SEM Investigation of Microbialites from Cenote Azul, Mexico, and Implications for the Search for Biosignatures on Mars

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ABSTRACT

A biosignature is any phenomenon for which a biological process is a known explanation, and can therefore be used as evidence for past or present life on a planet. On Mars, the geological record has provided evidence for an environment similar to an Archean Earth's in the planet's distant past. Consequently, Earth-analogue structures such as microbialites, which have been found on Earth since the Archean period, may be a key target for biosignature detection. Microbialites are organosedimentary structures that form due to the activities of microorganisms in mineral-saturated aqueous environments. To better understand the microbe-mineral interactions that cause them to form, the surface characteristics of microbialites from Cenote Azul, Mexico, were examined using Scanning Electron Microscopy (SEM). Microbialite samples were collected from a range of depths and prepared for SEM by fixing in 2.5% glutaraldehyde, mounting on aluminum stubs, and sputter-coating in gold. The outer surfaces of samples were imaged at various resolutions using a JEOL 6610LV SEM. Both qualitative and quantitative observations in diatom count, surface area colonized by microbial mats, and phospholipid fatty acid analysis show decreasing abundance of microorganisms and organics on the outer surfaces of microbialites with increasing depth. This may be due to the decreasing light availability and changing environmental conditions with depth, which have been observed in Cenote Azul. Characterizing the surfaces of microbialites such as those from Cenote Azul will provide valuable information in evaluating how microbialites form and grow on Earth. This knowledge can be extended to analogue systems such as Mars to gauge whether microbialite-like structures could be formed and preserved on other planets. Ultimately, this will aid in future biosignature detection studies and help refine the search for life in our solar system and beyond.

Introduction

The question of whether life exists, or once did exist on Mars is one that has driven humanity's interest in the planet since it was first observed by Galileo in 1610. Despite recent efforts to improve remote observations and robotic exploration on Mars, this fundamental question remains unanswered. Although the harsh surface conditions on Mars make searching for biosignatures extremely challenging, the search for life on the Red Planet can be refined by uncovering details of Mars' past and studying Earth analogue materials. There is extensive evidence for the past presence of liquid water on Mars' surface, particularly during an era known as the Noachian period from 4.1-3.7 Ga (Rizzo et al. 2021). The Martian geological record has provided evidence that Mars' environment was similar to an Archean Earth's at this time, with higher atmospheric pressures and temperatures compared to present day, vigorous volcanic activity and heat flow, and widespread aqueous environments (Rizzo 2020). Due to these favourable conditions for life as we know it, the Noachian period is considered to be the era during which Mars was most likely to have supported the formation and evolution of living organisms (Bibring et al. 2006). This brings about the question: if life did exist on Mars during the Noachian period, what would it have looked like?

A biosignature is any object, pattern, or phenomenon for which a biological process or agent is a known explanation, and therefore can be used as evidence for past or present life (National Academies of Sciences, Engineering, and Medicine 2019). It is unlikely that life would form and evolve on other planets in the exact same way it did on Earth. However if the search for biosignatures included all hypothetical scenarios in which life could be formed, this criteria would be too broad and speculative. Thus, by necessity, the current criteria used to assess biosignatures on other

planets is solely based on the characteristics of Earth-like life. Examples of early life on Earth should therefore be used as analogues to understand whether life may have existed previously on other planets, especially terrestrial planets such as Mars.

On Earth the earliest evidence of life dates back to >3.7 Ga, in the form of stromatolites (Yanez-Montalvo et al. 2020). This places the emergence of stromatolites in the Archean eon, causing them to be highlighted as potential analogue targets for fossilized life on Mars that may have emerged during the Noachian period. Stromatolites are a category of structures known as microbialites, which have been studied in the context of Mars in the past. In fact, possible structural matches between microbialites and Martian sediments have previously been suggested based on visual imagery (Rizzo 2020). On Earth, microbialites are organosedimentary structures that form due to the activities of diverse microbial communities in mineral-saturated aqueous environments (Yanez-Montalvo et al. 2020; Yanez-Montalvo et al. 2021). These communities interact with their surrounding environment to trap and bind sediments, and/or create conditions that favour the inorganic precipitation of minerals such as carbonates, silicates, and sulfates (Omelon et al. 2013). Microbialites were likely abundant on Earth's surface throughout the Precambrian era, participating in the oxidation of the atmosphere and development of biogeochemical cycles that allowed for the evolution of eukaryotic life (Yanez-Montalvo et al. 2021). However modern occurrences of microbialites are more rare, with only 20-30 freshwater and marine sites currently documented worldwide, including Pavilion Lake, British Columbia, Great Salt Lake, Utah, Bacalar Lagoon, Mexico, and Shark Bay, Australia (Yanez-Montalvo et al. 2020; Águila et al. 2022).

How exactly microbialites are formed and maintained due to the influence of microorganisms remains a topic of intense study. Current understanding of microbialite formation and growth describes a delicate balance between physical, chemical, and biological reactions (Dupraz et al. 2009). There are three proposed mechanisms for microbialite formation, where the metabolisms of microbes can cause trapping and binding of sediments, formation of nucleation sites, and/or changing geochemical equilibria to favour mineral precipitation (Omelon et al. 2013). A wide variety of phototrophic microbes such as cyanobacteria and algae, as well as chemotrophs, reside on the surface of microbialites in an organic layer of self-produced material called 'extracellular polymeric substance', or EPS (Yanez-Montalvo et al. 2020). The EPS provides an adhesive surface for the trapping and binding of sediments, securing the carbonate materials that have formed beneath, catching settling sediment and cations in the surrounding aqueous environment, and regulating the formation of minerals (Águila et al. 2022). The EPS also provides a chemically-reactive organic matrix for carbonate precipitation, allowing the surfaces of microbialites to calcify (Dupraz et al. 2009; Omelon et al. 2013). As cyanobacteria and algae colonize the microbialite substrates, they can form large swaths of area covered in EPS known as 'microbial mats'. Processes such as oxygenic photosynthesis within microbial mats raise the pH and create an alkaline environment on the surface of microbialites. This alters the surface chemistry and leads to increased EPS production, the formation of nucleation sites, and the *in situ* precipitation of carbonate (Omelon et al. 2013; Águila et al. 2022). As photosynthesis and EPS production continue, the surface area colonized by microbial mat continues to grow. However, factors such as the alteration of mineral stability via microbe metabolism, the rate of microbial community growth versus carbonate precipitation, and the movement of organisms such as cyanobacteria towards stimuli and nutrients make the continued growth of microbialites extremely complex (Stal 2012; Yanez-Montalvo et al. 2020). By studying the biological and physical characteristics on the surfaces of modern microbialites, the extent of the relationship between microbes and minerals, the response of microbial communities to different environmental conditions, and how this affects microbialite formation and growth can be better understood.

Bacalar Lagoon is the freshwater system with the highest presence of microbialites in the world

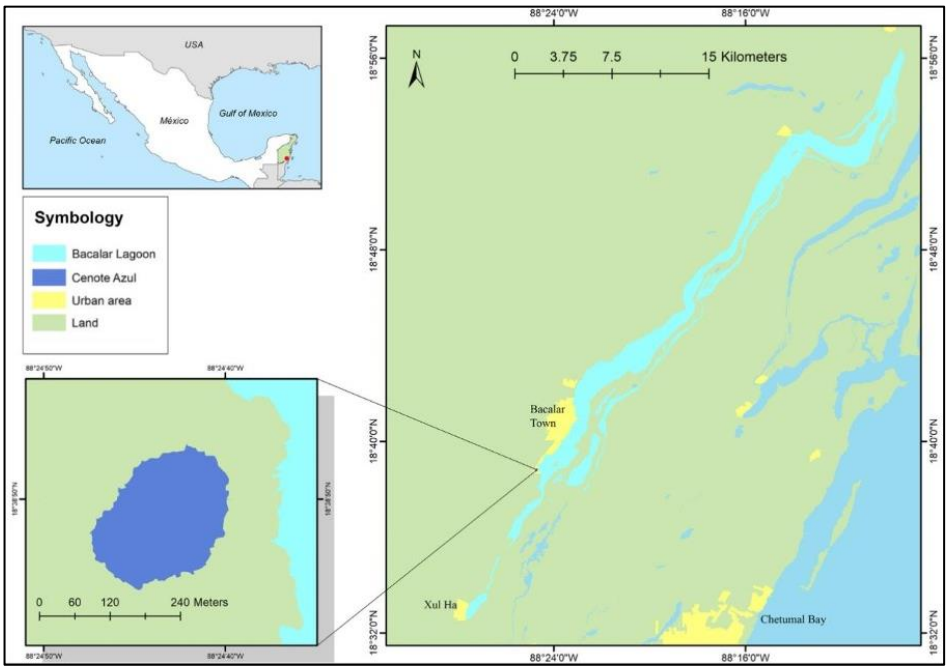

Figure 1. A map of Bacalar Lagoon, located in the state of Quintana Roo, Mexico. In the image in the upper-left corner, the location of Bacalar Lagoon is demonstrated by the red point, while the state of Quintana Roo is highlighted in green. The large image on the left shows the extent of Bacalar Lagoon, with the lagoon itself shown in light blue, and urban regions shown in yellow. The pop-out image in the bottom-left corner demonstrates the location of Cenote Azul relative to Bacalar Lagoon, and its general shape from a bird's-eye view, demonstrated by the darker blue area (adapted from Águila et al. 2022).

(Yanez-Montalvo et al. 2021), making this a popular area for the study of modern microbialites. Located in the state of Quintana Roo, Bacalar Lagoon consists of a ~40 km long lagoon and a series of interconnected sinkholes, also called 'cenotes'. These karstic environments form when existing subsurface caverns within the evaporitic bedrock collapse due to dissolution processes (Yanez-Montalvo et al. 2021). One particular cenote of note, Cenote Azul, is located 100 metres to the landside of Bacalar Lagoon, has a depth of ~71 m, a diameter of ~200 m, and is characterized by the extensive development of microbialites on its walls (Figure 1; Águila et al. 2022). Both Bacalar Lagoon and Cenote Azul are located in the Yucatan Peninsula, which is dominated by one of the largest karst aquifers in the world extending over an area of approximately 165,000 km^2 (Bauer-Gottwein et al. 2011). Although Bacalar Lagoon and Cenote Azul have been found to share hydrogeochemical characteristics, the formation Cenote Azul is believed to predate the formation of the Bacalar Lagoon system (Perry et al. 2009). Since the structure of the cenote causes and chemical factors such as light availability and water conditions to change with depth, the microbialites of Cenote Azul are prime targets for further investigation of how microbialites in system such as these form and continue to grow.

As a part of a larger body of work aiming to characterize Cenote Azul microbialites, this study focuses on a Scanning Electron Microscopy (SEM) investigation of the surface characteristics of these microbialites, and how this changes with depth. These powerful microscopes can acquire images of samples at extremely small resolutions on the scale of micrometres or nanometres. In the context of Cenote Azul microbialites, small-scale imagery can aid in surface characterization by visualising the interaction between bacterial populations, mineral structures, and organic material such as EPS. This is achieved by placing the sample in a high-vacuum environment, sweeping a focused beam of electrons across its surface, and recording the secondary electrons produced, and displaying these as Secondary Electron Imagery (SEI) in order to observe surface features and topography (Microscopy Australia 2023). By adjusting the working distance (WD) from the electron beam source to the surface of the sample, the intensity of the beam itself, and the spot size (SS) on the surface of the sample covered by the electron beam, more optimally focused images can be acquired at higher resolutions. By studying the surfaces Cenote Azul microbialites using SEM, the extent of microbe-mineral interactions, and how these may be affected by different depth conditions, can be assessed. Ultimately, an improved understanding

of the characteristics of microbialites on Earth can be extended to an understanding of potential analogue systems on Mars, which can inform future studies of such environments in the search for biosignatures of Martian life.

Methods

Microbialites were sampled from 15 depths within Cenote Azul by scuba diving, with a wide range of morphologies observed with changing depth. Hydrological conditions were also observed, with a thermocline located at 12 metres and a hydrogen sulfide layer at 61 metres (Figure 2). Eutrophic waters were found to lie above the thermocline, while oligotrophic waters were found to lie below. All samples were frozen on-site at -20°C for transportation back to McMaster University and storage until use. Representative samples of microbialites from depths of 4.5, 6, 10, 20, 30, 43.5, and 71 metres were chosen for the purposes of this study (Figure 2). It should be noted that the sample taken from a depth of 71m was found lying on the floor of the cenote, and may not have been recovered from its initial location of formation. Subsamples of roughly $10mm^3$ or less were taken from each depth using a saw sterilized with dichloromethane, methanone, and acetone between each use. The sample from the 10m depth was observed to have both 'regular' and 'green' layers, thus subsamples were taken from these individual layers to observe any differences that exist between them. These subsamples were then prepared for SEM in order to examine their outer surfaces. Subsamples were first defrosted overnight in the refrigerator in a 2.5% glutaraldehyde solution, and left to dry at room temperature for 24 hours. Fixed and dried subsamples were mounted on 25mm diameter aluminum stubs using double adhesive carbon tape, and edges were 'glued' down with nickel paint. The mounted stubs were baked at 400°F for 20 minutes to set, and sent for sputter-coating in a 2nm layer of gold. All samples were imaged at the Canadian Centre for Electron Microscopy on a JEOL 6610LV SEM using a SEI signal. Qualitative observations of microbe-mineral interactions were made by visual assessment of SEM images. Additionally, diatom count and microbial mat area measurements were taken in order to quantify change in surface organics with depth. Representative images at resolutions of 450x were chosen from each subsample depth for this analysis, except for the subsample at 6 metres which has a representative image at a resolution of 550x. Diatoms were identified based on their shape, with some identified on the genus level through correlation to a taxonomic key of diatoms in North America. Structures identified as diatoms were counted by hand from each of the representative SEM images. The percentage of image area covered by microbial mat and organic structures was assessed by visually separating mineral surfaces from organic surfaces and calculating area using the 'Measure' tool in ImageJ.

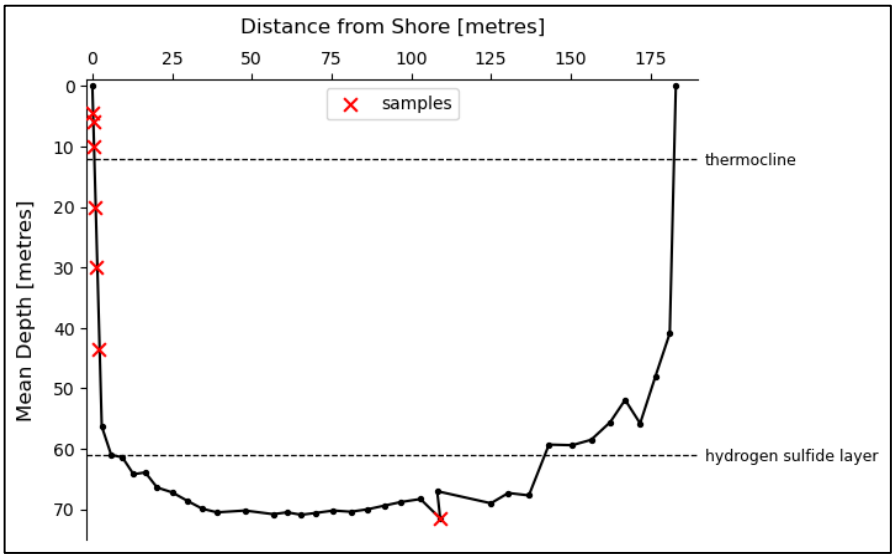

Figure 2. Depth profile of Cenote Azul, based on bathymetry data taken in 2023, provided by Julie Hartz. The coordinates of the left shore correspond to 18°38'50" N, 88°24'47" W, while the coordinates of the right shore correspond to 18°38'47" N, 88°24'40" W. The width of the cenote was measured to be 183 metres, while the depth of the cenote was measured to be ~71 metres. Locations along the depth profile where representative samples were taken are demonstrated by the red 'x's. The location of the thermocline at a depth of 12m and the hydrogen sulfide layer at 62m are represented by the dashed horizontal lines.

Additionally, diatom count and microbial mat area measurements were taken to quantify change in surface organics with depth. Representative images at resolutions of 450x were chosen from each subsample depth for this analysis, except for the subsample at 6 metres which has a representative image at a resolution of 550x. Diatoms were identified based on their shape, with some identified on the genus level through correlation to a taxonomic key of diatoms in North America. Structures identified as diatoms were counted by hand from each of the representative SEM images. The percentage of image area covered by microbial mat and organic structures was assessed by visually separating mineral surfaces from organic surfaces and calculating area using the 'Measure' tool in ImageJ.

Results

The outer surfaces of microbialites exhibit differences in organic content and mineral structure with depth. Outer surfaces of microbialites at 4.5m (Figure 3), 6m (Figure 4), and 10m (Figure 5) are colonized by microbial mats and a high presence of diatoms. On the other hand, outer surfaces of microbialites at 20m (Figure 6), 30m (Figure 7), 43.5m (Figure 8), and 71m (Figure 9) generally show little evidence of organic matter and are dominated by calcification or mineral structures. Microbialites at a depth of 4.5m visually exhibit the highest abundance of organics with the smallest amount of calcification, as evidenced by the presence of a widespread microbial mat and diatoms (Figure 3a) and filamentous algae and cyanobacterial structures (Figure 3b). There are almost no mineral structures visible on the surface of this sample, with only a few very small clusters of amorphous minerals. Microbialites at 6m also appear to have a surface dominated by a microbial mat, however an increased amount of calcification over this mat has occurred with an increased amount of amorphous mineral clusters (Figure 4a). Diatoms are present, but some appear to be more degraded compared to those at 4.5m (Figure 4b). At 10m, microbialite surfaces from both 'regular' and 'green' layering appear to be covered in organic mat, however these differ in the amount of calcification that has occurred (Figures 5a,c). When the surface of filamentous organic structures are observed at higher resolutions, there appears to be a higher presence of minerals on the surface of 'regular' layer EPS material while there appears to be a higher presence of organic structures such as diatoms on the surface of the 'green' layer EPS material (Figures 5b,d). Microbialites at a depth of 20m exhibit almost an entirely mineral surface, with very little microbial mat visible (Figure 6a). There are no diatoms present, and mineral structures appear as crystalline dendritic structures, or small clusters of amorphous minerals (Figure 6b). Microbialites from 30m exhibit similar features to those at 20m, with a surface dominated mostly by minerals. There appear to be larger and more crystalline mineral structures alongside amorphous mineral clusters (Figure 7a). However, there is a slight visible increase in the amount of organics visible at 30m, as evidenced by the presence of filamentous cyanobacteria on the surface (Figure 7b). Microbialites at a depth of 43.5m appear to be dominated almost entirely by amorphous mineral structures, with no organic structures or microbial mat visible (Figure 8a). However, certain areas of the 43.5m sample do appear to contain deflated organic structures and degraded diatoms (Figures 8b,c). Finally, the microbialite sample recovered from a depth of 71m has a surface mostly composed of crystalline platy mineral structures, with some amorphous and columnar minerals (Figures 9a,b). There is very little evidence of EPS or microbial mat colonization, and no diatoms.

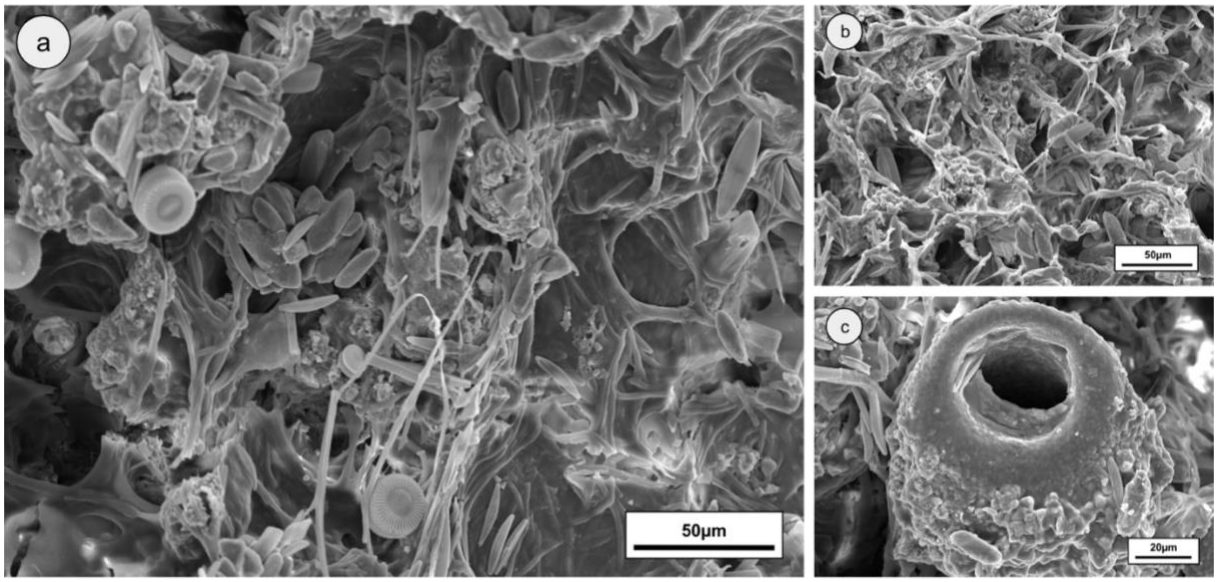

Figure 3. Sample from 4.5m depth. SEM images were acquired at a WD of 14mm, with a beam intensity of 15kV and a SS of 50. **(a)** 450x resolution image demonstrates an outer surface dominated by microbial mat and diatom structures. **(b)** 450x resolution image taken at a different location demonstrates filamentous cyanobacterial or algae structures. **(c)** 900x resolution image demonstrates the buildup of EPS material into a 'tunnel-like' structure, with the presence of calcification and diatoms on the surface.

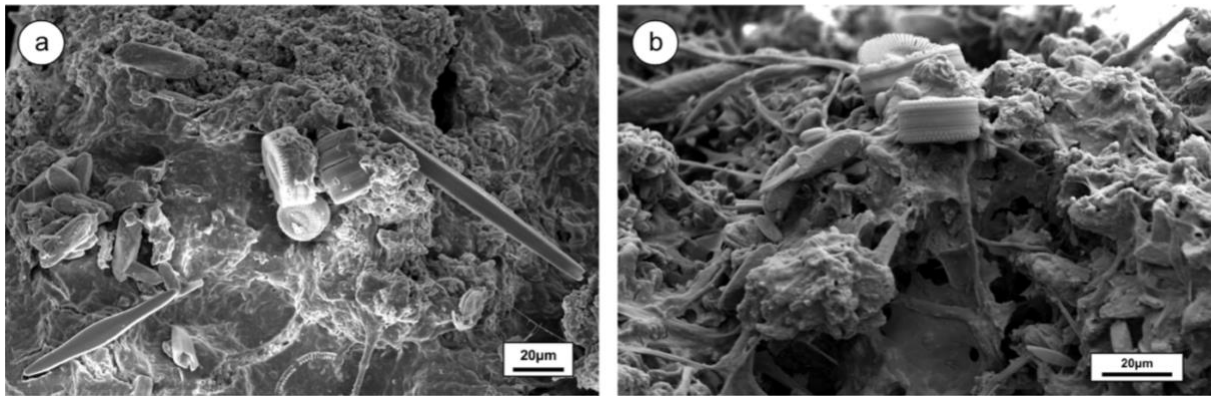

Figure 4. Sample from 6m depth. SEM images were acquired at a WD of 14mm, with a beam intensity of 10kV and a SS of 50. **(a)** 550x resolution image demonstrates a higher amount of calcification, but still a high presence of diatoms and microbial mat. **(b)** 750x resolution image demonstrates filamentous algae and cyanobacteria structures, while diatoms appear to be more degraded or fractured.

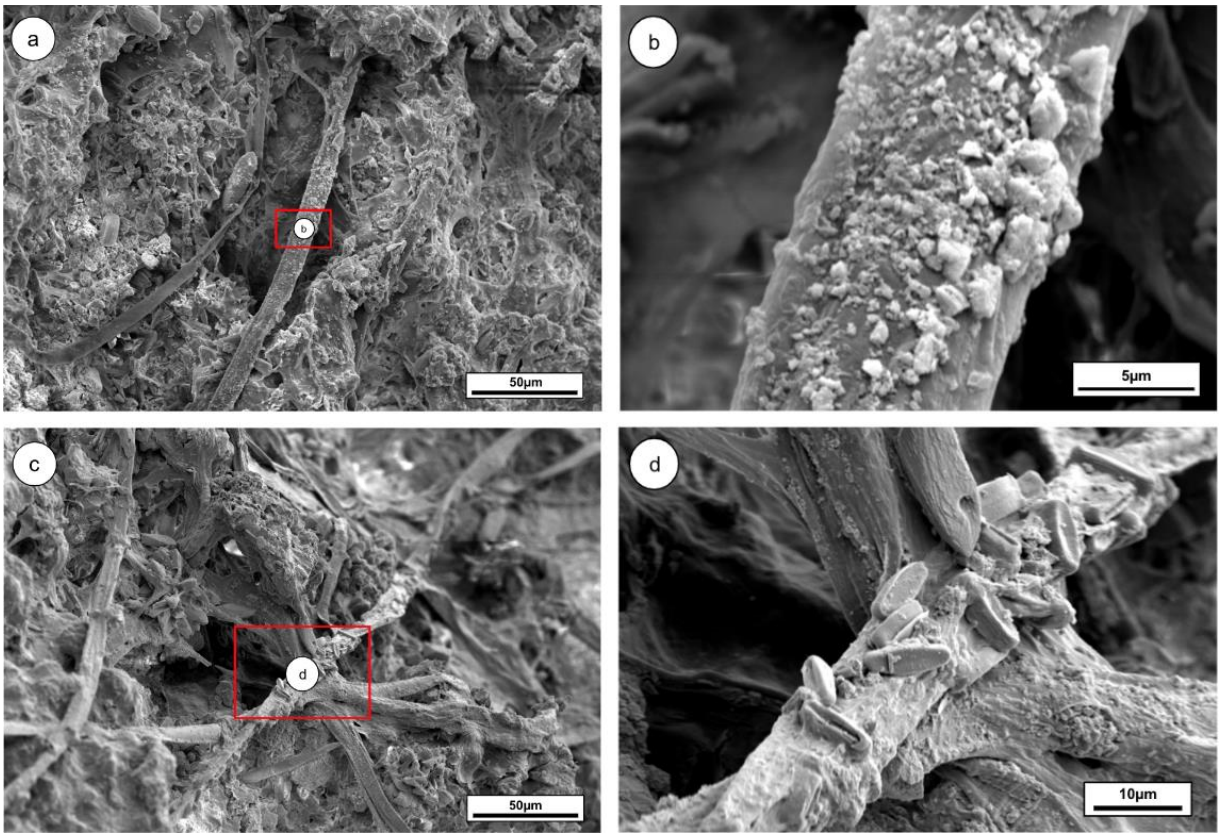

Figure 5. Samples from 10m depth. SEM images (a) and (b) represent the subsample taken at 'normal coloured' layering within the microbialite, which were acquired at a WD of 10mm, a beam intensity of 10kV and a SS of 50. SEM images (c) and (d) represent the subsample taken at 'green coloured' layering within the microbialite, which were acquired at a WD of 11mm, a beam intensity of 5kV, and a SS of 50. **(a)** 450x resolution image demonstrates a surface covered in highly calcified microbial mat and filamentous structures, with a decreased number of diatoms. **(b)** 5000x resolution image shows a closer look at the calcification occurring on the surface of the filamentous organic structures. **(c)** 450x resolution image demonstrates a surface still covered by microbial mat, but less calcified and with a higher presence of diatoms on the surface. **(d)** 2000x resolution image shows a closer look at the diatoms clustered on the surface of the filamentous organic structures.

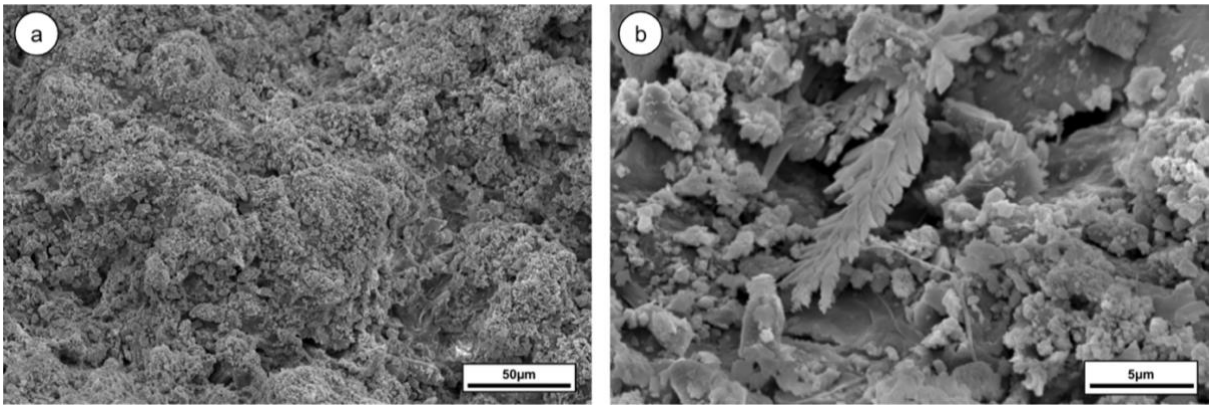

Figure 6. Sample taken from 20m depth. SEM images were acquired at a WD of 11mm, with a beam intensity of 10kV and a SS of 50. **(a)** 450x resolution image demonstrates an almost entirely mineral surface, with amorphous and crystalline mineral structures and no diatoms. **(b)** 4300x resolution images demonstrates both a highly crystalline dendritic mineral structure, and more amorphous clusters of minerals.

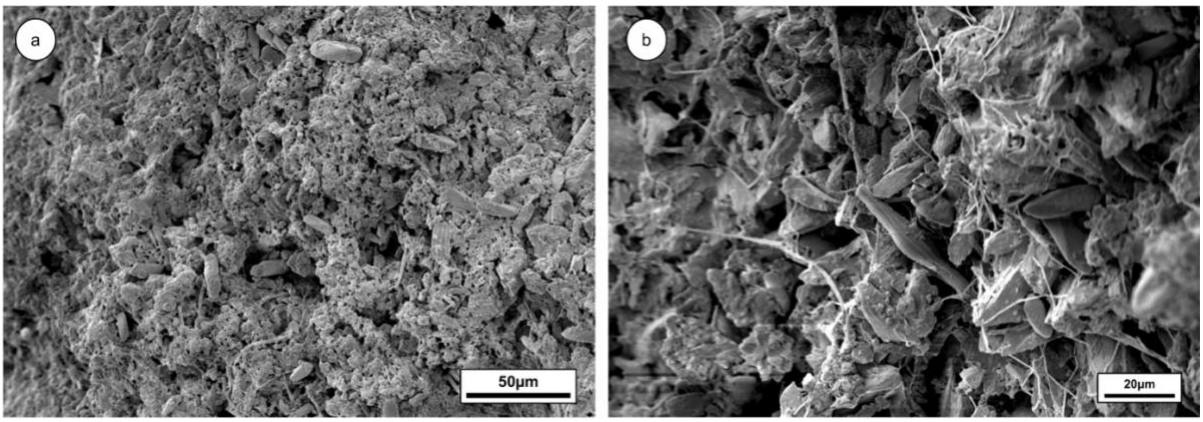

Figure 7. Sample taken from 30m depth. SEM images were acquired at a WD of 11mm, with a beam intensity of 5kV and a SS of 50. **(a)** 450x resolution image demonstrates a surface dominated by crystalline and amorphous mineral structures, however some small organic filaments and a diatom are visible. **(b)** 750x resolution image demonstrates filamentous cyanobacteria and diatoms present on the surface.

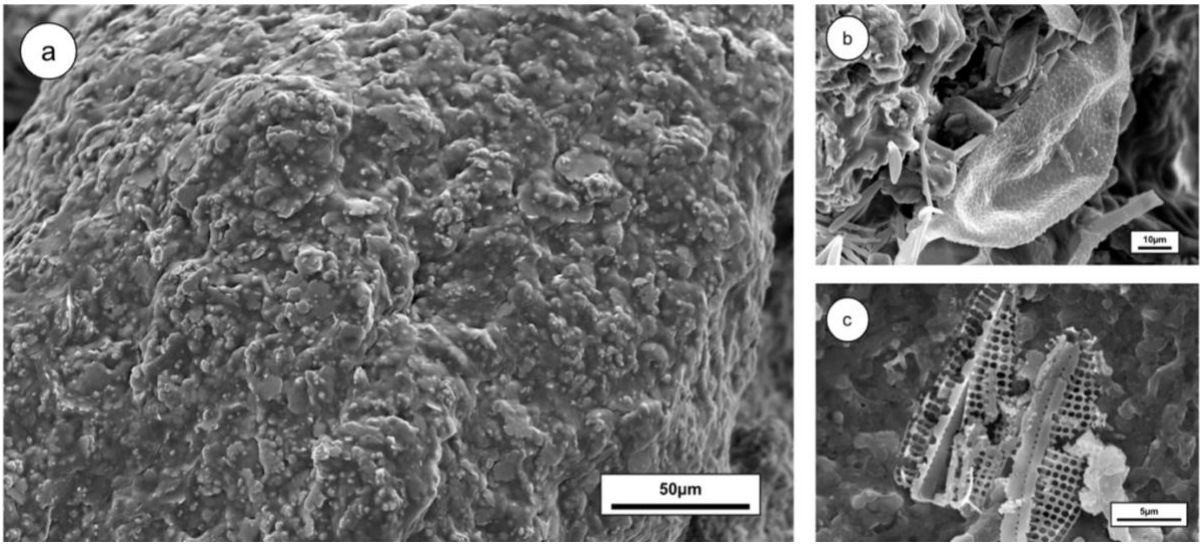

Figure 8. Sample taken from 43.5m depth. SEM images were acquired at a WD of 12mm, with a beam intensity of 15kV and a SS of 50. **(a)** 450x resolution image demonstrates an entirely amorphous mineral surface, with no presence of organics. **(b)** 1100x resolution image shows a deflated organic structure connected to fibrous material. **(c)** 4300x resolution image shows highly degraded diatom structures.

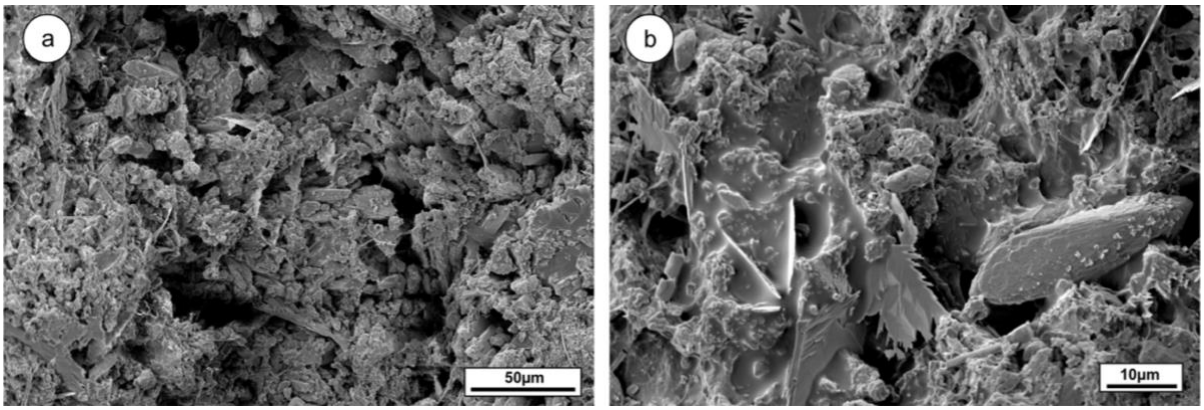

Figure 9. Sample taken from 71m depth. SEM images were acquired at a WD of 12mm, with a beam intensity of 5kV and a SS of 50. **(a)** 450x resolution image demonstrates a surface dominated by highly crystalline mineral structures, many in thin, platy, fragmented sheets. **(b)** 1500x resolution image shows the platy mineral structures alongside clusters of amorphous minerals, joined together by a potential microbial mat or EPS binding.

This visual assessment can be supplemented with numerical information about the amount of organic content that is present on the surfaces of these microbialites. It should be noted that although this analysis is quantitative, it is largely biased by the subjective nature of which structures qualify as diatoms/organics. Additionally, these results are heavily impacted by the fact that each SEM image only depicts a very small area of the sample surface, and cannot be considered to be perfectly representative. As such, some level of error should be expected on the two numerical analyses performed on the SEM images: diatom count and microbial mat surface area. A count of the number of diatoms present on the surface from images of the same (or similar) resolution generally shows a decreasing number of diatoms with increasing depth (Figure 10a). Microbialites at a depth of 4.5m had the highest diatom count of 52, while microbialites at depths of 20m, 43.5m, and 71m had the lowest diatom counts of 0. There does appear to be a small rise in the amount of diatoms present on the sample taken from a depth of 30m, with a count of 3, however this does not affect the overall decreasing trend. A similar trend can be observed when considering the percentage of microbialite surface area covered by microbial mat, with the percentage decreasing with depth within Cenote Azul (Figure 10b). The highest percentage of area covered by microbial mat occurs at a depth of 4.5m with ~80%, while the lowest percentage occurs at a depth of 45.5m with 0%. There is only a very slight increase in microbial mat area percentage at 71m, to ~0.86%. The largest difference between the diatom count and microbial mat area analysis is at depths of 6m and 10m. In the diatom count diagram, there is a steady decline in the number of diatoms present on the surface between these two depths. However in the microbial mat area diagram, the percentage of surface area covered by microbial mat is very similar between microbialites at 6m and 10m (~57.9% and ~57.2% respectively).

Discussion

The qualitative analysis of SEM imagery and quantitative analyses of diatom count and microbial mat surface area all demonstrate a trend of decreasing organics with increasing depth within Cenote Azul. In addition to the observed trend of a decreasing number of diatoms with increasing depth (Figure 10a), there also appears to be increasing diatom degradation and possibly dissolution with increasing depth within Cenote Azul. This is evidenced by comparing the SEM images of diatoms on the surfaces of microbialites

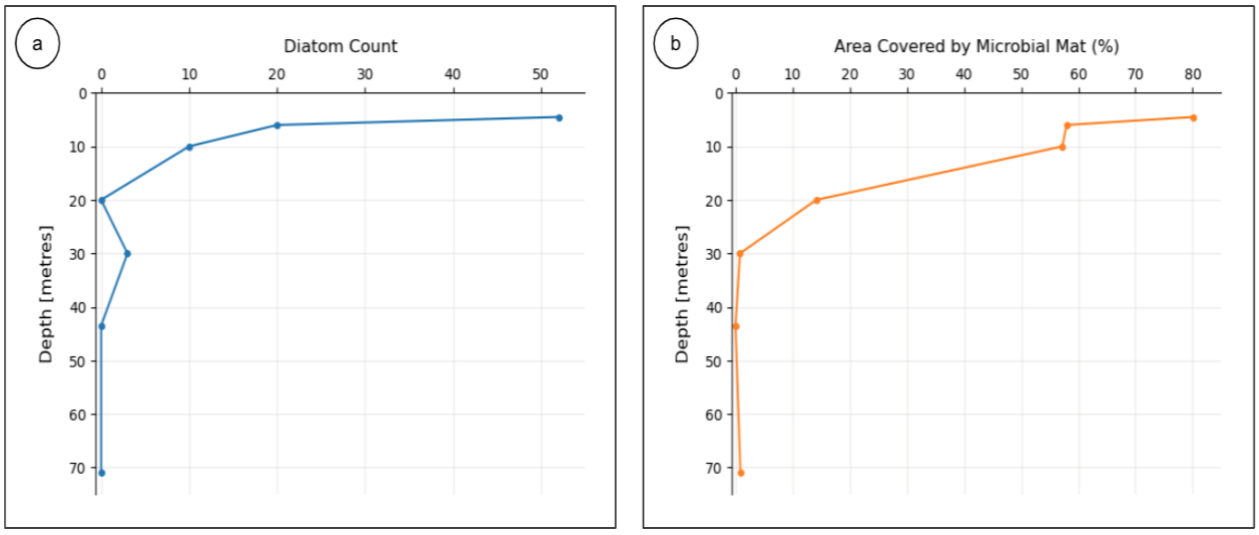

Figure 10. (a) Plot displaying the number of diatoms present on the surface of microbialites (x-axis) with decreasing depth in metres (y-axis) in Cenote Azul. The maximum diatom count occurs at [52, 4.5m] while the minimum diatom count occurs at [0, 20], [0, 43.5], and [0, 71]. **(b)** Plot displaying the percentage of microbialite surface area (x-axis) with decreasing depth in metres (y-axis) in Cenote Azul. The maximum area covered occurs at [80.06, 4.5] while the minimum area covered occurs at [0, 43.5].

from 4.5m, 6m, and 43.5m. As can be seen in Figure 3, diatoms on the surface of the 4.5m sample appear to be undamaged. Some degradation of the diatoms on the surface of the 6m sample is visible in Figure 4b, with the top disk of one of the diatoms broken and raised above where it should be. The increasing extent of degradation is especially visible in Figure 8c, where the diatoms on the surface of the 43.5m sample appear to be completely void of organic material and have very little of the silica cell wall remaining. This could indicate dissolution of the organic component of the diatoms at increased depth. Although the extent of diatom degradation on the surfaces of microbialites was not numerically assessed, this visual trend is evident in the SEM imagery. There are numerous environmental and chemical conditions that could result in the dissolution of diatoms including changing temperature, light availability, pH, and nutrient availability (Stevenson and Pan, 1999). The presence of a thermocline at 12 metres and a hydrogen sulfide layer at 61 metres both suggest changing temperature and chemical conditions within Cenote Azul, which may contribute to this observed increase in diatom degradation.

To confirm decreasing trends in organics with increasing depth seen in Figure 10, comparisons to phospholipid fatty acid (PLFA) content in Cenote Azul microbialites can be made. PLFAs are components of lipid layers (ie. cellular membranes) of bacterial and eukaryotic cells (White et al. 2020). Due to their polarity, PLFAs are highly reactive and have been shown to rapidly degrade within days or weeks after cell death (Boschker and Middelburg 2002). This causes PLFAs to be a very good indicator of active biomass, and to be very useful as tracers of biomass content and for making parallels to quantity of cells in a sample (White et al. 2020). Figure 11 demonstrates preliminary results for PLFA content of Cenote Azul microbialites from depths of 6m, 43.5m, and 49m (Hartz, n.d.). A general trend of decreasing PLFA content is observed with increasing depth in Cenote Azul (Figure 11), which aligns with trends observed in decreasing diatom count and microbial mat area with increasing depth (Figure 10). This also aligns with the visual trends observed from SEM imagery, with a higher amount of uncalcified organics visible at 4.5m, a general decrease in surface organics and increase in mineralization as depth increases to 20m, and mineral-dominated surfaces at depths of 20m and beyond. Overall, it can be said that there is a decrease in the organic content on the outer surfaces of microbialites with increasing depth within Cenote Azul. The increasing amount of mineralization and decreasing PLFA content with depth also serves to suggest that there is less active biomass in deeper microbialites in the cenote. The most likely reason

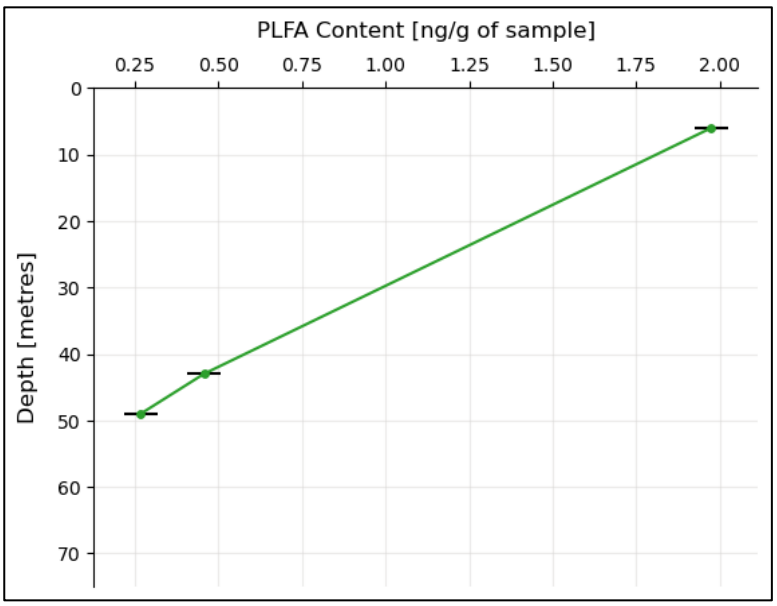

Figure 11. Plot displaying the PLFA content of Cenote Azul microbialites in units of ng/g (x-axis) with decreasing depth in metres (y-axis). The maximum PLFA content occurs at [1.974, 6] and the minimum PLFA content occurs at [0.268, 49], demonstrating a decreasing trend with depth. Black error bars represent the 5% error associated with PLFA content measured by gas chromatography mass spectrometry methodology (Hartz, n.d).

for this decline in organic content and active biomass is due to decreasing light availability in Cenote Azul. Previous studies have shown that light availability and temperature rapidly decline in Yucatan Peninsula cenotes after a depth of 10m (Schmitter-Soto et al. 2002; Yanez-Montalvo et al. 2021). In Cenote Azul, this temperature decline is evidenced by the presence of a thermocline at 12m. Qualitative and quantitative analyses show an apparent decline in organics on microbialite surfaces below 10m, which aligns with past observations in trends of light availability in cenotes. Decreased light availability causes less photosynthetic activity by cyanobacteria communities on the surface of microbialites, resulting in a decrease in the rate of biomass activity and EPS production. It has been hypothesized that EPS serves as calcification inhibitor, with areas of active cyanobacterial growth and abundant EPS production preventing calcification (Stal 2012). Microbialites located deeper in the cenote with less light would therefore have less EPS to act as an anti-calcification agent, which may suggest decreased microbial activity and increased calcification rates deeper within Cenote Azul. This, paired with the observed decline in organics with depth, may indicate that deeper microbialites are forming and growing more slowly and much less actively than shallower microbialites in Cenote Azul.

However, organic content may not just be a factor of depth, but could also be dependent on the location in the microbialite structure itself. This is evident when considering the difference between 'regular' and 'green' coloured layering present in the 10m sample. These regions were both oriented towards the surface of the cenote, located on different areas of the microbialite sample. As seen in Figure 5, filamentous structures on the surface of the 10m microbialite showed more calcification in the regular layering and a higher presence of diatoms in the green layering. This suggests that microbialite material from the green layering has a more active microbial community on its surface compared to the regular material. This implies that certain areas on Cenote Azul microbialites are forming and growing at different rates, which may affect their structure and morphology.

In the context of biosignature detection on Mars, this information may prove useful in the selection of sites for rovers and future manned Mars missions to investigate for signs of past life. Investigations into sinkhole-like depressions on Mars with comparable dimensions to those on Earth have been linked to formation by subsurface dissolution and collapse of evaporites, suggesting paleohydrological conditions with underground circulation of water (Parenti et al. 2020). The presence of sinkholes such as these may warrant an argument for further investigation into fossilized microbialites that could have formed in Mars' distant past. Trends in microbialites surface characteristics with depth in Cenote Azul, such as decreasing organics and biomass activity, may provide clues on where microbialite-like structures might be found and expectations for their relative age in Mars sinkholes.

Limitations and Next Steps

There are a number of limitations to this study that should be noted. Due to the nature of SEM, microbialite areas with as little variation in surface topography as possible were selected for subsampling and imaging. This is because the working distance of the electron beam is set to the highest point of the sample. When there are drastic variations in surface topography, areas of the sample surface that are lower than others are further away from the electron beam and are not able to be resolved as precisely. Additionally, areas of varying topography can cause surface charging of electrons to occur. This is due to an inability of electrons to flow off the sample surface, causing them to collect in areas of low topography. The need for a flat topography to obtain optimal SEM images resulted in some degree of bias in the selection of subsample locations, which may have limited the representativeness of these subsamples. However, the effects of surface topography are still visible in the resulting SEM images, with some portions of the microbialite surface affected by charging or blurring. Additionally, the resolution of images that was able to be achieved was limited to the micrometre scale, due to the technical limitations of the JEOL 6610LV SEM instrument. A more powerful instrument

would be able to resolve down to the nanometre scale and possibly show individual bacterial cells, allowing for additional organic content analysis.

Next steps for related future studies could include the imaging of thin sections of Cenote Azul microbialites. This would not only eliminate the detrimental effect of varying surface topography on SEM image resolution, but would allow for more thorough surface characterization and for any organic content beneath the surface of microbialites to be observed. Additionally, it may be useful to compare SEM imagery of modern microbialites such as those from Cenote Azul to SEM imagery of fossilized microbialites. Since there is no longer a widespread presence of liquid water on Mars' surface, it is highly unlikely that active microbialite-like structures could be found; microbial communities as we know them on Earth would simply be unable to survive. However, as evidenced by the existence of fossilized microbialites on Earth, microbialites that may have formed and grown on Mars' surface in the distant past could have been preserved and have the potential to be detected. In order to understand how microbialite surfaces change upon fossilization, a comparative analysis of SEM imagery between modern and fossilized microbialites could be conducted. This will help determine if fossilized microbialites can unequivocally be deemed as biosignatures despite the lack of organics on their surface. Ultimately, this would confirm whether microbialites may be good potential targets for biosignature detection on planets such as Mars.

Conclusion

Microbialites from Cenote Azul, Mexico show trends in decreasing surface colonization, organic content, and biomass activity with increasing depth. This is evidenced by visual observation of SEM imagery, diatom count, and percentage of surface area covered by microbial mat, and is further supported by preliminary PLFA results. These results suggest that deeper microbialites in Cenote Azul are less actively forming and growing in comparison to shallower microbialites, which aligns with past observations in the change in environmental conditions such as light availability and temperature in Yucatan Peninsula cenotes. With evidence for the past presence of widespread liquid surface water during the Noachian period, and investigations into Earth-like sinkhole structures on Mars, microbialites may be a primary target for further astrobiological investigations into possible biosignatures on Mars' surface. Understanding how microbialites form and grow on Earth in karstic environments such as Cenote Azul can inform us on how their biological signatures are preserved in Earth's geologic record, and will bring us one step closer to understanding how life may have formed, evolved, and could be preserved in analogue environments on Mars.

Acknowledgments

There are several people I would like to thank for the support and contributions they have provided in the completion of my thesis. My supervisor, Dr. Greg Slater, has been an extremely valuable source of guidance and insight throughout this thesis process. Thank you for sharing your passion for geochemistry with me, and for inspiring and allowing me to explore its connections to my own interests. Julie Hartz also deserves a huge thank-you for allowing me to be a part of this exciting project at Cenote Azul, and for sharing her knowledge and samples with me. Julie has been a constant support from the beginning to the end of my thesis… I wouldn't have been able to complete it without you! Recognition to Dr. Richard Léveillé at the Department of Earth and Planetary Sciences at McGill University is also deserved. Thank you for helping me get started on this project, and for fostering my enthusiasm for biosignatures on Mars. Finally, this thesis would not have been completed without the use of the facilities and instruments at the Canadian Centre of Electron Microscopy (CCEM). In particular, Jhoynner Martinez at CCEM, was a huge source of help and encouragement during my training on the SEM instruments. Thank you!

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