dorn 2007). Such evidence for organo-mineral interactions can be found in fossil rock varnish millennia after the decay of the

SAB community as accumulations of biomineralization products and/or amorphous organics, or as casts of pristine biological features as hyphae (Dorn 2007, Zerboni 2008, Mergelov et al. 2012); in both cases, the result is the stabilization of the color of the rock surface over time.

Many of the rocks on Mars' surface display reddish to dark shiny surface coatings resembling Fe- and/or Mn-rich terrestrial rock varnish. In desert rock varnish, a combination of dark pigments with metal oxi-hydroxides has been often observed (Dorn, 1998). It has been demonstrated that most of the color of rock coatings is produced by microbial pigments firmly bound to the rock particles (Dorn and Oberlander 1981, Dorn 2007). Thus, pigments produced by varnish microorganisms may play important roles in coating morphogenesis and distribution (Kuhlman et al. 2006). This is not surprising since some pigments, especially dark phenol-containing pigments, are associated with the accumulation of metals (Hong and Simon 2007). Krumbein (Krumbein and Jens 1981, Krumbein et al. 2003) studied the accumulation of dark pigments and Fe/Mn oxi-hydroxides over time, confirming that it takes 10^3 years for a thick desert varnish to form. Based on these considerations, if desert varnish on Earth has mostly a biological origin, the identification of corresponding biomarkers for extraterrestrial life could foster studies on Mars. These biomarkers can be the biopigments. Preserved biopigments not only yield interpretations on the appearance of ancient microorganisms but can also potentially elucidate their ecology and behavior, giving clues to potential targets in the search for extraterrestrial life (Lindgren 2016). Colored microorganisms have been identified in many extreme environments of our planet, which are often considered analogous to Mars. Scholars are investigating extreme ecological niches on Earth (in hot and cold deserts, namely the Dry valleys of Antarctica and the Atacama) as analogous to Mars's surface. For instance, desert-inhabiting microcolonial fungi—which were discovered in the early 1980s (Krumbein and Jens 1981, Friedmann 1982, Staley et al. 1982)—are examples of eukaryotes with the bewildering capacity to adapt to extraterrestrial conditions. Microcolonial fungi synthesize a mix of UV-absorbing and antioxidant pigments (carotenoids, melanin, and mycosporines) that convey multiple stress resistance to desiccation, temperature, and irradiation changes. Recent work reported the persistence of fungal melanin in Martian rock analogs after exposure to space and Mars-like conditions (Pacelli et al. 2020). This finding suggested that the production of UV-protecting pigments can be part of microbial survival strategies on Mars. Bacteriorhodopsin may potentially serve as remote biosignatures for life outside the solar system. Since the bacteriorhodopsin shows a strong well-defined peak of absorbance at 568 nm, which is complementary to that of Chl pigments (~700 nm), it might be used to compare the remote signatures of rhodopsin-like phototrophy to those produced by Chl-based photosynthesis analogs on exoplanets (DasSarma and Schwieterman 2021). Thus, these retinal-based proteins are potential biosignatures for extraterrestrial life, whose unique spectral profiles can be caught by next-generation space-based telescopes. Non-photosynthetic pigments in the extremophiles can also be used as potential surface biosignatures for the exoplanets research (Schwieterman et al. 2015). Recently, Sharma (2020) used computational models to invent novel pigments and simulate their spectral profiles for the identification of extraterrestrial life.

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Chapter 6

Insights on bacterial structural and functional diversity related to the formation of coatings on Ethiopian rock art[5]

Abstract

Two open-air shelters with rock paintings in Ethiopia were investigated for bacterial structural and functional diversity related to rock coatings formation. Rock coating samples were taken close to but not directly from the rock art and prepared as thin sections for petrographic microscopy and scanning electron microscope (SEM) observation, or directly observed by SEM. Thin section petrography showed that all samples have a surface coating distinguishable from the substrate rock, with different thickness and textures. SEM coupled with energy dispersive X-ray analysis (EDS) revealed that chemical compositions of the substrate rocks are silicatic minerals, while some of the coatings were identified as gypsum, calcium oxalate, calcium carbonate and phosphorus containing minerals. Genomic material were extracted from the coating samples and 16S rRNA genes were sequenced to identify bacterial communities. 16S data was processed with PICRUSt to predict gene functions. Gypsum coating samples have the least bacterial diversity or no community at all, thus there is the possibility that the gypsum was formed abiotic or maybe anthropogenic. However, one gypsum coating sample has high number of genes for ureolysis that leads to the precipitation of calcium carbonate. Bacterial taxa and genes capable of calcium carbonate precipitation are also found in most of the other coating samples. The results indicate that bacterial communities on rock art coatings contain genes capable of mineral dissolution and precipitation, thus the bacteria are potentially involved in the formation of rock coatings, which points out their importance in the long-term preservation of rock art.

Keywords: Rock coatings, thin section petrography, SEM-EDS, 16S rRNA sequencing, functional gene prediction, biomineralization

6.1. Introduction

Open-air rock art is an important cultural heritage that allows us to understand people from past societies. These works, including pictographs and petroglyphs, are usually located in open shelters that are exposed to natural and anthropogenic weathering. Natural weathering is a combination of abiotic and biotic agents, deteriorating the pigments and the rock substrate, sometimes accompanied by the formation of coatings over the remaining art which obscure its visual appearance. Rock coatings can be centimeters to nanometers in thickness, and varies in appearance and elements, such as dark desert varnish containing Mn and Fe, rock surface case hardening caused by silica glaze, iron oxides and organics embedded dark streaks, to name a few. Origins of coatings are biotic and abiotic, and the formation of coatings could be an ongoing process of deterioration or protection of the rock surface

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[5] Wu, Y.-L.; Villa, F.; Zerboni, A., Gallinaro, M.; Cappitelli, F. “Insights on bacterial structural and functional diversity related to the formation of coatings on Ethiopian rock art.” (in progress)

(Dorn, 1998; Dorn et al. 2017). Water, temperature and pH interact with microorganisms in the environment and eventually lead to lithic alteration. To understand the state of the rock surface and preserve the rock art, it is essential to investigate the microbial community living on the rock art.

From the geological point of view, mineral crusts and weathering residues from rock art and open shelters have been investigated with a variety of geochemical and mineralogical techniques including Raman, SEM-EDS and XRF (Green et al. 2017; Hernanz et al. 2014). (Peña-Monné et al. 2022) explained weathering of sandstone and accretions in rock art shelters mostly through abiotic factors, while increasingly authors are concerned about microbiome influence on rock art. In Australia, petroglyphs were carved into rock varnish that contains Mn and Fe from environmental or bacterial precipitation, but acid rain formed during industrial development could change the pH and microbiome of the rock surface (Gleeson et al. 2018). Also in the Kimberley region of Australia, (Green et al. 2021) recognized microbial origins of oxalate-rich dark-colored accretions from rock art shelters and suggested their use for radiocarbon dating.

In the context of built stone heritage conservation, microorganisms have been held responsible for decomposing the lithic surface and generating colored biofilms. Previous research emphasized biochemical functions related to the deterioration of stone monuments (Warscheid and Braams 2000). Over the decades, the influence of microbial communities has been recognized and an increasing amount of literature have implemented next-generation sequencing (NGS) in a wide range of cultural heritage (Ding et al. 2020). These studies characterize biofilms as agents of deterioration on stone, focus on sulfur and nitrogen cycles that potentially produce mineral dissolving acids, and analyzed the microbial communities' functions with metagenomic methods (Ding et al. 2022; Gu and Katayama 2021). However, microorganisms are also capable of encrusting the weathering rock surface and providing some form of stability. For example, on built stone monuments, lichens and fungi are noticed that after initial colonizing, the organisms and hyphae bind with the mineral that improved surface stability, and decreased water penetration which is also a cause of stone erosion. Lichen and fungi are also known to produce oxalate crusts which are almost insoluble in water, providing long-lasting surface protection (Concha-Lozano et al. 2012; de la Rosa et al 2013; Gadd and Dyer 2017; Pinna 2014).

On the other hand, the development of NGS technology enabled researchers to extract taxonomic and genomic data for non-culturable microorganisms that could not be isolated with culture-dependent methods in the past. Metagenomic and functional gene analysis were also used in geomicrobiology research of cave basalts, and indicated that prokaryotic communities are involved in the biogeochemical cycling of major elements (Gonzalez-Pimentel et al. 2021). We acknowledge that abiotic precipitation of minerals such as calcium sulfates and phosphate sulfates occur largely in nature (Sauro et al. 2014; Van Driessche et al. 2019), while at the same time researchers are also exploring bacterial participation, for instance the mobility of silica deposit in caves (Sauro et al. 2018) and biomineralization of calcitic cave speleothems (Dhami et al. 2018). Unlike cave rock art, open-air rock arts are located in drier environments and are assumed to host a different set of microbial communities from inside the cave (Gonzalez et al. 1999), such as more resistant to heat and UV radiation. Take rock varnish, for example, a dark-colored rock coating that usually forms in arid desert environments. (Esposito et al. 2015) compared the microbiome of varnished and non-varnished rock samples and suggested that the high Fe and Mn varnish favors anoxygenic autotrophy and highly specialized bacteria. In their research, structures that indicate mineral deposition mediated by microorganisms were found on both types of samples. (Lang-Yona et al. 2018) employed DNA shotgun analysis and reported the microbiome mainly consisted of bacteria, but suggested an indirect rather than direct involvement of the bacteria in desert varnish formation. In the Negev Desert, (Nir et al. 2021) applied shotgun analysis to petroglyphs carved into rock varnish, and found that 95.7% of the sequencing data were phylogenetically assigned to bacteria. Their results indicated that the microbial community was potentially associated with the dissolution and precipitation of minerals. The bacterial taxonomy of open-air rock art in Spain was identified by 16S rRNA sequencing, and suggested the dominating phylum Firmicutes might have a protective effect (Roldán et al. 2018). These data indicate that rock coatings and open-air rock art host bacterial communities with multiple roles that are worth further investigation.

Our goal with this research is to further investigate the bacterial structure and function from biofilms inhabiting Ethiopian rock art, with emphasis on how these bacteria are associated with the formation of mineral rock coatings. With this study we wish to understand more about biogenic rock coatings on open-air rock art that would establish the basis to understand its long-term preservation, and to the best of our knowledge the only research line of eastern African rock art carried out from a biological perspective, by a collaboration of archaeologists, geologists and biologists.

6.2. Materials and methods

6.2.1. The rock art sites

The open-air rock art shelters are located in southern Ethiopia of the Borana region. One of the shelters is close to the town of Yabelo and therefore will be referred to as Yabelo in this text. The other rock art shelter will be referred to as Bor. More rock art sites are situated in this region and archaeological missions in recent years proved their potential for further research (Gallinaro et al. 2018). The Yabelo site is included in a series of granite shelters named Dhaka Kura (Crow's Rock) by the local people, who are still actively using the sites for animal herding. Rock paintings on the wall of the shelters exhibit an array of geometric shapes along with domestic and wild animals. The preservation of the paintings is urgent due to deterioration of pigments and rock substrate, while running water and accretions cover the visibility of paintings. The Bor site is another rock shelter south of Yabelo developed in low-grade metamorphic rock, with camel, anthropogenic figures and wild fauna painted in black or white. This site is more severely damaged by modern graffiti made of charcoal and chalk that covered the paintings.

Both sites show the advantage of hosting rock coatings with a variety of colors and texture in close proximity to the same shelter, which makes them naturally made labs that provide the opportunity to investigate different types of rock coatings from the same bulk rock under the same environment. To understand the preservation state of the paintings, several samples were removed from the rock art shelters with chisels and scalpels.

6.2.2. Thin section petrography and SEM-EDS

Yabelo samples 1, 3, 5, 7, 8 and 9 were cut perpendicularly to the surface and polished into thin sections mounted on glass slides according to the protocol described in (Zerboni 2008). Micromorphology of rock coatings in the thin sections was examined with an optical petrographic microscope (OPTIKA B-1000 POL) under plane-polarized light (PPL) and cross-polarized light (PXL). Afterward the thin sections were carbon coated and observed with a JEOL JSM-IT500 scanning electron microscope (SEM) coupled with energy dispersive X-ray analysis (microprobe, EDS) for chemical characterization with accelerating voltage 20kV. All measurement data normalized to 100 weight % element. For samples Yabelo 1, 3, 5, 7, 8, 9 and Bor 1, 2, 7, small pieces of coating samples were directly mounted and carbon coated to observe surface and profile morphologies with SEM and EDS measurements.

6.2.3. DNA extraction and 16sRNA sequencing

Total genomic DNA from the surface coating samples was extracted with DNeasy® PowerBiofilm® Kit (QIAGEN) according to manufacturer's protocol and the quantity of DNA was measured with a Qubit 4 Fluorometer. Amplification was performed with nested PCR using CS1_515FB/CS2_806RB primers for bacteria. The 16S rRNA genes were sequenced using the NGS Illumina platform.

6.2.4. Functional gene prediction with PICRUSt

Bacterial calcium carbonate precipitation could be induced by photosynthesis, ammonification, denitrification, dissimilatory sulfate reduction and ureolysis (Zhu and Dittrich 2016). Bacterial ureolysis is a well-understood mechanism that increases the pH by releasing ammonia which creates a favorable environment for calcium cation and carbonate anion precipitation (Hammes and Verstraete 2002). The urease nickel metalloenzymes are composed of three subunits encoded by genes *ure*A, *ure*B and *ure*C, while accessory proteins and urea transporters are encoded by *ure*D, *ure*E, *ure*F, *ure*G and *ure*H (Hoffmann et al. 2021). Genes encoding ammonification, denitrification and dissimilatory sulfate reduction (Llorens-Marès et al. 2015) were added with urease encoding genes as indicators of calcium carbonate precipitation, but ammonification genes did not yield significant reads and were removed. Genes encoding carboxysome proteins are included because calcium carbonate formation is associated with carboxysome proteins in cyanobacteria (Blondeau et al. 2018; Kamennaya et al. 2012; Li et al. 2016; Walter et al. 2021).

Genes associated with other types of bacterial biomineralization found in the literature were selected as indicator genes. In the case of calcium phosphate minerals such as apatite, phosphate transport system protein *pho*U was included because a random transposon mutant failed to precipitate apatite when there was an insertion in this gene (Fishman et al. 2018). (Cosmidis et al. 2015) observed that the production of hydroxyapatite was upregulated in *Escherichia coli* when the alkaline phosphatase *pho*A was overexpressed, and heterologously expressed *pho*X1 in *E. coli* induced the precipitation of calcium-phosphate mineral (Skouri-Panet et al., 2018). Table 6.1 lists the mineralization genes that could be assigned KO numbers and had positive reads from PICRUSt results.

Table 6.1. Genes involved in mineral precipitation based on literature

Precipitation	Method	Protein (gene)	Reference
Calcium carbonate	Dissimilatory sulfate reduction	adenylylsulfate reductase subunit A (*apr*A)	(Llorens-Marès et al. 2015)
		adenylylsulfate reductase subunit B (*apr*B)	
		sulfite reductace (*dsr*A)	
	Denitrification	nitrous oxide reductase (*nos*Z)	(Llorens-Marès et al. 2015)
		nitric-oxide reductase (*nor*C)	
		nitric-oxide reductase (*nor*B)	
	Ureolysis	urease subunit gamma (*ure*A)	(Hoffmann et al. 2021)
		urease subunit beta (*ure*B)	
		urease subunit alpha (*ure*C)	
		urease accessory protein (*ure*E, *ure*F, *ure*G, *ure*D, *ure*H)	
	Cyanobacteria photosynthesis	carboxysome protein CcmL (*ccm*L)	(Walter et al. 2021)
		carboxysome protein CcmM (*ccm*M)	
		carboxysome protein CcmN (*ccm*N)	
		carboxysome shell protein CsoS1 (*eut*M)	
		putative carboxysoe peptide A (*eut*N)	
Calcium phosphate	Influence expression of calcium phosphate precipitation	phosphate transport system protein (*pho*U)	(Fishman et al. 2018)
		alkaline phosphatase (*pho*A)	(Cosmidis et al. 2015)
		uncharacterized protein (*pho*X1)	(Skouri-Panet et al. 2018)

6.3. Results and discussion

6.3.1. Thin section petrography

Thin sections of Yabelo samples under a petrographic microscope showed a dark colored coatings growing on the surface of rock substrates, that could be grouped into three different morphologies. The first type of coating is exceptionally thick and exceeds 100µm on Yabelo 3 (Fig. 6.1a), appearing black and untransparent. The second type of coating on Yabelo 5 (Fig. 6.1b) is also dark and fluffy and grows on another transparent surface layer corresponding to an older coating. The third type of coating can be found on Yabelo 7 which is approximately 30µm thick (Fig. 6.1c), which is similar in morphology to the coatings on Yabelo 1, 8 and 9.

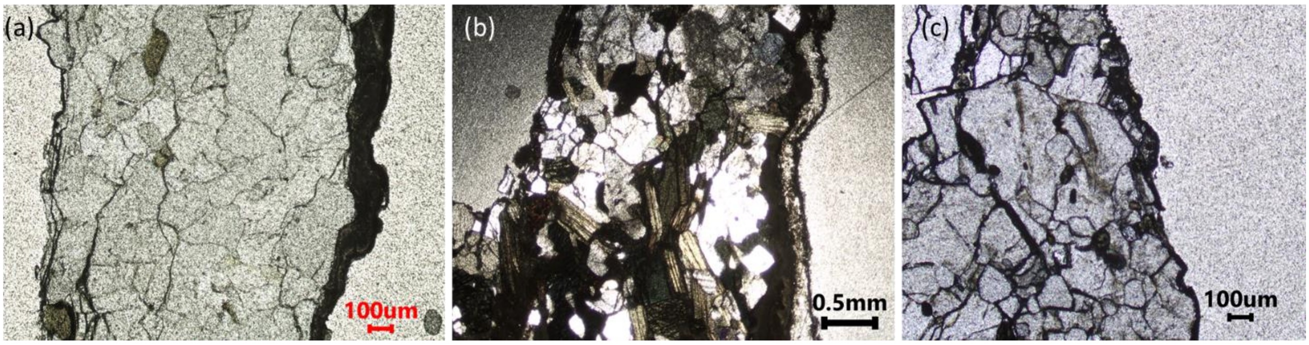

Figure 6.1. Thin sections of rock samples under plane polarized light (PPL), are listed as Yabelo 3 (a), Yabelo 5 (b) and Yabelo 7 (c). Surface coatings are seen on the right side of the figures.

6.3.2. SEM imaging and EDS analysis

SEM observations on the surface, profile and thin section of samples revealed coatings with different morphologies. EDS analyses indicated that these coatings have a variety of chemical constituents. On the profile of Yabelo 1 mineral grains of the coating could be seen growing on a homogenous substrate (Fig. 6.2a), EDS analysis showed the base rock on the left is aluminosilicate while the coating on the right (Fig. 6.2a, spc 21) contains a mineral that is mostly Ca. The surface of Yabelo 3 is covered with a coating that is consistent with thin section analysis in thick and homogenous in texture. This coating is high in Ca and S (Fig. 6.2b, spc 36) which could be gypsum. The surface of Yabelo 5 has a coating containing Si, Al, Mg, K and Fe (Fig. 6.2c, spc 48), but materials composed of mainly C and N were also found on the surface, which could be organic (Fig. 6.3a). This sample was taken from the lower part of the shelter and possibly contaminated by human and animal activities. The high N compound could also be derived from the urine of hyraces active at the Yabelo site (Gallinaro and Zerboni 2021) and South African rock art sites (Prinsloo 2007).

The surface of Yabelo 7 has a smooth morphology (Fig. 6.2d, spc 23) containing Si, Al, Mg, P, Ca, K and Fe. Interestingly, the surface also has small oolitic balls (figure 6.3b) that are high in Ca amid the aluminosilicate base rock. Diaz et al. (2014) assessed functional gene diversity of oolitic sands from Great Bahama Bank and suggested that calcium carbonate precipitation in oolitic environments is probably biologically influenced by microorganisms with diverse physiologies including oxygenic and anoxygenic photoautotrophs, oxygenic and anoxygenic heterotrophs, denitrification, sulfate reduction and ammonification. It would thus be interesting to consider that besides rock coatings, bacterial community on Yabelo 7 might also affect the formation of these oolitic balls.

The surface of Yabelo 8 has a coating with high Ca growing over the rock substrate. EDS analysis of the thin section showed that the base rock of Yabelo 8 is an aluminosilicate, while the coatings contain a high amount of Ca, a lesser amount of P and noticeable amount of Fe (Fig. 6.2e, spc 14). There are also organic structures such as filaments and membrane found on the surface of the sample whose origin is biological (Fig. 6.3c). The surface of Yabelo 9 has several different features. EDS analysis on a profile, in this case an area scan, showed the coating (Fig. 6.2f, spc 6) is mainly Si with Al, P, Ca, K and Fe on a silicate substrate. Other surface attachments include organic-looking matters containing K, Cl and S (Fig. 6.3d). The surface coating of Bor 1 has a homogenous appearance consisting mainly of S and Ca that indicate gypsum. White crystals containing P, Zn and lesser amount of Mg are embedded in the surrounding gypsum (Fig. 6.2g, spc 44). The surface of Bor 2 is also covered in gypsum-like coating (Fig. 6.2h, spc 9). Bor 7 has a silicate substrate and surface coating (Fig. 6.2i, spc 21) high in Ca with some Fe.

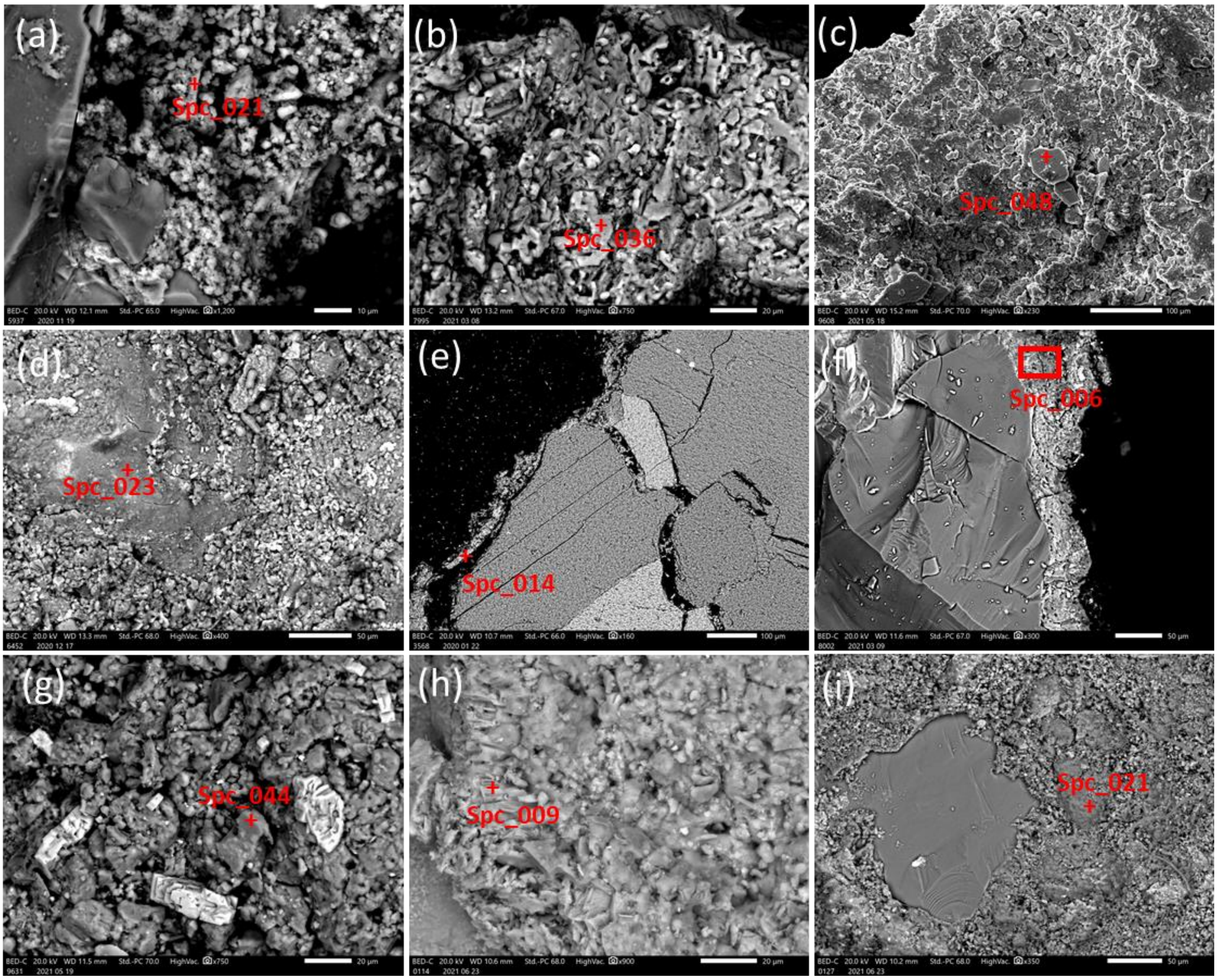

Figure 6.2. SEM images and locations of EDS spot analyses of profile on Yabelo 1 (a) with the coating on the right and rock substrate on the left, and different morphologies of surface coatings from Yabelo 3 (b), Yabelo 5 (c) and Yabelo 7 (d). A thin section of Yabelo 8 (e) reveals the coating partly separated from the substrate, while the profile of Yabelo 9 (f) showed the coating tightly connected. The surface coating of Bor 1 (g) is embedded with white crystals, coatings on Bor 2 (h) are heavily layered and Bor 7 (i) has coating with the substrate rock underneath revealed.

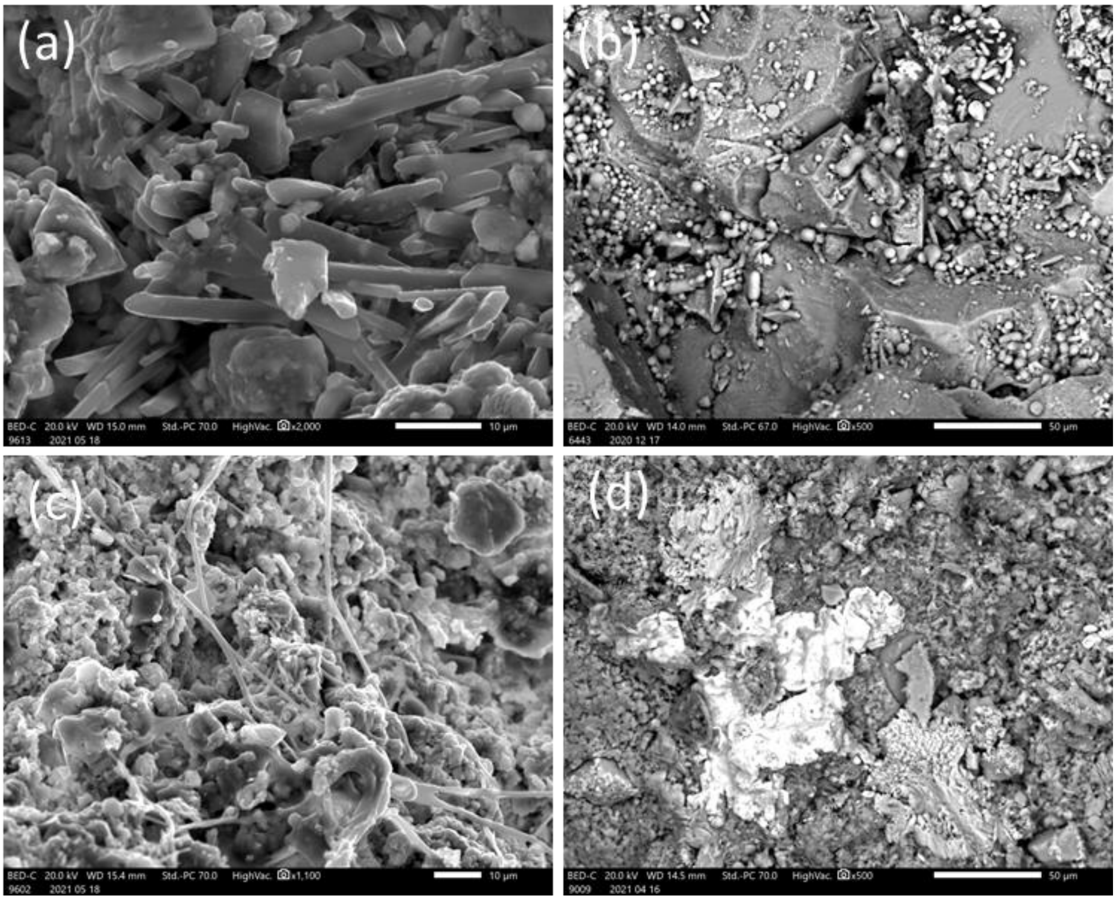

Figure 6.3. SEM images of different features on the surface of samples. Rod-shaped organic materials made of C and N were found on Yabelo 5 (a), small balls of high Ca on the rock substrate of Yabelo 7 (b), organic and biological filaments on Yabelo 8 (c) and attachments on Yabelo 9 (d).

Major elements detected by EDS and their relative abundance are shown in Table 6.2 as weight % elements. The surface coatings of Yabelo 3, Bor 1 and Bor 2 are mainly composed of Ca and S close to the atomic weight ratio of 40:32 which suggest it is a calcium sulfate, and the morphologies resemble that of gypsum. Coatings from Yabelo 1 and Bor 7 have distinguishing high level of Ca (31.70% and 26.45%) while Yabelo 7, Yablo 8 and Yabelo 9 show moderate content of Ca (5.36~14.11%), with crystal structures that resemble calcium carbonate or calcium oxalate. In the case of P, Yabelo 8 has the highest amount (9.51%) while Yabelo 9 contains 2.07% and most other samples contain minor amounts from 0.22% to 0.73%. Fe is present in noticeable amounts from 0.92% to 5.69%. Based on these observations, bacterial genes involved in elemental cycles of Ca, P, Fe, and S, especially calcium carbonate precipitation, were sought as marker genes within the functional genes predicted by PICRUSt.

Table 6.2. Major elements in surface coatings detected by EDS analysis, normalized to 100% weight element. nd=not detected

		Si	**Al**	**Mg**	**K**	**P**	**Ca**	**S**	**Fe**	**Ti**	**Cl**
Yabelo 1	spc 21	0.19	nd	0.28	0.63	0.60	31.70	0.18	nd	nd	nd
Yabelo 3	spc 36	0.26	nd	0.52	nd	0.73	18.38	15.92	nd	nd	nd
Yabelo 5	spc 48	12.28	6.15	5.95	3.28	nd	nd	nd	5.69	0.99	nd
Yabelo 7	spc 23	8.55	4.68	1.32	2.57	0.64	12.56	nd	4.39	0.26	1.91
Yabelo 8	spc 14	12.26	3.03	1.36	1.18	9.51	14.11	nd	2.23	nd	nd
Yabelo 9	spc 6	18.26	1.58	0.45	2.84	2.07	5.36	0.33	0.92	nd	0.25
Bor 1	spc 44	0.54	0.26	nd	nd	nd	32.52	24.00	nd	nd	nd
Bor 2	spc 9	0.24	nd	nd	0.27	nd	19.83	16.79	nd	nd	nd
Bor 7	spc 21	2.98	1.48	0.35	nd	0.22	26.45	0.52	1.09	nd	0.44

6.3.3. Bacterial community

On the Phylum level (Fig. 6.4), Yabelo 1 and Bor 7 have the most diversity with Actinobacteria and Proteobacteria as the most prominent phyla, while Yabelo 1 is the only sample hosting a significant community of cyanobacteria. In samples Yabelo 5, Yabelo 7, Yabelo 8, Yabelo 9, and Bor 1 the most abundant phylum is Firmicutes, followed by Proteobacteria in Yabelo 5, Yabelo 7, and Bor 1, Actinobacteria in Yabelo 8 and Deinococcus-Thermus in Yabelo 9. In conclusion, Actinobacteria, Proteobacteria and Firmicutes are present in all samples. The phyla Actinobacteria, Proteobacteria, Cyanobacteria, Firmicutes, Bacteroidetes, Deinococcus-Thermus and Chloroflexi have also been reported on desert varnish and biological rock crusts from arid environments (Lang-Yona et al. 2018; Wieler et al. 2018).

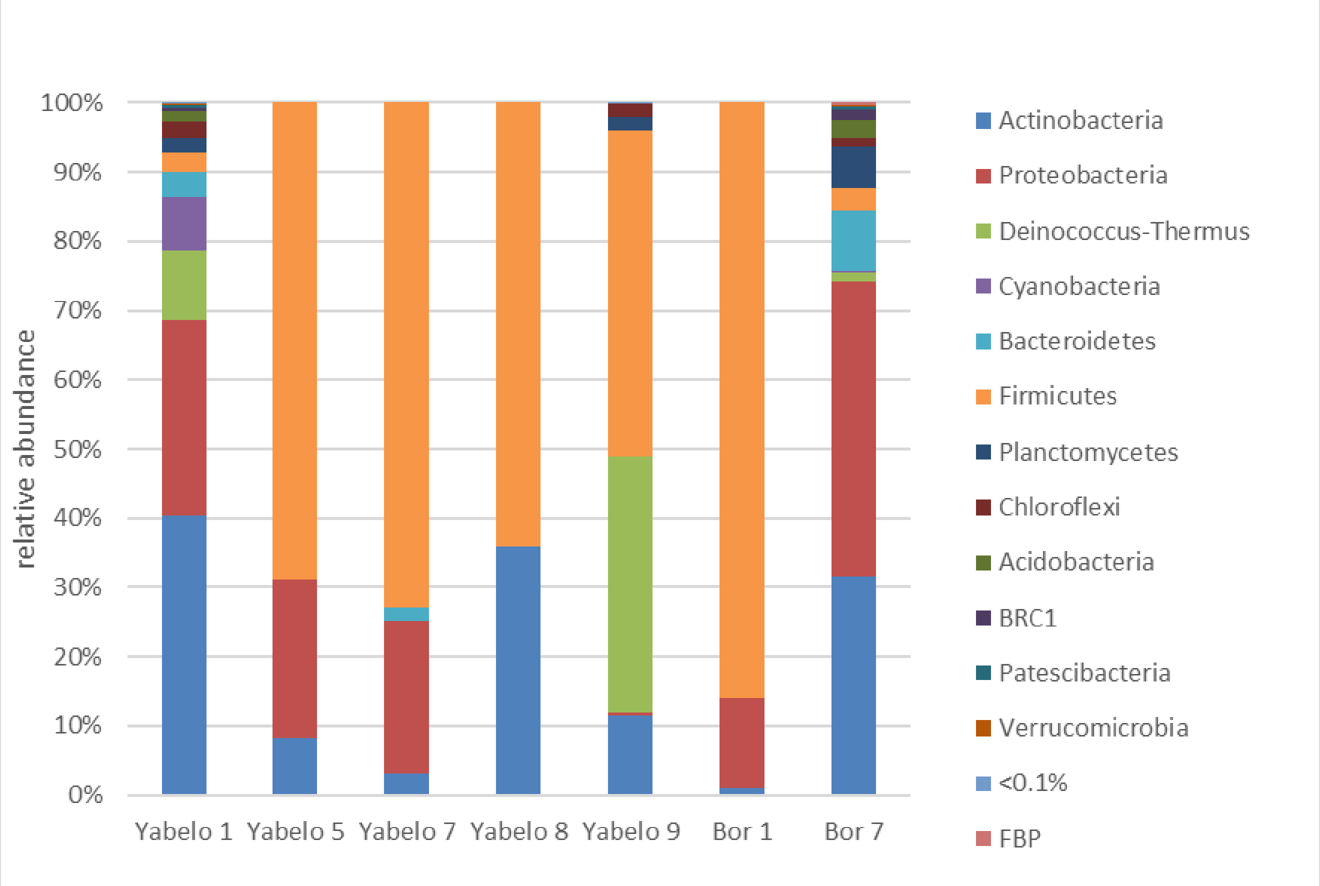

Figure 6.4. Taxonomic distribution of the bacterial communities at the phylum level, only phyla with relative abundance over 0.1% were represented.

On the genus level (Fig. 6.5), Yabelo 1 has a high diversity in which bacteria genera with a relative abundance of less than 1% constitute up to 20.57% of the whole community. The single most abundant genus is *Ornithinimicrobium* (13.74%), followed by *Truepera* (10.23%), *Citricoccus* (10.09%), *Gloeocapsa*_PCC-7428 (7.66%), *Amaricoccus* (7%), *Luteimonas* (6.62%), *Paracoccus* (4.24%), unidentified genera in families Intrasporangiaceae (3.95%) and Rhodobacteraceae (2.96%). *Ornithinimicrobium* was isolated from a mold colonized wall indoors (Kämpfer et al. 2013), *Truepera* was found in gypsum-rich hypersaline lake sediments (Sirisena et al. 2018), *Citricoccus* have been isolated from medieval wall painting (Altenburger et al. 2002a). The cyanobacteria *Gloeocapsa* has been found in black biofilm staining the building of the National Museum of the American Indian in the US (Cappitelli et al. 2012) and stone temples in India (Rossi et al. 2012), *Luteimonas* was isolated from hypogeum walls (Grottoli et al. 2020), *Paracoccus* was isolated from white biofilm colonizing show caves (Gutierrez-Patricio et al. 2021). Unclassified Intrasporangiaceae were found in Maya lime plasters (Jroundi et al. 2020), while genera from Rhodobacteraceae were found in black patina of travertine river banks (Antonelli et al. 2020) and biofilms collected from chapels (Coelho et al. 2021). Of special interest is the genus *Brevibacterium* with the relative abundance of 1.06%, which was also isolated from a limestone monument and was found capable of precipitating calcium carbonate (Andrei et al. 2017), and biomineralizing the phosphate mineral struvite (Coşgun et al. 2022; Soares et al. 2014).

The bacterial community of Yabelo 5 was most different in comparison to other samples because the dominant genera were mostly animal and human pathogens or gut microbiome, including *Enterobacter* (22.94%), *Lactobacillus* (17.96%), *Streptococcus* (15.94%), *Gemella* (14.81%), *Abiotrophia* (13.32%), *Rothia* (7.75%) and *Staphylococcus* (6.34%). However, halophilic *Rothia* was found in low abundance at sediment-ground water interface (Glamoclija et al. 2019). *Staphylococcus* was also one of the dominant bacteria identified in Spanish Levantine Rock Art located in an open air shelter (Roldán et al. 2018), and described by others with mineral weathering properties (Genderjahn et al. 2021).

The most dominant genus in Yabelo 7 is *Bacillus* (29.85%), well known for many species that biomineralize calcium carbonate (Andrei et al. 2017; Jroundi et al. 2020) as well as struvite (Soares et al. 2014), and found frequently in patinas of Spanish Levantine Rock Art (Roldán et al. 2018). Other dominant genera include *Enterobacter* (9.64%), *Domibacillus* (5.71%), *Paenibacillus* (5.56%), unidentified genera from families Ruminococcaceae (5.47%) and Lachnospiraceae (5.34%), *Solibacillus* (3.30%), *Enhydrobacter* (2.94%), *Virgibacillus* (2.89%), *Delftia* (2.84%) that was predominant in hypersaline sediments (Sirisena et al. 2018), *Sorangium* (2.67%), *Oceanobacillus* (2.40%), unidentified genus from the order Bacillales (2.09%), and the genus *Diaphorobacter* (2.06%) that could precipitate calcium carbonate through nitrate reduction activity when oxygen is deficient (Lin et al. 2021).

Yabelo 8 is dominated by *Planococcus* (54.14%) that is known to produce orange pigmented carotenoids (Moyo et al. 2022), followed by *Brevibacterium* (13.02%) known for biomineralization of calcium carbonate and struvite, *Citricoccus* (9.43%), and the radioresistant *Rubrobacter* (5.98%) common in desert lithic environments and colonized granite (Genderjahn et al. 2021) as well as producing carotenoids that caused rosy discoloration on monuments (Cojoc et al. 2019). *Kocuria* (4.22%) was also isolated from weathering rocks (Xi et al. 2018) and other bacteria in the community include *Salinicoccus* (2.81%), unidentified genera from families Planococcaceae (3.49%), Micrococcaceae (1.98%) and the order Bacillales (2.51%).

Yabelo 9 is dominated by *Truepera* (37.04%), which was found as one of the major bacteria in gypsum-rich hypersaline sediments (Sirisena et al. 2018), and *Virgibacillus* (33.16%) that have been isolated from deteriorated mural paintings (Heyrman et al. 2003). Other genera include

Alkalibacterium (6.74%), *Nesterenkonia* (4.32%), *Georgenia* (2.43%) which was isolated from a medieval wall painting (Altenburger et al. 2002b) and *Ornithinimicrobium* (1.71%). Unidentified genera were found from the families Carnobacteriaceae (3.76%), Bacillaceae (2.17%), Isosphaeraceae (1.88%) and the order Thermomicrobiales (1.97%).

Bor 1 has relatively less diversity in their bacterial communities. Bor 1 is 86% *Staphylococcus* and 11.38% *Delftia*, with minor percentages of *Halomonas* (1.73%), *Lawsonella* (0.83%) and *Cutibacterium* (0.05%). As mentioned in previous samples, *Staphylococcus* was found in other rock art sites and mineral weathering processes, while *Delftia* were found in gypsum-rich sediments (Sirisena et al. 2018), which corelates to the SEM observation that the surface coating of Bor 1 is gypsum.

The largest proportion of genera in Bor 7 are unidentified genera, constituting 17.87% of the total community. Similar to Yabelo 1, Bor 7 also has a higher number of genera with low relative abundance, the proportion of genera less than 1% of relative abundance is 13.73%. The most dominant genus is *Luteimonas* (16.56%) that has been isolated from macroalga surface as epiphytic bacteria (Verma et al. 2016) and farmland soil (Wang et al. 2015), followed by *Citricoccus* (13.58%), *Ornithinimicrobium* (8.28%), *Amaricoccus* (7.46%), *Paracoccus* (5.41%), *Limnobacter* (3.45%) a potential chemolithoheterotroph capable of oxidizing thiosulfate to sulfate (Spring et al. 2001), *Rubrivirga* (2.19%), *Luteitalea* (2.03%), *Oceanobacillus* (2.03%), *Rhodopirellula* (1.40%), *Ellin6055* (1.35%), *Niabella* (1.30%), *Truepera* (1.25%), *Pedobacter* (1.08%) and *Echinicola* (1.00%).

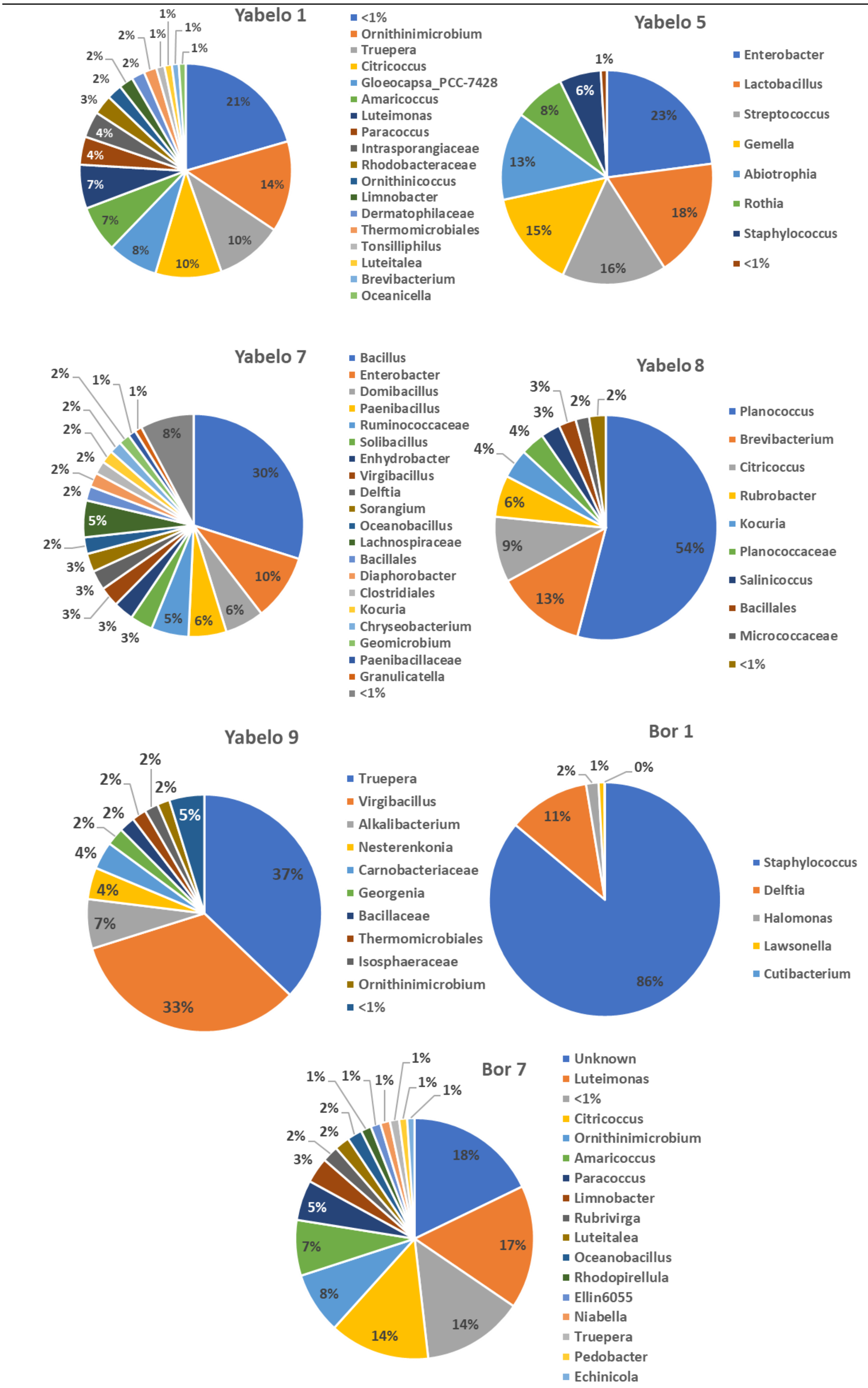

Figure 6.5. Taxonomic distribution of the bacterial bacterial communities at the genus level and unidentified genera at family or order level.

6.3.4 Functional gene prediction (PICRUSt)

Biologically induced precipitation of calcium carbonate is a widespread phenomenon performed by both autotrophic and heterotrophic bacteria. Autotrophic mechanisms include cyanobacteria photosynthesis while heterotrophic precipitation of calcium carbonate mostly happens through the sulfur and nitrogen cycles. The sulfur cycle relies on the dissimilatory reduction of sulfate by sulfate reducing-bacteria (SRB), for example, it could start with abiotic dissolution of gypsum to provide sulfate and calcium ions, then sulfate is reduced to sulfide by SRB and subsequently bicarbonate ions are formed and precipitate with calcium ions as calcium carbonate (Seifan and Berenjian 2019).

The nitrogen cycle mechanisms include ammonification of myxobacteria, dissimilatory nitrate reduction (denitrification) and urea degradation, in which all three produce metabolic carbon dioxide (CO_2) and ammonia (NH_3). Denitrification happens when nitrate and organic carbon are available. This metabolic pathway is achieved when nitrate is being used as an electron acceptor by denitrifying bacteria. Denitrification creates N_2 and CO_2 as by-products, and this process is expected to predominantly happen under O_2-limited conditions. The consumption of protons increases the pH and favors the precipitation of calcium carbonate in the presence of calcium ions (Seifan and Berenjian 2019). Bacterial ureolysis is a well-understood mechanism that increases the pH by releasing ammonia which creates a favorable environment for calcium cation and carbonate anion precipitation (Hammes and Verstraete 2002).

Table 6.3 shows a normalized abundance of genes to total reads of each sample. Bor 1 has the highest abundance of calcium carbonate precipitation genes (0.45%) that all came from ureolysis, which could be associated with the most abundant bacteria in the sample, *Staphylococcus* (86%). While *Staphylococcus* was also present on Spanish open air rock art (Roldán et al. 2018), 90% of the strains of the human pathogen *Staphylococcus aureus* produced urease, the enzyme that generates ureolysis (Zhou et al. 2019). Urease catalyzes the hydrolysis of urea into ammonia (NH_3) and carbon dioxide (CO_2) resulting in an increase in pH that creates a favorable environment for calcium carbonate precipitation in the presence of calcium cations (Hammes and Verstraete 2002).

Yabelo 1 is second most abundant in calcium carbonate precipitating genes (0.18%), this sample harbors the genus *Brevibacterium* (1.06%) that is known to precipitate calcium carbonate (Andrei et al. 2017) and the phosphate mineral struvite (Coşgun et al. 2022; Soares et al. 2014). However, Yabelo 1 is the only sample with a cyanobacteria community (*Gloeocapsa*_PCC-7428, 7.66%) and has the highest input of genes from carboxysome proteins. Bor 7 has a 0.14% abundance in calcium carbonate precipitation, this sample share similar abundance of other mineralization genes with Yabelo 1, and the two samples share some core microbiome as well. Coatings from Yabelo 1 and Bor 7 also have the highest levels of Ca (31.70% and 26.45%, respectively) that are not related to gypsum and they contain the highest amount of calcium precipitation genes besides Bor 1 (0.18% for Yabelo 1 and 0.14% for Bor 7), therefore we suggest the possibility of active calcium carbonate precipitation on these two samples.

Yabelo 7 also has a notable abundance in calcium carbonate precipitation (0.11%), the most dominant genus on this sample is *Bacillus* (29.85%) which is known for high production of urease and is often chosen for the restoration of historic buildings because of its biomineralization ability (Jroundi et al. 2017). Biogenic calcium carbonate shows advantages to consolidating stone in comparison to artificial acrylics. Inoculation of fragile stone with urease-producing bacteria and customized medium could generate growth of calcium carbonate minerals in cracks and fissures, while acrylics do not integrate into the stone and eventually peel off or even cause damage (Ortega-Villamagua et al. 2020). The second largest genus on Yabelo 7 *Enterobacter* (9.64%) was isolated from Ethiopian soil and characterized as a rapid urease producer (Mekonnen et al. 2021).

Samples Yabelo 3, Bor 1 and Bor 2 have coatings that resemble thick and homogenous gypsum and the three samples also have the lowest microbial diversity. Yabelo 3 did not provide enough genomic material for sequencing and the DNA sample extracted from Bor 2 was below the detection limit on the Qubit fluorometer. These results likely suggest that the gypsum coatings were not biogenically produced by microorganisms and have the possibility of being anthropogenic, as gypsum has been used as white pigment in south African rock art (Bonneau et al. 2022), and in Argentinian rock art gypsum was used in mixtures of paint and independently as white pigment (Gheco et al. 2020). It is however worth noting that Bor 1 has the highest amount of urease genes, in the occurrence of abiotic gypsum dissolution there would be a source of Ca^{2+} for calcium carbonate precipitation, while sulfate could be assimilated by the bacteria community as PICRUSt results suggest with the highest amount of assimilatory sulfate reducing genes.

The denitrification pathway can induce calcium precipitation and the genes are most prominent in Yabelo 1 and Bor 7, less in Yabelo 5 and not found in other samples. In the nitrogen cycle, denitrification reduces nitrate into N_2 that could be released as gases. However, more abundant genes in nitrogen assimilation and nitrogen mineralization present in all samples suggest that most of the bacterial community could assimilate nitrate into amino acids such as glutamate (Huang et al. 2020;

van Heeswijk et al. 2013), and then metabolize the organic N into ammonium (Llorens-Marès et al. 2015).

Concerning genes in sulfur metabolism, although dissimilatory sulfate reduction is an alternative pathway to induce calcium carbonate precipitation, it has negligible results from PICRUSt. On the other hand, there are a higher amount of assimilatory sulfate reduction genes that incorporate sulfate into amino acids, as well as a higher amount of sulfur mineralization genes that release organic sulfur as sulfide (Llorens-Marès et al. 2015). This indicates that the reduction of sulfate to sulfide on these samples mainly goes through assimilation of heterotrophic bacteria instead of dissimilatory reduction by sulfate reducing bacteria. The sulfide produced could be released as a gas (H_2S), converted to elemental sulfur by sulfur oxidizing bacteria or combine with iron to produce pyrite (Hammes and Verstraete 2002). Sulfide oxidation is mediated by electron transport chain short-circuiting in the periplasmic space where the perchlorate reductase (*pcr*AB) directly oxidizes sulfide to elemental sulfur (Mehta-Kolte et al. 2017), however *pcr*AB genes are not listed in KEGG database and could not be used for PICRUSt.

Genes related to calcium phosphate precipitation are present in all samples but did not yield significant differences between each other. These genes are known to regulate the expression of calcium phosphate precipitation and are part of phosphorus metabolizing pathways, their presence indicates the potential of microbial communities utilizing phosphorus and precipitating related minerals (Fishman et al. 2018; Cosmidis et al. 2015; Skouri-Panet et al. 2018). Coating samples of Yabelo 5, 7, 8, 9 and Bor 7 were detected with iron but associated genes were not prominent. Although ferrous (Fe^{2+}) minerals are subject to nitrate-dependent microbial oxidation to form various biogenic ferric (Fe^{3+}) oxide minerals in anoxic environments (Weber et al. 2006), the ubiquinol cytochrome c reductase (cytochrome *bc1*) complex in this mechanism (Bird et al. 2011; Bryce et al. 2018) did not yield presentive reads and was left out.

The results of functional gene prediction by PICRUSt discussed above gave insights into the potential of bacteria communities involved in bioprecipitation of coatings, but the possibilities still exist that a coating was precipitated by bacterial communities long gone and the current methodologies used were unable to find any trace. This research amplified the 16S rRNA that is common in all bacteria DNA to identify taxa and used PICRUSt to predict gene functions with the same data. Results show the bioprecipitation genes predicted in the DNA extracted from the coating samples, unlike RNA transcriptions which represent the genes that are actively working. Both DNA and RNA are snapshots of the microbial community at the time of sampling and metagenomic data would better correlate with the most recent coating formation.

It could be possible to investigate older bacteria communities in the future with techniques developed for ancient DNA (aDNA) research, which retrieved ancient pathogenic bacteria from bones, teeth and mummies. Environmental aDNA has been recovered from permafrost and ice to find bacteria including *Bacillus*, *Actinomycetes*, *Alphaproteobacteria*, *Acidobacteria* and *Firmicutes* by amplifying 16S rRNA (Arning and Wilson 2020, Arriola et al. 2020). Bacterial amplicon sequencing of aDNA extracted from subglacial calcite from the Boggs Valley found endolithic communities including the phyla Chloroflexi, Actinobacteria, Cyanobacteria and Proteobacteria. Other bacteria are associated with sulfur-reducing and organic matter oxidizing communities known to dissolve silicate bedrock, or autotrophic, heterotrophic, and phototrophic taxa that promote calcite precipitation (Frisia et al. 2017). However, there are still challenges to aDNA research. Ancient bacterial DNA are prone to contamination from human handling samples, suboptimal storage, laboratory contaminants, and false positives. Interpretation of bacterial communities could be problematic in paleomicrobiology, for example GC-rich Gram-positive bacteria persist more successfully, and mycobacteria are better protected against enzyme degradation because of membranes rich in mycolic acid (Arning and Wilson 2020).

We also cannot exclude the hypothesis of biological activity in concert with concomitant chemical/physical processes. The observed morphologies could provide clues to the biotic or abiotic nature of the coatings. For example, the presence of calcium oxalates could be recognized with SEM imaging, which are often formed from biomineralization of lichen and fungi. SEM images of our coating samples also reveal organic materials with filaments and membranes that could be derived from fungi or lichen (Fig. 6.3c, d), in this case we can consider the possibility of bioprecipitation from non-bacterial sources (Gallinaro and Zerboni 2021).

Table 6.3. Relative abundance of PICRUSt reads normalized to 100% total reads per sample.

	Yabelo 1	Yabelo 5	Yabelo 7	Yabelo 8	Yabelo 9	Bor 1	Bor 7
Calcium carbonate precipitation*	0.18%	0.08%	0.11%	0.03%	0.02%	0.45%	0.14%
Calcium phosphate precipitation	0.12%	0.09%	0.12%	0.12%	0.11%	0.14%	0.12%
Carboxysome protein	0.02%	0.00%	0.01%	0.00%	0.00%	0.00%	0.02%
Dissimilatory sulfate reduction	0.00%	0.00%	0.0011%	0.00%	0.00%	0.00%	0.00%
Denitrification	0.02%	0.01%	0.00%	0.00%	0.00%	0.00%	0.03%
Ureolysis	0.13%	0.07%	0.10%	0.03%	0.01%	0.45%	0.09%
Nitrogen assimilation**	0.22%	0.14%	0.15%	0.20%	0.17%	0.14%	0.20%
Nitrogen Mineralization***	0.03%	0.10%	0.08%	0.09%	0.04%	0.06%	0.03%
Assimilatory sulfate reduction**	0.08%	0.02%	0.05%	0.02%	0.06%	0.08%	0.07%
Sulphur Mineralization***	0.09%	0.02%	0.05%	0.10%	0.11%	0.02%	0.08%

***** Calcium carbonate precipitation is the sum of Carboxysome protein, Dissimilatory sulfate reduction, Denitrification, and Ureolysis

****** Assimilation of nitrate and sulfate by microorganisms into organic matter for example amino acids

******* Mineralization here refers to organic nitrogen or sulfur that are released by heterotrophic microorganisms as soluble inorganic forms, such as ammonium and sulfide

6.4. Conclusions

Our research site, the rock art panels in Yabelo from southern Ethiopia showed loss of surface including erosion, flaking and spalling. On the other hand, growth of overlying coatings was visible and occurred in a variety of colors such as white, red and black. Examination of surface coatings with microscopy allowed us to identify coatings and morphology on each sample. SEM imaging coupled with EDS chemical analysis is efficient in recognizing certain minerals such as gypsum. EDS analysis is semi-quantitative but could point out elements for mineral marker genes to carry on genomic investigation in bacterial communities. Chemical and taxonomy analysis revealed that the same rock art panel could harbor coatings with a variety of textures, morphologies, chemical compositions and different microbial communities, all within close proximity to the supposedly same mineral substrate and identical environmental conditions. This observation reflected results from arctic-alpine rock coatings that hosted significantly different bacterial communities despite being sampled from the same transect (Marnocha and Dixon 2014). The same paper also identified bacterial-mineral interactions and processes that facilitated the formation of diverse rock coatings.

Functional analysis of the coatings puts forth the possibility of multiple reactions happening in the subaerial biofilm at the rock-air interface, including mineral dissolution that generates cations and anions, reprecipitation of the ions to form new minerals, and microbial metabolism of major elements C, N and S that assimilate sulfate and nitrate into amino acids. Sulfur and nitrogen cycles in bacterial metabolism could actively induce mineral precipitation, therefore organic nutrient cycles could not be left out when discussing the formation of inorganic mineral coatings. From this perspective it would be worth investigating in the future how other microbial pathways influence mineral coatings on rocks, especially those that involve both organic and inorganic elements such as iron and manganese oxidation. Whether the microbial communities are deterioration or protective is case specific and will change through time with changing dynamics, or when the communities are replaced.

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Chapter 7

Conclusions

We acknowledge the importance of rock art and ensuring its existence is the only way to maintain access to this resourceful information. Therefore, all forms of analysis must be based on safeguarding the integrity of the rock art, thus the priority of using non-invasive methods first. When there is need to acquire crucial data that involves invasive sampling (e.g., dating), the decision depends on if the outcome could justify the loss from micro-sampling. In our case studies from both Ethiopia and Oman, we demonstrated that with accurate geomorphological observation it is possible to perform micro-sampling and acquire data for analysis or even dating. Gallinaro and Zerboni (2021) removed tiny quantities of pigments from Ethiopian rock art for SEM and EDS analyses and suggested the red pigment consists of iron oxides. The rock varnish growing over petroglyphs in Oman were radiocarbon dated and rendered the age of 2600±60 uncal. years BP, which corresponds to the local Early Iron Age (overall commonly dated between 1300 and 300 BCE). This age refers to the growth of the rock coating; thus it is a limit *ante quem* for the making of engravings and we can try to delineate a tentative relative chronology of events (Zerboni et al. 2021). These examples showed that micro-sampling of rock art is crucial for studies in physicochemical analysis, dating and preservation. Physicochemical analyses not only reveal the raw materials, techniques and châine operatoires of rock art. These information could further interpretate the provenance and trade of materials was well as sociocultural decisions made by the artists, and generate a broader archaeological view of the people and society that made them. Dating rock art remains a challenging task but is essential to establish the chronology of cultural contexts, only then can we understand the change of technological practices over space and time, or even aspects of environmental changes (Domingo and Gallinaro 2021).

Open air rock arts almost inevitably have some forms of rock coatings that coexist with microbial communities. Rock coatings are known to have a protective role under certain circumstances, but whether the microorganisms are protective or deteriorative is still uncertain. Another problem that microorganisms cause is the biofilm they form usually contains pigments that discolor stone surfaces. Initial analyses on colored rock coatings from Ethiopian rock art revealed a broad range of bacteria diversity and possible functions, including biomineralization, pigmentation, major element cycling, and human and animal pathogens. It is worth pointing out from this research that coating samples from the same site under similar circumstances hosted completely different sets of bacteria, which is in line with arctic-alpine rock coatings that contained significantly different bacterial communities although being sampled from the same transect (Marnocha and Dixon 2014). These results indicate that what we view as ‘close proximity’ and ‘same environments’ are still huge spatial and chemical differences for microorganisms that could develop unexpected diversities. This also points out the complexities of microbial communities outside laboratories and prompts more consideration when dealing with them. As an example, discoloration by SAB is generally disliked and often dealt with by mechanical washing or biocide treatments, these actions have consequences in the long term and could not be considered sustainable. Therefore, we made an attempt to discuss the ecological role of biogenic pigments in nature, and viewing these pigments as part of the environment evoke us to rethink decisions made for conservation treatments and whether it is necessary to remove them.

The role of bacterial communities on rock coatings requires joint expertise in geomorphology, molecular biology and bioinformatics for investigation. Functional analysis of the bacterial communities on rock coatings suggested a complex web of interactions in SABs, where biomineralization and biodeterioration are both possible depending on where the dynamics are shifted. It should also be kept in mind that the microbial communities identified are only representative of the present time, it could be possible that microbial activities in the past caused alterations to the rock art, but the communities have been replaced over time and therefore were not detected. These findings are consistent with our initial proposal in the first chapter that rock art should be viewed in a broader sense of ecosystem, where microorganisms are important inhabitants that generate metabolic cycles which result in alteration of the rock surface. The outcomes of bioprotection or biodeterioration rely on how the reactions sum up and does not work in merely one direction. Thus the “rock art ecosystem” is alive with micromorphology and microbial communities that changes with time and can reflect the greater outside environment.

The methodologies we proposed in this research could be carried out on other immobile works of art for analytical and preservation purposes, namely frescoes, wall paintings, murals or even large sculptures and monuments. These methods provide advantages to field work for remote sites. From the social-economical point of view, preservation of rock art and all forms of culture heritage eventually rely on the local community which at the same time is the largest stakeholder (Agnew et al. 2022). The initiative of preserving a relic linked to their past will generate a sense of place for the population and help create a unique identity. This social experience can also bring economic benefits with carefully planned tourism. Under sustainable management, the publicity and income made by tourism can improve the local economy and preservation resources in a positive feedback.

From the scientific point of view, preservation of rock art could ensure a source of valuable information that could be extracted in the future. Technological advancement could unveil information not accessible in the present. New dating and analytical methods with higher sensitivity and more precision that require less or no sampling are the trend and could be achieved in the near future. These techniques could reveal data to understand past climate changes, how ancient people lived, or even ancient microbial communities that were replaced. Climate change and air pollution definitely has impact on microorganisms, as the change of precipitation, temperature, emission of SO_2 and nitrous oxides all effect growing conditions and available nutrients (Schröer et al. 2021). By preserving rock art, we could further explore how people lived in the past, and how microorganisms were influenced by the changing environment.

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