Untangling ambiguities in the microbial fossil record

Experimental abiotic and biological approaches

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Dissertation presented at Uppsala University to be publicly examined in Norrland I Gm118, Geocentrum, Villavägen 16, Uppsala, Friday, 26 May 2023 at 10:00 for the degree of Licentiate of Philosophy. The examination will be conducted in English. Chairman of the grading committee: Doctor Barbara Cavalazzi (Department of Biological, Geological, and Environmental Sciences, University of Bologna).

Abstract
Huld, S. 2023. Untangling ambiguities in the microbial fossil record. experimental abiotic and biological approaches. 33 pp. Uppsala: Uppsala universitet.

Life on early earth has long been the topic of discussion for many researchers: how did it come to be? Which cells came first? Where can we find them? The most ancient rocks on our planet may hold some of the answers to these questions, but many may only be answered in laboratories. Chemical and morphological traces can be found from Archaean deposits, tantalisingly similar to modern day prokaryotes. Often, they are interpreted as the fossilised remains of bacteria or archaea. However, the caveat remains the abiotic mechanisms with which many similar traces and markers can be formed. The purpose of this thesis was to look into the similarities and differences in abiotic and biological formation of filamentous structures in rocks and observe whether there are chemical or morphological factors that allow for distinguishing between the two. Various laboratory methods were used: chemical gardens to form filamentous abiotic structures and experimental mineralisation of a filamentous methanogen in carbonate, phosphate, and silicate in order to compare and contrast the various mineralisation mechanisms in the fidelity of preservation of the microbes. In the former experiment, analysis with electron paramagnetic resonance (EPR) spectroscopy was carried out to identify potential chemical biomarkers. A combination of scanning and transmission electron microscopy, energy dispersive X-ray (EDX) analysis, X-ray diffraction (XRD) and Raman spectroscopy were also used to analyse the minerals and precipitates formed in both sets of experiments. The results of this research indicate that morphology of filamentous structures and the chemical signatures in biominerals may not be reliable as biogenic indicators. Furthermore, the work on experimental mineralisation reveals the possible biases in the rock record of microbial preservation which is highly dependent on the structure of the cell wall, chemistry of the environment, and the mineral formed. Finally, this work has important outcomes for the search for biomarkers on earth and on other planets and for the recognition of pseudofossils versus microbial fossils in the rock record.

Keywords: chemical gardens, pseudofossils, experimental mineralisation, microbial fossils, methanogens

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URN urn:nbn:se:uu:diva-500732 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-500732)

Dedicated to my father

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. Huld, S., McMahon, S., Sjöberg, S., Huang, P., Neubeck, A. (2023) Chemical Gardens Mimic Electron Paramagnetic Resonance Spectra and Morphology of Biogenic Mn Oxides, *Astrobiology,* 23(1): 24-32.

II. Huld, S., Willman, S., McMahon, S., and Neubeck, A. Experimental mineralisation in carbonate, phosphate, and silicate of the filamentous hydrogenotrophic methanogen *Methanobacterium oryzae*, [Manuscript in preparation]

Contents

Introduction

The thought of early life on Earth around four billion years ago conjures up images of a hot, dark, and barren rock world, where the air is thick with black smoke and the stench of sulphur and boiling red streams of lava flow over the ground. Many artists have depicted this scene and it can really only be described as something out of a fantasy book. This is the very earliest image we have of planet Earth, and it may seem at first to be completely inhospitable and devoid of life. But organisms very different from us might think otherwise (if they *could* think that is), and where the members of the animal kingdom would choke on the deadly fumes, these would thrive and delight in the veritable smorgasbord of available energy treasure troves. I refer, of course, to the prokaryotes. These single-celled organisms have really fulfilled the idiom "left no stone unturned" and have exploited almost every possible niche the world has to offer. Their short turnover time and prolific asexual reproductive methods have allowed them to evolve time and time again to adapt to new habitats, giving new meaning to the Darwinian term "survival of the fittest" and indeed providing the experimental basis to test Van Valens evolutionary "Red Queen Hypothesis" (Morran *et al.,* 2011). They are ubiquitous on earth's surface and today make up the second largest chunk of biomass after plants (Bar-on *et al.,* 2018). Habitats for bacteria and archaea range from symbiotic and parasitic relationships with plants, animals, and fungi to the deepest and most remote parts of the planet like hydrothermal vents, the sediment/soil subsurface and polar regions. Biogeochemical cycling of elements in earths litho-, bio-, and atmosphere is largely controlled by microbes and their distribution can have profound effects on local climate. If ever the word "primitive", colloquially used to refer to something underdeveloped, could be misapplied, it would be so for the prokaryotes.

So how do we know that microbes ruled the earth several billions of years ago? Well, though invisible to the naked eye, microbes can intensely alter their immediate surroundings either actively by, for example, boring into rocks to scavenge for what they consider food, or passively, through metabolic or mechanical processes that may interact with the environment. The most impressive of these is of course the stromatolites. These sticky biofilms trap sediments in the water column and, once coated, the producers climb on top of the sediment layer, and proceed in the formation of a new microbial mat. Thus, the typical domed and layered shape is born, and its lithification gives rise to

the name (stroma = layers; lithos = rock). Stromatolites were much more abundant in the Proterozoic, and a few Archaean samples potentially constitute some of the oldest evidence for life on Earth (Nutman *et al.,* 2016). However, this has been disputed (Allwood *et al.,* 2018) due to the possibility of forming stromatolites without the influence of biology. This type of disagreement has come up many times before when searching for evidence of early life on earth and it stems from the difficulty in interpreting structures in the rock record as biological versus abiotic. Rocks from the Archaean are already scarce due to the steady recycling of the tectonic plates and the few that remain are often heavily metamorphosed. Sedimentary structures are rarely discernible and the possibility of preserving organic matter comes close to nil. Despite this, many claims have been made over the years of putative fossil microbes (e.g., Schopf, 1993; Sugitani *et al.,* 2009; Cavalazzi *et al.*, 2021 among others), some of which have been disputed (e.g., Brasier *et al.,* 2002). What's more, abiotic formations of minute structures similar in morphology to microbes have been created in the lab (García Ruiz *et al.,* 2002; Cosmidis and Templeton, 2016; McMahon, 2019). Microbes can also leave chemical traces that can be used to identify them, and these include biominerals (Johannessen *et al.,* 2020) isotope fractionation signatures, and organic molecules (Peckmann and Thiel, 2004). Many of these can also have an abiotic origin (McCollom *et al.,* 2006).

It is this ambiguity in the interpretation of microbial signatures in the rock record that I wish to dive into with my doctoral thesis. However exciting it may be to be the researcher who finds the oldest fossil evidence for life on earth it is of vital importance that that conclusion is undeniably correct. The implications of finding life in the Archaean are vast and not to be taken lightly. In fact, it constrains the time of the origin of life, tells us about the communities and habitats available on early earth, and allows us to extrapolate these findings to the search for life on other planets. My own work has looked at both the abiotic and biological side of things. Paper I focused on an abiotic method called chemical gardens that can produce lab-grown biomorphs that may be incorrectly interpreted as microbial fossils in the rock record. In addition, this paper looked into the use of biominerals as useful chemical biomarkers. Following this, paper II was focused on the use of experimental mineralisation on a strain of filamentous methanogen so as to investigate the early stages of mineral encrustation on this microbe, and its potential for long-term preservation. The two papers together essentially examine two faces of the same coin: filamentous traces in the rock record, are they abiotic or biogenic?

Background

Chemical gardens: what are they and how are they relevant?

I would like to start by bringing up a perhaps less attractive area of research in the search for early life on Earth: the abiotic procedures. In the last 20 years or so, doubt has begun to arise as to the authenticity of many microbial fossils and traces found in the rock record (Brasier *et al.,* 2002; Allwood *et al.,* 2018; McMahon, 2019). With mechanisms like Fischer-Tropsch reactions that can produce organic molecules from abiotic volcanic precursors (McCollom *et al.,* 1999) and even abiotic reactions that can fractionate isotopes in ways similar to biological fractionation (McCollom *et al.,* 2006), the race to find and interpret biological signatures has become ever more difficult.

More recent research has provided evidence that abiotic self-assembly structures can form relatively easily that mimic the morphology and even at times the chemistry and structure of microbes and microbial fossils (García Ruiz *et al.,* 2002, 2003; Cosmidis and Templeton, 2016; McMahon, 2019; Huld *et al.,* 2023). Some of these lab-grown structures are known as chemical gardens. They are relatively simple to make. Grains of a seed metal salt are added to an alkaline solution, usually silicate or carbonate. As the salt begins to dissolve it creates a small pocket of acidic solution. The interaction between metal cations from the dissolving salt and carbonate or silicate ions in solution create a semipermeable gelatinous membrane that surrounds the salt grain. As the salt continues to dissolve, pressure builds up inside the membrane until eventually it ruptures and jets of fluid will stream out that are almost immediately coated once more in a membrane (Barge *et al.,* 2015). These jets often have uniform and filamentous shapes and can form several times from the original grain or along existing filaments, thus creating branching structures. The membranes are semipermeable and allow for the passage of hydroxyl ions to the interior where they react with metal cations and precipitate as metal (oxy)hydroxides onto the inside of the membrane, which acts as a template (McMahon, 2019; Huld *et al.,* 2023).

The resulting structures have many morphological characteristics in common with bacterial filaments and some doubt has arisen as to whether or not these abiotic structures could form in nature and be wrongly interpreted as fossil microbes (McMahon *et al.,* 2021). The morphology of chemical gardens

is also somewhat varied, since it depends on initial alkaline solution concentrations, the seed salt used or flow rates in the case of injected solutions (Barge *et al.,* 2015).

Chemical gardens are also a plausible mechanism for the origin of life at hydrothermal vents. Therefore, they undoubtedly represent an important branch of research in the topic of origin and early life on Earth and Mars (Ding et al., 2016; Sainz-Díaz et al., 2021).

Experimental fossilisations: history

Experimental fossilisation has been very useful in increasing our understanding of the mechanisms with which mineralisation of organic matter occurs. The first experiments of this kind on microbes were performed on cyanobacteria (Oehler and Schopf, 1971; Francis *et al.,* 1978). Since then, a whole host of microbes have been experimentally fossilised like Gram-negative and Gram-positive bacteria (Ferris *et al.,* 1988, Westall, 1997), iron oxidisers (Schieber *et al.,* 2008), thermophilic bacteria (Lalonde *et al*., 2005) and archaea (Orange *et al.,* 2009, 2011, 2012). The information that can be acquired from such experiments concerns the method, location and timing of nucleation and growth of the crystals. By controlling the parameters, such as concentration, pH, pressure, and temperature, it is possible to investigate a wide range of scenarios that could imitate environmental conditions during the life of the organism or throughout the process of taphonomy and diagenesis. For example, Westall *et al.,* (1995), silicified a few samples of microorganisms under different conditions of pressure and time. They showed that silicification starts differently whether it is on the cells themselves or on the extracellular polymeric substances (EPS) that they form. Studies such as these, and many more that have followed, have indicated that the varying conditions of formation, as well as the nature of the organic matter and the intricacies of the different metabolisms and activities of the microbes produces very different precipitates. This is reassuring since it tells us that many microbes may produce very specific organo- or biominerals that could be used to identify them in the rock record. However, there is also an important taxonomic bias in that there will be preferential preservation of some over others, leading to underrepresentation of certain taxa in the fossil record. For example, Gram-positive bacteria tend to silicify better than Gram-negative, due to the thicker peptidoglycan wall of the former (Westall, 1997). Therefore, in siliceous environments such as hydrothermal vents or hot springs, assuming a mixed population of Gram-positive and Gram-negative bacteria present, the latter will be underrepresented in the final death assemblage. Similarly, Archaea have yet another type of cell surface structure and therefore mineralise differently from bacteria (Orange *et al.,* 2009; Kish *et al.,* 2016). It is this lesser-known domain of life which has been the focus of large parts of my PhD.

Archaea and their cell wall structure

This domain was first described by Carl Woese and George E. Fox in 1977 and was recognised as separate from bacteria due to properties of their cell wall, coenzymes, and ribosomal RNA. The first known representatives were the methanogens that, as the name suggests, generate methane through the use of various substrates. Commonly they are divided into hydrogenotrophs that use hydrogen as an electron donor and carbon dioxide as the carbon source or acetotrophs that use acetate, where the former are chemotrophs and the latter heterotrophs. It is in fact this ability of theirs that gave the name to the Archaea, meaning 'ancient things' in Ancient Greek, since their metabolism was plausibly one of the first to be viable in the primitive Earth atmosphere. Archaea are particularly well-known for being extremophiles tolerating extreme temperatures (Erauso *et al.,* 1993), salinities (Magrum *et al.,* 1978) or pH (Miot *et al.,* 2017). They have also been found in more moderate habitats and play important roles in many ecosystems including but not limited to, soils (Chow *et al.,* 2022), microbial mats (Orphan *et al.,* 2008), lakes (Yang *et al.,* 2020), ocean sediments and even the human gut (Bang and Schmitz, 2015). Despite their structural similarities to bacteria, they actually share more genetic information with eukaryotes (Brochier-Armanet *et al.*, 2011) which has led many to the conclusion that Eukarya may have evolved from within the Archaeal line (e.g., Williams *et al.,* 2013; Raymann *et al.,* 2015; Gribaldo and Brochier-Armanet, 2020). Their differences with the other two domains are highlighted not just genetically however, but also in their cell membranes and cell walls. In fact, Archaea often have what is called an S-layer which is made of proteins that form a paracrystalline structure that is hydrophobic and is often referred to as a 'molecular sieve' (Schultze-Lam *et al.*, 1996) (Fig. 1). Most archaea seemingly have an S-layer, but a few are missing one. Several anionic groups on this layer render the surface charge negative and allows for interaction with cations. A few archaea, like some types of methanogens, may have additional cell wall structures that are similar to the peptidoglycan found in bacteria like, for example, pseudomurein (Meyer and Albers, 2020). It is often these cell wall structures that determine the extent, if any, of mineral precipitation onto the surface of the microorganism which directly affects their preservation potential in the rock record.

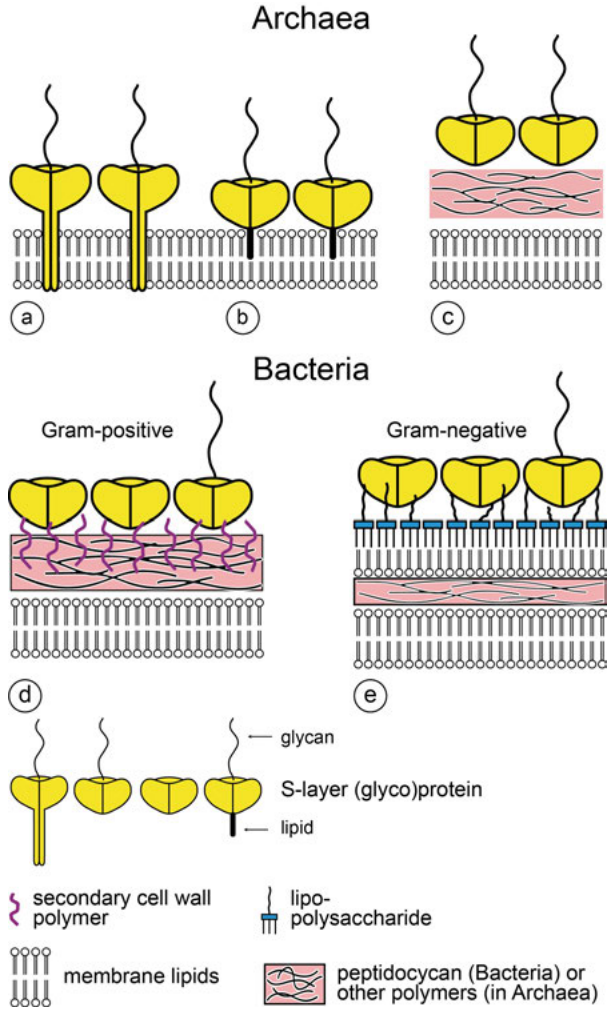

Figure 1. Cell wall structural differences between archaea (a-c), Gram-positive (d), and Gram-negative bacteria (e). The proteinaceous S-layer is often but not always present in archaea and bacteria, whereas a peptidoglycan layer is always found in bacteria, either thick and external (Gram-positive) to the membrane or thin and sandwiched between two membranes (Gram-negative). Some archaea also have a pseudopeptidoglycan layer between the membrane and the external S-layer (Sleytr *et al.* 2014).

Microbial fossils: bio- or organomineralisation?

Mineralization is often considered one of the first and crucial steps in microbial fossilisation (Oehler and Schopf, 1971; Toporski *et al.,* 2002; Orange *et al.,* 2009; Li *et al.,* 2013; Schiffbauer *et al.,* 2014). The rapid entombment or encrustation of the microscopic organism in various types of minerals means subtracting the microbe from its surrounding environment, thus protecting it from chemical and physical degradation (Konhauser *et al.,* 2003; Alleon *et al.,* 2016). Minerals are much more resistant than organic matter and can therefore better withstand the increase in pressure and temperature that come with metamorphism and diagenesis (Briggs and Wilby, 1996; Briggs, 2003; Benzerara and Menguy, 2009; Kremer *et al.,* 2012; Muscente *et al.,* 2015). The original microbe can potentially be preserved as entire cellular structures and become real body microfossils (e.g., Benzerara *et al.,* 2004), or the organic components may remain as molecular traces that linger through time and record their former presence on earth (Ueno *et al,* 2006; Orange *et al.,* 2012). What's more, the precipitation of minerals on, around or even internally to microbes can be a biomarker in itself, forming fossils where organic matter has long since vanished (Jimenez-Lopez *et al.,* 2010; Johannessen *et al.,* 2020). The composition and/or structure of the precipitate may have unique characteristics due to its biologically or organically influenced formation, and this can sometimes be both chemically and morphologically interpreted as evidence for the prior presence of biological entities.

It is, perhaps, opportune to begin with defining the differences in the terms biomineral and organomineral. The latter was first proposed by Trichet and Defarge in 1995 at the 7th International Symposium on Biomineralization and was meant to separate those mechanisms with which living entities control the precipitation of minerals intra- or intercellularly for a specific scope from those minerals formed simply in association with organic matter, both biological and abiotic.

The formation of organominerals can occur in several different ways. One of the primary mechanisms is the alteration of the chemistry and redox conditions in the immediate vicinity of the microbes in question. This often occurs due to metabolic processes of the microbe itself where waste products that are pumped out may increase or decrease pH and/or may locally increase the concentration of certain ions that can influence the saturation index of particular minerals (Zhu and Dittrich, 2016). For instance, boring microbes that etch

away at a surface to get at a certain resource tend to release other ions (Bennett et al., 2010). An increase in concentration may lead to supersaturation and nucleation can occur. On the contrary, microbes using and thus removing certain compounds from the environment can also affect redox conditions. A prime example is the production of carbonates in response to removal of carbon dioxide from the atmosphere by photosynthesizing microbes (Dupraz *et al.,* 2004).

Another passive mechanism is due to polymers on the surface of the cells that function as nucleation sites for precipitating minerals like carboxyl, phosphoryl or hydroxyl (Ferris *et al.,* 1988; Benning *et al.,* 2005). Most minerals require the formation of a nucleus in the form of nanometre-scale particles to initiate precipitation, and, even at saturation point of the solution, there is a free energy barrier to overcome for this to occur (Sear, 2007). The presence of charged organic molecules in microbial cell walls can attract ions. These locally concentrated charged ions can act to overcome the kinetic reaction barrier by subsequently attracting other ions that lead to the precipitation of minerals (Fig. 2). Consequently, depending on the chemistry of the outer wall of the microbe, cell wall-specific precipitation may occur. The cell walls of bacteria are generally subdivided into two structural varieties called Gram-positive and Gram-negative, which is based on their reaction with a stain commonly used for light microscopy (Schultze-Lam et al., 1996). The former, have a much thicker wall of peptidoglycan, polymers that are rich in carboxylate groups with an internal lipid membrane (Fig. 1). Additional secondary polymers add to the overall electronegative charge density. Gram-negative bacteria on the other hand, have a much thinner peptidoglycan layer and an outer lipid bilayer membrane as well as an internal one (Fig. 1). The outer membrane contains lipopolysaccharides that are anionic making the surface of the cell strongly electronegative. So, Gram-positive bacteria are more likely to collect a thicker sheath of precipitate than gram-negative bacteria since the cations in solution are more attracted to the larger number of available binding sites (Westall *et al.,* 2000). It has also been shown that metal binding to negatively charged surface groups can induce precipitation and can therefore also occur on Gram-negative bacteria, albeit usually in a thinner crust (Schultze-Lam *et al.,* 1996; Warren and Ferris., 1998; Fein *et al*., 2002, 2006). In the case of archaea, as mentioned previously, they often have an S-layer, a paracrystalline protein layer, whose negative surface charge allows for the interaction with ions in the environment (Fig. 2). These can also concentrate ions and thus favour precipitation (Orange *et al.,* 2009), even preserving the cell wall at times (Kish *et al.,* 2016).

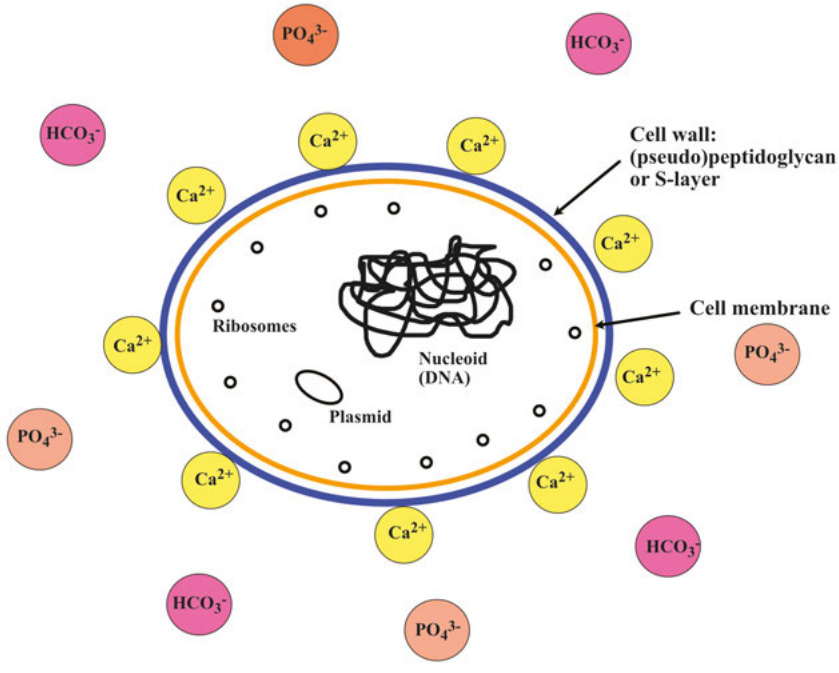

Figure 2. Cell surface biomineralisation. Negatively charged polymers in the cell wall like carboxyl, phosphoryl, or hydroxyl attract cations which in turn attracts anionic compounds like, for example, phosphate or bicarbonate. The resulting local concentration of ions can cause the precipitation of minerals.

Whereas the above-mentioned mechanisms of biomineralization are *induced* in the case of metabolic changes of the environment or *influenced* (Dupraz *et al.,* 2009) in the case of the passive precipitation on cell surfaces and other extracellular substances (Zhu and Dittrich, 2016), some bacteria may *control* the precipitation of minerals (Benzerara *et al.,* 2005; Benzerara and Menguy, 2009). In the latter case, the biominerals that form serve a particular purpose for the organism and therefore have distinct structures, composition, size ranges, and location (e.g., within the cell). These are therefore generally good biomarkers. An example of controlled precipitation might be that of a coccolithophore that, through cellular processes, induces the precipitation of ordered calcium carbonate to form the characteristic external plates that make up their skeleton and from which their name derives. There are no known examples of controlled mineral precipitation by archaea.

Considering that the mineralisation of a microbe depends a lot on the chemistry of the environment, the localised alteration due to metabolic products, and the nature of the cell surface, the range of possible biominerals is vast.

Carbonate mineralisation: mechanism, biological influence, and fossils

The formation of carbonates is largely attributed to high pH and high alkalinity (Soetaert *et al.,* 2007; Zhu and Dittrich, 2016). An increase in the dissolved inorganic carbon (DIC) as carbonate or bicarbonate ions, combined with the removal of carbon dioxide and protons creates a favourable environment that is supersaturated with respect to calcium carbonate (Soetaert *et al.,* 2007). The metal constituents for the formation of carbonate minerals, like calcium and magnesium cations, are provided in various ways. Although they are relatively common in modern seawater (~400ppm and ~1300ppm respectively), some locations can have higher concentrations such as hydrothermal vents (Kelley *et al.,* 2005) or zones of basalt weathering (Roberts *et al*., 2004; Kenward *et al.,* 2009). Weathering of rocks may occur through the chemical dissolution by microorganisms that delve into the rock in search of nutrients, such as phosphorus (Rogers *et al.,* 1998). In the process, the silicate minerals of the basalt are destroyed, and cations released into the water. The negatively charged functional groups such as carboxyl, phosphoryl, or hydroxyl of the cell wall or EPS will attract the newly released metal cations from solution. Consequently, an influx of carbonate or bicarbonate ions will cause precipitation of carbonate crystals onto the cell walls as has been seen in many cases (Bosak and Newman, 2003; Roberts *et al,* 2004; Kenward *et al.,* 2009; Zhu and Dittrich, 2016). Thus, the cell walls of microbes act as sites for heterogeneous nucleation of carbonate minerals.

Carbonate precipitation is often influenced by microbes, either passively or actively. Many microbial metabolisms may increase the alkalinity of their surrounding environment. Oxidation of organic matter produces bicarbonate thus increasing alkalinity. Where oxygen is unavailable, several other metabolisms may oxidise organic matter through the use of nitrates, iron or manganese oxides, or sulphate. Anaerobic respiration mechanisms are also known to consume protons thus increasing pH (Soetaert et al., 2007). Use of nitrate, sulphate, and methane in microbial metabolisms all produce bicarbonate ions (Turchyn et al., 2021). Anaerobic methanogens can induce the precipitation of calcium carbonate through the reduction of bicarbonate species (Visscher and Stolz, 2005). Furthermore, they consume carbon dioxide thereby driving up the pH (Kenward et al., 2009). It could be that anywhere where there are increased amounts of calcium and carbonate or bicarbonate ions in the water, the localized rise in pH caused by the consumption of carbon dioxide from methanogens drives precipitation. A mechanism like this was previously proposed in the formation of dolomite, where microbial erosion locally released calcium and magnesium ions into the waters which, combined with methanogens removing carbon dioxide drove precipitation in otherwise unfavourable circumstances (dilute, low temperature, low magnesium) (Roberts *et al.,* 2004). In addition to this, the methanogens may provide nucleation sites on

their cell surfaces to help overcome the kinetic energy barrier (Roberts *et al.,* 2004). In 2021 Turchyn *et al.* carried out a study on the porewaters of marine sediments and found that where there were larger amounts of methane there was also a higher overall alkalinity and a higher propensity for calcium carbonate precipitation. The limiting factor was often the presence of phosphates which may act as a kinetic barrier to the nucleation of calcium carbonate.

Calcified microbes are perhaps not the most common of mineralized microorganisms but they are nonetheless to be found in the rock record. They can be found as late as the Pleistocene, in palaeosols (Monger *et al.,* 1991) or in Precambrian calcite veins (Bons and Montenari, 2005). It is not always possible to tell whether or not microbes are involved in the precipitation of carbonate or whether they are simply entombed in a flow of carbonate rich fluids (Trewin and Knoll, 1999). Entombment of microbes in calcite has been noted also in cave carbonates (Melim *et al.,* 2016).

Phosphate mineralisation: mechanism, biological influence, and fossils

Geographical factors on phosphogenesis include both upwelling from deeper waters (Glenn *et al.,* 1994) or weathering from surface glaciation or orogeny (Föllmi, 1996). On a more local scale, several mechanisms that concentrate phosphate in sediment pore-waters may interplay. Generally, an increased burial of organic matter, skeletal parts and authigenic apatite will decrease the phosphate availability since this will remove the phosphorus from oxidation processes (Muscente *et al.,* 2015). If the sedimentation rate in a basin is low, the organic matter can become relatively concentrated, and P-remineralisation may occur which produces phosphate ions (Glenn *et al.,* 1994). Redox conditions of the bottom waters and sediment pore waters also affect the P-remineralisation. If the water column is oxic, more phosphorus (in organic matter for example) can be transported into the sediment where it can be remineralised to phosphate. The remineralisation in the upper sediment layers that are oxic will occur through aerobic respiration. In deeper sediments, the electron acceptors will be different (not oxygen) and the conditions more suboxic/anoxic. In this case, a more likely scenario for P-mineralisation is through anaerobic heterotrophic respiration like, for example, bacterial sulphate reduction (Muscente *et al.,* 2015).

Phosphates released into pore waters are also subject to adsorption. In particular, a strong redox gradient produces an iron cycle which can affect phosphate availability (Glenn *et al.,* 1994; Nelson *et al.,* 2010). When iron oxyhydroxide reaches deeper into anoxic sediments it will get reduced to ferrous ions and consequently release any adsorbed phosphates. This ferrous iron may diffuse upwards into oxic bottom water, where it will form iron oxyhydroxides

once more and adsorb more phosphate. Similarly, the phosphates may also reach oxic waters and be once more adsorbed. This iron cycle can only occur where there is an oxic/anoxic transition zone. Microbial mats may limit the diffusion of phosphates to oxic bottom waters and therefore concentrate phosphates in the sediment pore water. Furthermore, underneath microbial mats where there are suboxic/anoxic conditions and hydrogen sulphide present, sulphur oxidising bacteria may metabolise polyphosphates and release more phosphates, concentrating it to a point where precipitation can occur. Finally, heterotrophic reactions in the microbial mat may alter pH conditions to favour phosphogenesis (Briggs, 2003).

Experimental phosphatisation of soft tissues has previously been studied, often in terms of decay (Briggs and Kear, 1993; Briggs, 2003) since it is the decay of organic matter through various microbial metabolisms that releases phosphorus and locally concentrates phosphates which allows for the precipitation of minerals. According to Briggs (2003), decay largely occurs in anoxic or suboxic conditions, and the electron acceptors used by microbes can be depth dependant, with the deepest being carbon dioxide. Other studies on microbial precipitation of calcium phosphate have looked at bacteria (Benzerara *et al.,* 2005) where they studied the orientation and nature of crystals precipitating on and inside the microbe, and at archaea (Kisch *et al.,* 2016; Miot *et al.,* 2017). The latter studies showed that the precipitation of iron phosphates was closely associated with the S-layer, thus preserving it very well and also looked at the diagenetic factors of fossilisation.

Phosphatized deposits are often classified as Lagerstätte since the preservation of both inorganic and organic structures is of very high and three-dimensional detail. As a phenomenon, it is largely limited to the Phanerozoic (Muscente *et al.,* 2015). The most spectacular known deposit of phosphatisation is of course the Doushantuo formation in South China that has remarkable three-dimensional fossils of what are thought to be various microbes, acritarchs, and possible animal embryos (Zhang *et al.,* 1998; Xiao and Knoll, 2000). Other phosphatized remains of microbes are more recent, like phosphatic coprolites in the Palaeocene (Cosmidis *et al.,* 2013) or phosphatic chalks from the Cretaceous (Lamboy, 1990). In both these papers, the role of bacteria in the precipitation of phosphates was inferred. Similar to the phosphatisation of the S-layer, the microbes in coprolites showed mineralisation of the periplasm in Gram-negative bacteria which aided the exceptional preservation of such intimate details as cell wall layers in bacteria.

Silica mineralisation: mechanism, biological influence, and fossils

Silica precipitation, as with other minerals, requires the formation of a nucleus in the form of nanometre-scale particles to initiate precipitation, and, even at saturation point of the solution, there is a free energy barrier to overcome for this to occur. The free energy barrier of quartz is higher than that of amorphous silica, so it is more difficult for quartz to nucleate. Hence, most precipitates in silica sinters are amorphous silica like opal-A. Diagenesis may alter it to other forms of silica depending on time and physicochemical conditions and the ratio of various products may indicate the diagenetic age of the sinter (Herdianita *et al.,* 2000; Benning *et al.,* 2005). Silica in solution is made of monomers in the form of silicate tetrahedrons or orthosilicic acid ($Si(OH)_4$). These molecules are stable as long as the concentration is lower than that required for the precipitation of amorphous silica. Amorphous silica is an inorganic polymer made of silica monomers bound by a siloxane bond (Si-O-Si) with various levels of hydration. Typical areas of silica deposition are at hydrothermal vents. At 25ºC the equilibrium concentration of silica is a lot lower than at hydrothermal vents, where the waters that were formed below the surface are extremely enriched in silica. Here it remains soluble due to the particular conditions of pressure, temperature, and pH. When the water reaches the surface however, the rapid cooling and changes in pressure and pH stimulate the precipitation of silica (Benning *et al.,* 2005).

Organic molecules, in particular free hydroxyl (OH^-) groups on the surface of microbial cell walls, may act as nucleation sites or surfaces for monomeric silica (Benning *et al.,* 2005). Consequently, more silica monomers like silicic acid or silicic salts may attach to the bound silica anions through their Si-OH groups and create siloxane bonds, whilst expelling water. Some may also form covalent bonds with organic molecules (Si-O-C) through the hydroxyl groups (Kolb and Liesch, 2008). Exopolymeric substances (EPS) may provide nucleation sites and are often better preserved than the microbes themselves (Westall *et al.*, 2000).

Several experiments have shown that microbes do not significantly speed up silica precipitation (Fein *et al.,* 2002; Yee *et al.,* 2003) but that some microorganisms will more readily silicify than others, like Gram-positive bacteria as opposed to Gram-negative bacteria or archaea (Francis *et al.,* 1978; Westall *et al.,* 1995, 1997). It seems likely that organic molecules act as a template and nucleation point for the precipitation of silica (Benning *et al.,* 2003). Peptidoglycans in the bacterial cell wall have previously been shown to form bonds with silica monomers. Archaea, on the other hand, don't contain peptidoglycans in their cell wall and it is potentially the glycoproteins that are present in the archaeal S-layer that bind silica (Orange *et al.,* 2009). It has been shown that metal cations may act as bridges between the organic polymer

and the silica in solution for bacteria (Warren and Ferris, 1998; Fein *et al.,* 2002; Phoenix *et al.,* 2003) and archaea (Orange *et al.* 2011).

Silica is one of the most common types of mineralization, particularly in the Precambrian, before the advent of silicifying organisms in the ocean (Siever, 1992). Precipitation of silica is well known to occur at hot springs and silica sinters both today, for example in New Zealand (Handley *et al.,* 2008) or Iceland (Schultze-Lam *et al.*, 1995) where geothermal activity is abundant, and in the past, like the Rhynie Chert in Scotland which contains silicified organisms (Trewin, 1996). Many studies have been carried out to settle the debate as to whether or not the silica that is precipitated at silica sinters and hot springs is deposited as a consequence of biological or abiotic processes (Benning *et al.,* 2005; Gong *et al.,* 2022). More typical findings are of EPS and biofilms (Westall, 2000) or biomolecules that can be indicative of what type of organism originally existed like bacterial or archaeal lipids (Benning *et al.,* 2005; Kolb and Liesch, 2008). Silicified microfossils are relatively abundant and have been found in a variety of different localities around the world. These include silicified carbonates and peats (Knoll, 1985), cherts (Westall, 2001; Chongyu, *et al.,* 2003; Cavalazzi *et al.,* 2021), and silicified stromatolites (Fairchild and Subacius, 1986) to name just a few.

Filamentous fossils: biogenic or not?

The purpose of this dissertation was to delve into the ambiguities that lie in the interpretation of microfossils in the rock record. The focus was not just on morphology but also on the use of bio- or organominerals and their analyses with various instruments.

In paper I we focused on the differences to be found between biological and abiotic manganese oxides. Previously, the use of electron paramagnetic resonance (EPR) spectroscopy had been proposed as a way to distinguish between biologically produced and abiotic Mn oxides in nature, and that it therefore constituted a good biomarker (Kim *et al.,* 2011). Manganese are paramagnetic ions and according to the crystal lattice structure of Mn oxides and the interactions between paramagnetic ions in one substance, different signals may be produced. In our project we grew chemical garden filaments composed of Mn oxides and carried out EPR analysis to test this. The filaments themselves had morphological characteristics that fell into criteria previously used to identify fossil microbes (Dodd *et al.,* 2017). The possibility for chemical gardens to imitate biology morphologically has previously been studied (García Ruiz *et al.,* 2002; McMahon, 2019). The EPR spectra obtained from our manganese chemical gardens were within the range previously described as biological (Kim *et al.,* 2011). As mentioned previously, various crystal lattice structures, like cation vacancies, and spin interaction systems accounted for

this similarity. We concluded that EPR alone was not a reliable method to characterize the biogenicity of natural Mn oxides.

Paper II took the perspective of biological fossils. Experimental mineralisation of microbes has been studied on many occasions (Oehler and Schopf, 1971; Westall, 1997, 2000; Briggs & Wilby, 1996; Briggs, 2003; Kisch *et al*., 2016; Miot *et al.,* 2017). The purpose of this project was to investigate the mineralisation of the hydrogenotrophic filamentous methanogen *Methanobacterium oryzae* in three different solutions separately: carbonate, phosphate, and silicate. The choice of organism was in part due to its metabolism which is considered as one of the oldest on earth (Wolfe and Fournier, 2018), in part due to its filamentous morphology, which is often found in rocks, and in part due to its interesting cell wall properties. This methanogen does not have an S-layer like most other Archaea but instead has pseudomurein which is structurally similar to the bacterial peptidoglycan layer (Meyer and Albers, 2020). The three fossilisation methods were chosen on the basis that they are three common minerals found in nature that may encapsulate microorganisms as discussed above. These experiments produced methanogens somewhat coated in minerals for silica and phosphate, with precipitates creating smooth to globular sheaths around the methanogens and thus creating a relatively faithful representation of the morphology, albeit larger in dimensions. Carbonate experiments resulted in the formation of larger crystals of calcite but without replicating the methanogens' morphology. The precipitation mechanisms of minerals onto the methanogens may be a result of passive nucleation onto electronegative polymers of the cell walls due to the pseudomurein composition, or it may be a result of the methanogenic metabolism which removes carbon dioxide and therefore creates a locally alkaline environment that favours precipitation (at least for phosphates/carbonates). The results indicate that the filamentous methanogen *M. oryzae* can be coated in minerals that preserve its outer morphology and leaves moulds in phosphates. However, continued long term precipitation may swamp the fossil and leave no traces. In any case, if filamentous fossils are found in the rock record, this work shows that the size of the filaments can become much larger and no longer reflect the true initial size of the microbe, thus constituting a taphonomic bias. This may also be the reason for many filaments being recognised as bacterial fossils and almost no body fossils of archaea recorded in the ancient rock record. The results also indicate that *M. oryzae* may have a role in the precipitation of phosphate since phosphates were precipitated from just the phosphate-buffered medium in all experiments even where no phosphate fossilising agent was added. Overall, the fossil representation of this organism (or other microbes with a similar cell wall structure/metabolism) may be more significant than previously recognised.

Future work

Since work on abiotic procedures is somewhat underrepresented with respect to the biological approach in microbial fossil research, further studies on chemical gardens, their formation in natural conditions, and their preservation potential could be valuable. Similar mineralisation experiments to the ones conducted here could be carried out on filaments formed from chemical gardens. In this way, it may be possible to see whether or not these structures can mineralise further and compare them to biological mineralisation experiments.

Some other interesting questions have arisen during the course of this work. First of all, the mineralisation experiments were carried out on methanogens with a very peculiar cell wall. They do not have an S-layer which is considered an almost universal trait for Archaea, but instead have a layer of pseudopeptidoglycan. Future experiments could look into comparative mineralisation of archaea with different types of cell walls to investigate how they may interact with ions in solution. This could be valuable in evaluating taxonomic biases we may see in the rock record. Furthermore, to see the extent to which the microbes are still alive at the various mineralisation times, it would be of use to analyse the methane production in the mineralised samples throughout time.

Another curiosity that has come up is the role of *M. oryzae* in the precipitation of phosphates. It seems that phosphates precipitated in all experiments, from phosphate already present in the buffered medium. It would be interesting to investigate the role that these methanogens may have in the precipitation of phosphates and how they might influence it.

My work on anaerobic archaea and formation of minerals associated with organic matter and microbes, has also led me to consider the role of these organisms within natural mineral-microbe systems and their influence on weathering. The use of bacteria to increase weathering of silicate minerals and concomitantly sequester carbon dioxide emissions is a new technology that strives to lessen the emission of greenhouse gases from industries. Recently, there has been an increased interest in anaerobic silicate weathering and carbon dioxide sequestration in marine sediments. Methanogens potentially have a fundamental impact on carbonate precipitation due to a coupled increase in alkalinity in the sediments that they occupy. I am curious therefore, to investigate which anaerobes may be present in these kinds of systems and their role in silicate weathering. Additionally, phosphorus starvation could induce bacteria (or possibly archaea) to attack minerals that contain phosphorus. If this

is the case, weathering of silicate minerals would significantly increase, thereby improving the outcome of carbon dioxide sequestration. This would tie in with my work on low phosphate buffered mediums for methanogens, and how they react to these conditions.

Acknowledgements

First and foremost, I would like to thank my main supervisor Anna Neubeck for her wonderful enthusiasm in all my work. The many in-depth conversations about the projects were always engaging, helpful, and at times funny. Her open-door policy has always made me feel at ease with asking for advice. Of course, I would also like to thank my co-supervisors Sebastian Willman and Sean McMahon for their valuable input to both my manuscripts and thesis, and, not least, for help with university bureaucracy.

My thanks also go out to all the people in the Palaeobiology department (and beyond) who have put up with my relentless knocking on doors to ask for help, for their advice on teaching and research, for wonderful field trips, and of course for great lunchtime and afterwork company.

Finally, my gratitude goes out to my partner Rufo for his unwavering support through the ups and downs that come with a PhD (and life in general) and to all my friends.

A large part of the experiments for the second manuscript were carried out at the Swedish University of Agricultural Sciences (SLU) in Uppsala. Here, I would in particular like to thank Anna Schnürer and Simon Isaksson for their valuable help in anaerobic culturing and analyses. Furthermore, TEM analyses were carried out at the Rudbeck laboratory, Uppsala.

This project was funded by Helge Axson Johnsons Foundation Grant No. F21-0040 and the Swedish Research Council Grant No. 05018. In addition, this work was funded by The Royal Society of Edinburgh and the European Cooperation in Science and Technology (COST).

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