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ORIGINAL ARTICLE

Phototrophic biofilm communities and adaptation to growth on ancient archaeological surfaces

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Abstract

Purpose Hypogea can be considered under-examined environments as regards microbial biodiversity. New understanding has been gained about the predominant phototrophic microorganisms forming biofilms colonising archaeological surfaces in hypogea. In fact, the description of new taxa has remained elusive until recently, as many biofilm-forming phototrophs possess a cryptic morphology with a lack of specialised cells.

Methods A multiphasic study, including cytomorphological and ecological descriptions, genetic and biochemical analysis was carried out on the biofilms colonising hypogean environments around the Maltese islands. Molecular studies were imperative because biodiversity was found to be more complex than that indicated by classical taxonomy.

Results The dominant microbial life-form on archaeological surfaces is a compact subaerial biofilm. This study has led to new strains of the eukaryotic microalgal genus *Jenufa*, and the prokaryotic cyanobacteria *Oculatella*, *Albertania* and *Nodosilinea* being identified as the principal phototrophic biofilm-formers colonising the ancient decorated surfaces of Maltese hypogea. Complex morphologies and elaborate life cycles were eliminated as biodiversity was dictated only by the local contemporary microenvironment. The production of thick multilayered sheaths aided adherence to the substrate, concentrating microbial cells in biofilm formation. *Albertania skiophila* trichomes were able to glide inside the extracellular matrix. *Oculatella subterranea* exhibited phototaxis associated with a photosensitive apical cell containing a rhodopsin-like pigment.

Conclusion The biofilm provided a protective barrier and an improved chance of survival for cells growing in a low-nutrient, low-light environment. Effective strategies to prevent and control the growth of biofilms on the archaeological surface should take into consideration the adaptation of microorganisms to this particular mode of life.

Keywords Hypogea · Catacombs · Caves · *Oculatella* · *Albertania* · *Nodosilinea* · *Jenufa*

Introduction

The biodeterioration of built heritage has garnered considerable interest during the past 30 years due to an emergent need to preserve these sites for future posterity. Studies have employed different techniques and approaches, mainly depending on whether the archaeological surface is decorated and whether it is sheltered or exposed to weathering conditions. Even though sites, such as caves, hypogea or catacombs, are sheltered, the archaeological surface is still very sensitive to the slightest change in environmental parameters, with a case in point being the rock art in Lascaux and Altamira caves (i.e. Dornieden et al. 2000; Martin-Sanchez et al. 2012; Sáiz-Jiménez 2014).

The growth of microbial biofilms on the archaeological surface, especially when this is embellished by painting or sculpture, interferes with the visual integrity of the work of art and hinders its appreciation. Moreover, previous studies have also confirmed physical and chemical interaction due to the activity of different groups of microorganisms with the underlying substratum, further enhancing biodeterioration (Albertano and Urzì 1999; Cañaveras et al. 2006; Zammit et al. 2011b; Unković et al. 2018; Urzì et al. 2018).

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Contrary to a planktonic lifestyle, growth of microorganisms in a biofilm brings about several changes in gene expression that result in the production of an extensive extracellular polysaccharide (EPS) matrix as an adaptation for attachment to the archaeological surface. This mode of life brings about several advantages over free-living cells such as resistance to environmental stresses (Bharti et al. 2017) and improved cell–cell communication (Flemming and Wingender 2010).

Most of the research regarding biofilm growth on cultural heritage sites has been conducted with the intent of investigating the environmental parameters supporting microbial growth, the biodiversity of microorganisms and methods to control or prevent their growth. Few studies have tried to understand how these microorganisms are adapted to grow as biofilms on the archaeological surface.

This present contribution is concerned with the predominant phototrophic biofilm-forming taxa colonising valuable archaeological surfaces and a description of their adaptations to growth within a biofilm community.

Materials and methods

Sampling

Archaeological surfaces were monitored for the presence of phototrophic biofilms and these were periodically observed and mapped. Representative biofilm samples that were non-invasive to the underlying substratum were taken from St. Agatha's Crypt and Catacombs, St. Paul's Catacombs and the Abbatija tad-Dejr Catacombs in Rabat and the Ħal-Saflieni Hypogeum in Tarxien, Malta, as previously described (Zammit et al. 2008, 2009, 2011a, b). Relative humidity (RH, %), temperature (T, °C) and photosynthetic photon flux density (PPFD, μmol photons m^{-2} s^{-1}) were recorded.

Biofilm communities were grown in culture on agar-solidified BG11, BG110 and BBM media at 18 °C and with a light intensity of 20 μmol photons m^{-2} s^{-1} provided by cool-white fluorescent tubes with a dark–light cycle of 12:12 h. Non-axenic strains of cyanobacteria were isolated on the same media and after a few months of growth on agar media, cyanobacterial isolates were also transferred to liquid BG11, BG110 and BBM media.

Microscopy

The original biofilm samples, as well as biofilm communities growing in culture, were studied by means of light microscopy (LM), confocal laser scanning microscopy (CLSM), scanning (SEM) and transmission electron microscopy (TEM) to determine the predominant phototrophic taxa. The morphology of ubiquitous strains, as well as characteristic features and adaptation to the biofilm mode of life, were noted. Different stages in the life cycle were studied from isolated microorganisms growing in culture.

Samples were examined by a FV1000 confocal laser scanner system mounted on an IX 81 inverted microscope (Olympus, Milan, Italy) with a × 60 (1.32 NA) plan-apochromat oil immersion objective. The wavelengths of the excitation lasers were in the blue (405 nm and 488 nm; diode/Ar), green (515 and 543 nm; Ar/HeNe) and red (635 nm; diode) regions. Images were acquired in the three channels simultaneously. Fluorescence emission was collected in the blue (emission at 400–480 nm), green (emission at 490–530 nm), red and far red regions (590–800-nm emission range) of the spectrum. Data consisted of a series of two-dimensional (2D) cross-sectional images. Spectral analysis (CLSM-SA) of the natural fluorescence of chlorophyll pigments and phycobiliproteins was carried out on the cyanobacterial filaments. Spectra were obtained for a defined range of wavelengths, after excitation with each laser separately. For SEM, hydrated samples were affixed to aluminium stubs, using double-sided carbon tape, and gold sputtered. A Quanta 200 SEM (FEI, Hillsboro, OR, USA), coupled with secondary electron and backscatter detectors, was used to give a detailed and a highly magnified view of the surface. The SEM was operated at 30 keV using a large field detector (LFD) under low vacuum conditions. For TEM, microsamples fixed in 2.5% glutaraldehyde were post-fixed in a 1% osmium tetroxide solution, dehydrated in a graded ethanol series and embedded in epoxy resin (Epoxy 812 Resin Kit, Multilab Supplies, England). Thin sections were collected on copper grids and were observed using a H-7100 TEM (Hitachi, Tokyo, Japan) operating at 100 kV.

Molecular analysis

DNA was extracted from the predominant phototrophic morphotypes using the UltraClean Soil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) according to the manufacturer's protocol. The 16S rRNA gene, ITS region and the partial 23S rRNA gene were amplified using the primer pair: 27F (5′-AGAGTTTGATCCTGGCTCAG-3′ corresponding to positions 8–27 of the 16S rRNA gene of *Synechococcus* sp. PCC6301), after Wilmotte et al. 1993, and 591R (5′-TCGCCGGCTCATTCTTCA-3′) after Haugen et al. 2007. Polymerase chain reactions (PCRs) were carried out in 25-μl aliquots containing approximately 100 ng DNA, a deoxynucleoside triphosphate mixture (0.2 mM each), buffer (1/2 volume of the supplied 2× buffer), supplemented to give a final concentration of 2.5 mM $MgCl_2$, 1.25 U of *Taq* polymerase (Top-Bio, Czech Republic) and 0.5 pmol of each primer. Amplifications were run in a T3000 Thermocycler (Biometra, Germany). The profile used was as follows: 5 min at 94 °C, 40 cycles of 94 °C for 45 s, 57 °C for 45 s and 72 °C for 2 min, and a final elongation step of 10 min at 72 °C.

PCR products were verified on 1.4% (*w*/*v*) agarose gel. Successful amplifications were purified using JetQuick PCR Purification Kit (Genomed, USA) and cloned into pGEM-T Easy Vector System (Promega, USA) according to the supplier's manual and using competent *Escherichia coli* DH5 alpha cells. Competent cells were transformed by heat shock and aliquots of white colonies were transferred to 20 μl of sterile water and heated for 5 min at 94 °C; then, 0.4 μl was used as a template in 10 μl of PCR total volume. The PCR setup and cycling conditions were the same as for initial amplification.

The cloned genes were sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI 3730xl DNA Analyser (both from Applied Biosystems, Foster City, CA, USA) at GATC Biotech Laboratories (Constance, Germany). The partial 16S rRNA gene sequences were compared to other sequences available in GenBank and also to the sequence data of closely related strains isolated from hypogean environments, using the blast algorithm (Altschul et al. 1990). Sequences were aligned using CLUSTALX algorithm implemented in Bioedit 7.0.9.0 (Hall 1999), refined and used to generate a phylogenetic tree. *Gloeobacter violaceus* PCC7421 was used as the outgroup. Phylogenetic trees were inferred by neighbour-joining (NJ) and maximum-parsimony (MP) methods in PAUP v4.0b10 (Swofford 1999) and Bayesian inference (MB) in MrBayes v3.2.6 (Ronquist and Huelsenbeck 2003). MP analysis was carried out using the heuristic search with the tree-bisection-reconnection algorithm (TBR) of ten replicates with random stepwise addition and the MulTrees option. For neighbour-joining, corrected distances were calculated with the HKY85 model (Hasegawa et al. 1985). Robustness of branches was tested by bootstrap analysis on 1000 replicates. The likelihood model was set to a general time reversible model (Rodriguez et al. 1990) including estimation of invariant sites and assuming a discrete gamma distribution with six rate categories (GTR+I+G). No molecular clock was assumed. For Bayesian analysis, two runs with four chains each were run simultaneously for 300 million Markov Chain Monte Carlo (MCMC) generations starting with a random tree. The temperature of a hot chain was set empirically to 0.2 and trees were sampled every 100 generations. The standard deviation between the two MCMC runs was below 0.01, indicating convergence. The first 25% of trees were discarded as the burn-in phase and a 50% majority rule consensus tree was calculated including posterior probabilities.

Results

The archaeological surface of Maltese hypogea, including decorative stucco, mortar, intonaco and paint layers, was subject to stable T throughout the year (19–21 °C) and a RH superior to 95% with frequent condensation phenomena. The light intensity at sampling points was low, with a PPFD that never exceeded 15 μmol photons m^{-2} s^{-1}. The four man-made hypogea investigated were excavated into limestone, in which calcite was the dominant mineral (Zammit et al. 2011b).

Throughout this study, phototrophic biofilms were observed to grow as thin, compact, pigmented films over the archaeological surface (Fig. 1). The surface-attached microbial communities received light energy from lamps introduced into archaeological sites for the benefit of visitors. These biofilms were multispecies microbial communities (Figs. 2 and 3), in which the predominant phototrophic microorganisms were filamentous cyanobacteria (Figs. 2, 3, 4, 5 and 6), as indicated by the presence of phycobiliprotein pigments via CLSM-SA (Fig. 7), together with heterotrophic bacteria and green microalgae (Fig. 8). On the other hand, biomats formed thicker, heterogenous, multilayered communities in particular microenvironments due to the presence of natural light, such as at the entrance chambers to hypogea. Higher organisms such as slugs were observed to graze on them (Fig. 1).

Direct microscopic observation (LM, CLSM, SEM, TEM) of the biofilms and biomats revealed that the cells occurred in clusters (Figs. 2 and 3) with cyanobacterial filaments often surrounded by actinomycetes and heterotrophic bacteria (Figs. 2 and 3). Within the community, different cells possessed confluent sheaths and capsules which contributed to the formation of the EPS matrix (Fig. 3). The microorganisms were not sessile, and even though the community was attached to the archaeological surface, individual cells or thin simple filaments were regularly observed to move or glide in the EPS (Fig. 2a). Cells in the microbial community interacted closely together, and heterotrophic bacteria formed intimate associations with phototrophic microorganisms (Fig. 3). Specialised cells, as well as spores, could be identified, but these were better elucidated via microscopic observations of the same communities growing in vitro (Figs. 4, 5, 6, 7 and 8).

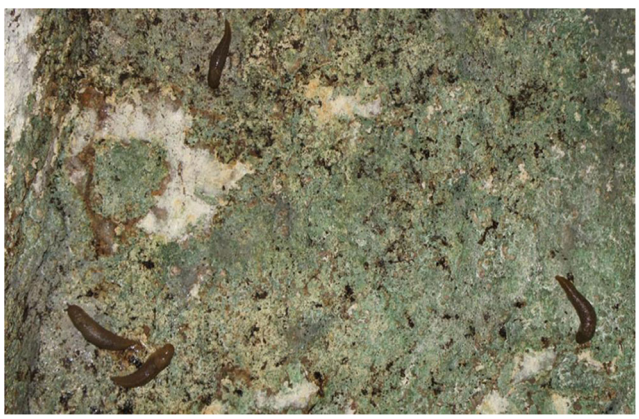

Fig. 1 Phototrophic biofilms composed predominantly of filamentous cyanobacteria in complex ecological interaction with higher organisms on an archaeological surface at St. Agatha Catacombs

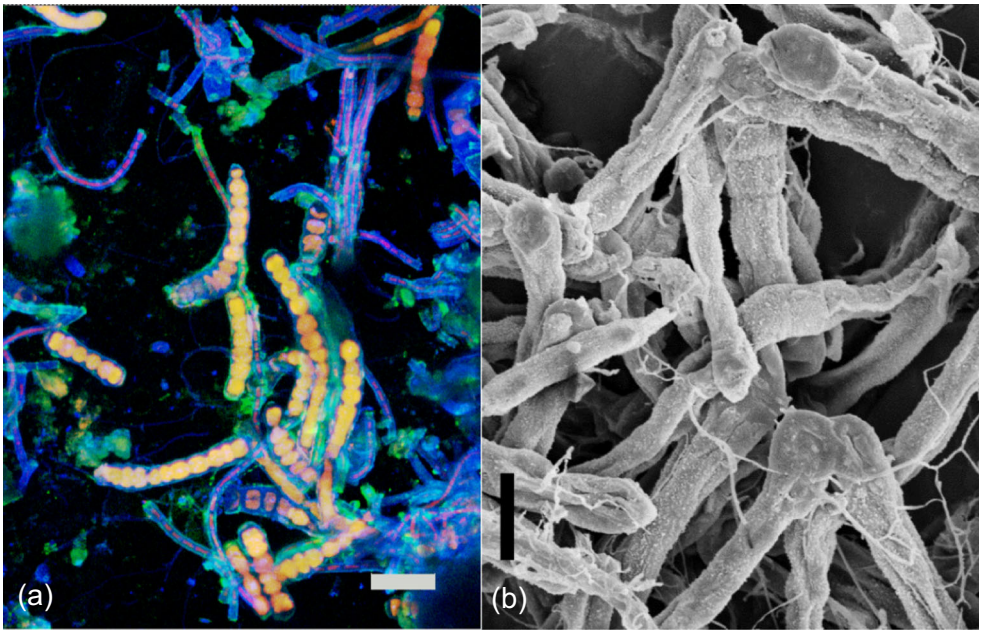

Fig. 2 Direct observation of phototrophic biofilms composed predominantly of filamentous cyanobacteria. **a** CLSM micrograph of biofilm from St. Paul Catacombs, showing *Spelaeonaias* sp. hormogonia and finer *Oculatella subterranea* filaments. Structures shown in blue are EPS sheaths and biofilm matrix. Scale bar = 20 μm. **b** SEM micrograph of biofilm from St. Agatha Catacombs composed of branches of *Spelaeonaias* sp. surrounded by actinomycete filaments. Scale bar = 10 μm

In fact, direct microscopic observation of the biofilm samples and the corresponding microbial communities growing in culture revealed small cells and cryptic morphologies (Figs. 2a, 3, 4, 8), which could be better understood by observing the isolated strains and studying their life cycle.

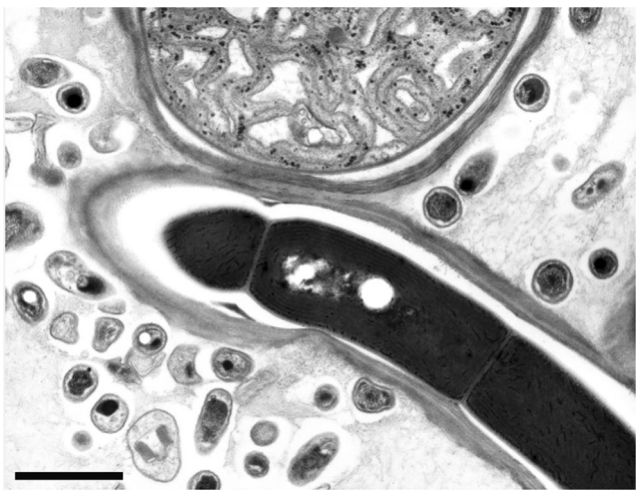

Fig. 3 Transmission electron micrograph of phototrophic biofilm from St. Agatha Catacombs, composed of Oscillatorialean and Nostocalean filaments with heterotrophic bacteria. Micrograph shows confluent cyanobacterial sheaths contributing to the formation of an exopolysaccharide matrix, in which smaller bacteria are also embedded. Scale bar = 2 μm

The prevalent filamentous cyanobacteria forming biofilms on the archaeological surface belonged to the Leptolyngbyaceae. Their simple filaments consisted of a fine trichome of pigmented cells surrounded by a thick transparent sheath (Figs. 2a and 3). Trichomes were frequently observed to glide towards the light and, of these, *Oculatella subterranea* filaments could be easily identified through the presence of a specialised apical cell. However, in the case of stains belonging to the genera *Albertania* Zammit and *Nodosilinea* Perkerson et Casamatta (Fig. 4), genetic studies had to be used to confirm these taxa.

Heterocytous cyanobacteria belonging to the genera *Nostoc* (Fig. 5) and *Spelaeonaias* (Fig. 6) were also present. Both were observed to produce these nitrogen-fixing cells in low-nitrate environments (Figs. 5 and 6). *Nostoc* strains produced resistant akinetes under conditions of low nutrient availability. *Spelaeonaias* filaments branched frequently, with erect filaments growing towards the light source (Fig. 6). These cyanobacteria were capable of growing in low-light environments due to the presence of the phycobiliproteins phycoerythrin, phycoerythrocyanin and phycocyanin (Fig. 7) inside their cells. Ubiquitous coccal green microalgae surrounded by thick capsules were observed to grow in all biofilms, and especially at the Ħal-Saflieni Hypogeum, and these belonged predominantly to the genus *Jenufa* Němcová, M. Eliáš, Škaloud et Neustupa (Fig. 8).

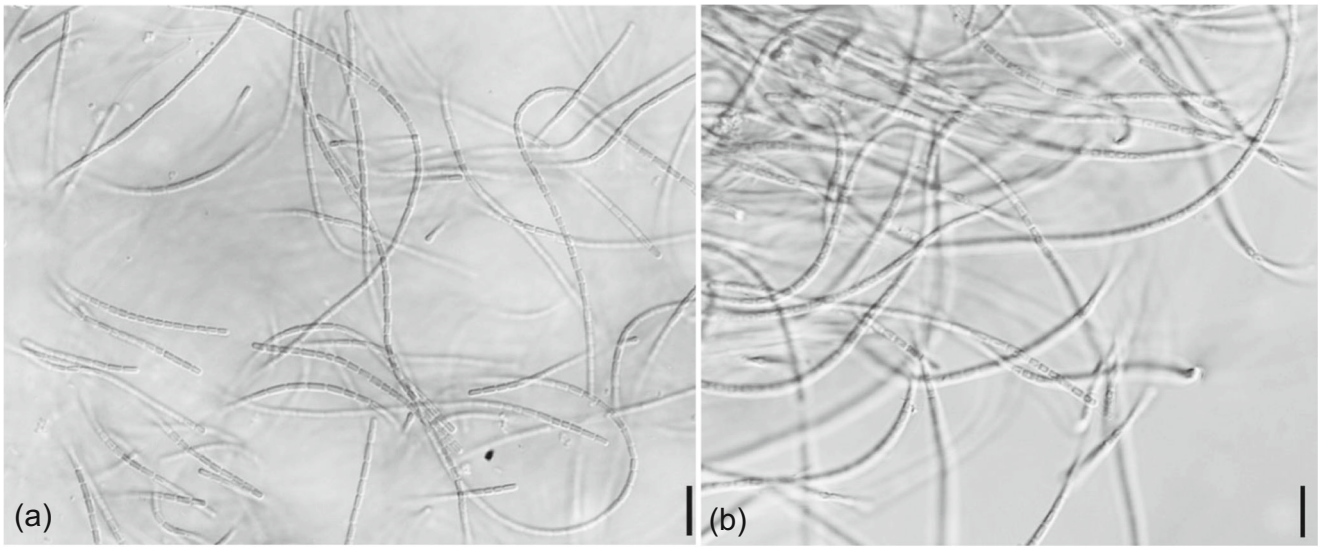

Fig. 4 Light micrographs of fine filaments of the *Nodosilinea* strains sequenced in this study, isolated in culture from Abbatija tad-Dejr and Saint Agatha's Catacombs respectively. **a** 2AD401 and **b** SA1303. Scale bars = 10 μm

A study of the corresponding isolated strains growing in culture revealed that complete life cycles were simple, with propagation and dispersal via the production of hormogonia in filamentous cyanobacteria (Fig. 5a) and autospores (Fig. 8) in green microalgae.

A genetic analysis of the predominant fine filamentous cyanobacteria helped confirm the identity of simple filaments with a cryptic morphology. The phylogenetic analysis is shown in Fig. 9. Different clades show sequences for strains belonging to *Oculatella* and *Nodosilinea* spp. sequenced as part of this study, as well as other *O. subterranea* and *A. skiophila* strains sequenced from biofilms colonising the same archaeological surfaces. All three taxa were distinct from the genus *Leptolyngbya*, in which they were originally classified, the type species of which, *L. boryana*, is also shown in Fig. 9. The sequences acquired as part of this study were submitted to GenBank under accession numbers MK02140 to MK02142.

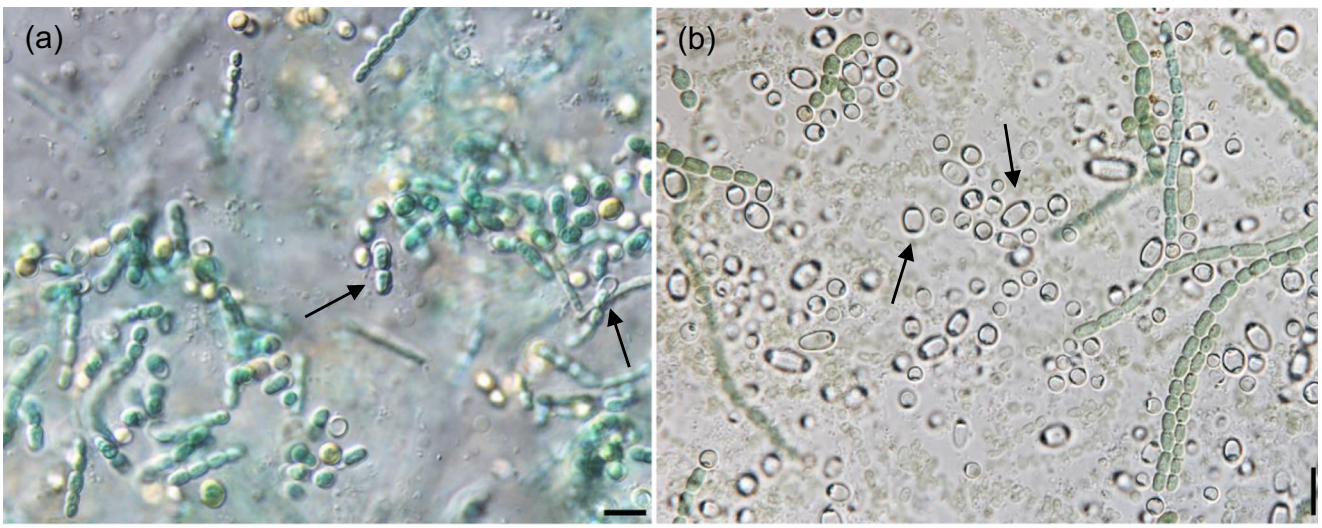

Fig. 5 Light micrographs of *Nostoc* strains isolated in culture from Abbatija tad-Dejr showing **a** germinating akinetes and hormogonia **b** numerous large heterocytes. Scale bars = 10 μm

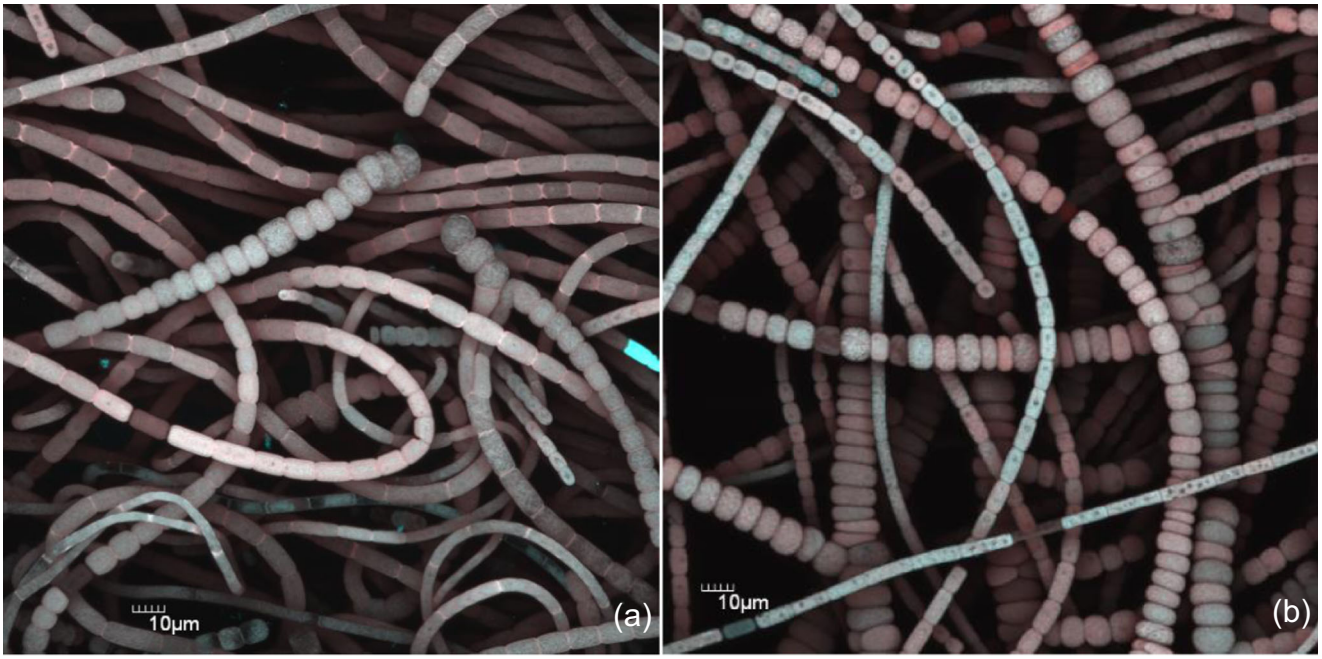

Fig. 6 Confocal laser scanning microscopy of *Spelaeonaias* strains from St. Paul's and St. Agatha's Catacombs respectively. **a** SP302, showing reverse Y-branching. **b** SA1301, showing T-type branching. The autofluorescence is of the phycobiliproteins phycoerythrin (light blue), phycocyanin and phycoerythrocyanin (pink). Scale bars = 10 μm

Discussion

During this study, a better understanding of the adaptations of microorganisms towards this particular mode of life has been gained through direct observation of biofilms, the biofilm community growing in culture and the isolated strains. This study presents evidence of unique morphological and ecological adaptation, with genetic data supporting the fact that the

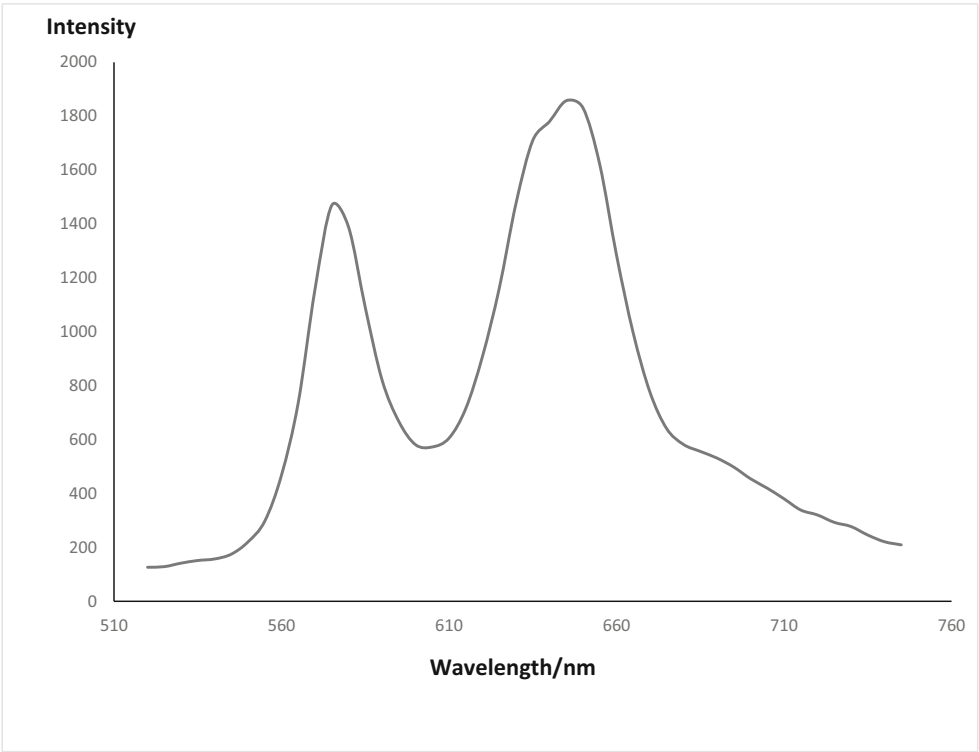

Fig. 7 Spectral analysis via CLSM-SA of *Spelaeonaias* strain SA1301 isolated from St. Agatha's Catacombs, showing emission maxima of phycoerythrin, phycoerythrocyanin (shoulder) and phycocyanin at 575, 635 and 650 nm, respectively

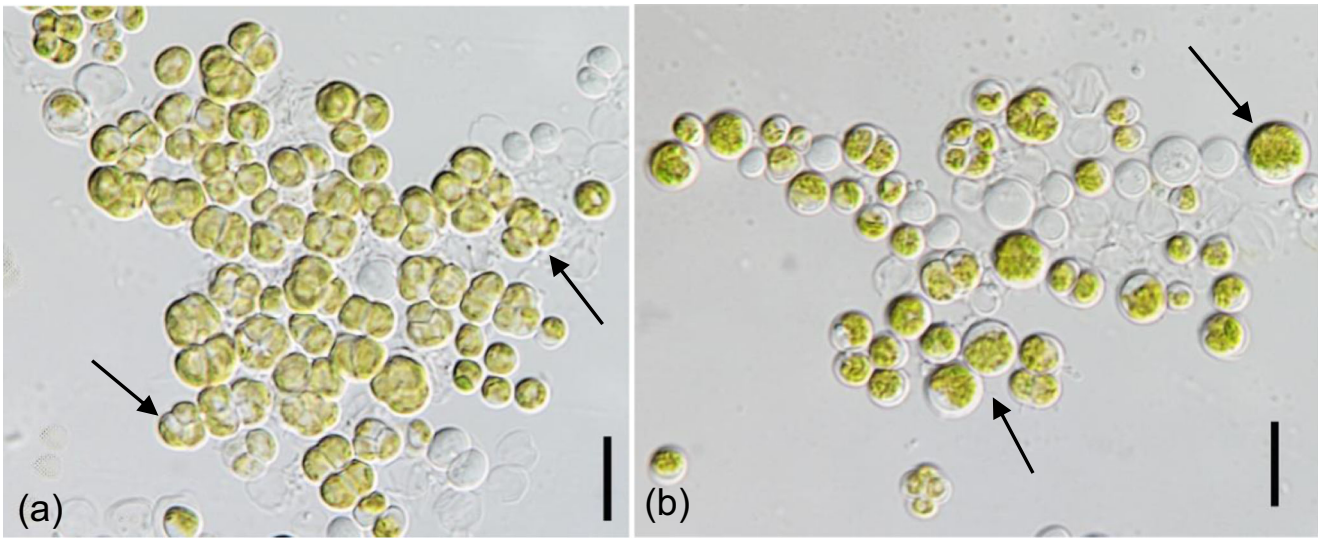

Fig. 8 Light micrographs of *Jenufa* strains isolated in culture from St. Agatha's and St. Paul's Catacombs, showing **a** formation of autospores and **b** thick extracellular capsules. Scale bars = 10 μm

microorganisms growing in these biofilms have evolved unique genetic adaptation to this mode of life. In fact, the phototrophic taxa found to be predominant biofilm formers on the archaeological surface were filamentous cyanobacteria, mostly belonging to the Leptolyngbyaceae: *Oculatella subterranea*, *Albertania skiophila* and *Nodosilinea* sp. (Fig. 9). Both *Oculatella subterranea* and *Albertania skiophila* were described using a polyphasic approach (Komárek 2016) and are recognisable due to the successful strategies that facilitate growth on archaeological surfaces, leading to a unique ecology when compared to closely related genera. They are sciaphilous microorganisms, growing both in sheltered hypogea, such as the ones studied here, as well as outdoors in sheltered areas of cultural heritage sites in various locations (Del Mondo et al. 2018; Vázquez-Martínez et al. 2018).

Since ubiquitous phototrophic strains were found to belong to genera that have all been recently described, i.e. *Oculatella*, *Albertania*, *Nodosilinea* and *Jenufa* (Němcová et al. 2011; Perkerson et al. 2011; Zammit et al. 2012; Zammit 2018), this fact and cryptic morphologies have, so far, probably contributed to an underestimation of the biodiversity occurring on archaeological surfaces. In fact, apart from archaeological surfaces in the Maltese islands, similar strains have also been recently characterised from biofilms colonising archaeological surfaces in Greece and Italy, among other locations (Christodoulou et al. 2015; Del Mondo et al. 2018; Vázquez-Martínez et al. 2018; Zammit et al. 2012; Zammit 2018).

Invaluable information was obtained from studying the biofilm communities directly and also from observation in situ. This study allowed the examination of biofilm using sampling techniques that were non-invasive to the underlying substratum (Urzì and De Leo 2001) and that preserved the biofilm structure formed in situ. Subsequent CLSM and SEM observation of biofilm and biomat samples provided a means for adequate three-dimensional inspection without significant distortion of sample architecture. Moreover, the direct observation of microorganisms by LM, CLSM and TEM helped elucidate cryptic morphologies and life cycles which contributed to correct identification. Finally, genetic investigation, especially the amplification and sequencing of the conserved 16S rRNA gene, provided confirmation of the evolution of species that were adapted to grow on archaeological surfaces, such as *Oculatella subterranea* (Zammit et al. 2012) and *Albertania skiophila* (Zammit 2018). This present study led to the identification of two new strains of *Nodosilinea*, SA1303 and 2AD401, from the archaeological surface at Saint Agatha's Crypt and Catacombs and Abbatija tad-Dejr respectively, as well as a new strain of *Oculatella*, SP043, from the Catacombs of St. Paul (Figs. 4 and 9). Further molecular studies are being conducted on these strains and a number of others because there is likely to be much more diversity than that indicated by classical taxonomy. The amplification and analysis of the 18S rRNA of coccal microalgae from the same archaeological sites, as well as biochemical investigation, has been carried out in a separate study (Agius 2018).

Apart from the pigmented phototrophic biofilms studied here, white biofilms too were observed to grow at the same sites on archaeological surfaces with different microenvironments, particularly in the absence of artificial or natural light, in chambers that were not open to the general public. The biodiversity of both types of multispecies biofilm and their interactions with the archaeological surface are described elsewhere (Zammit et al. 2009, 2010, 2011a, b; De Leo et al. 2012).

Heterogenous phototrophic biofilms, which contained a rich biodiversity of species, formed in response to environmental stresses on the archaeological surface, most notably

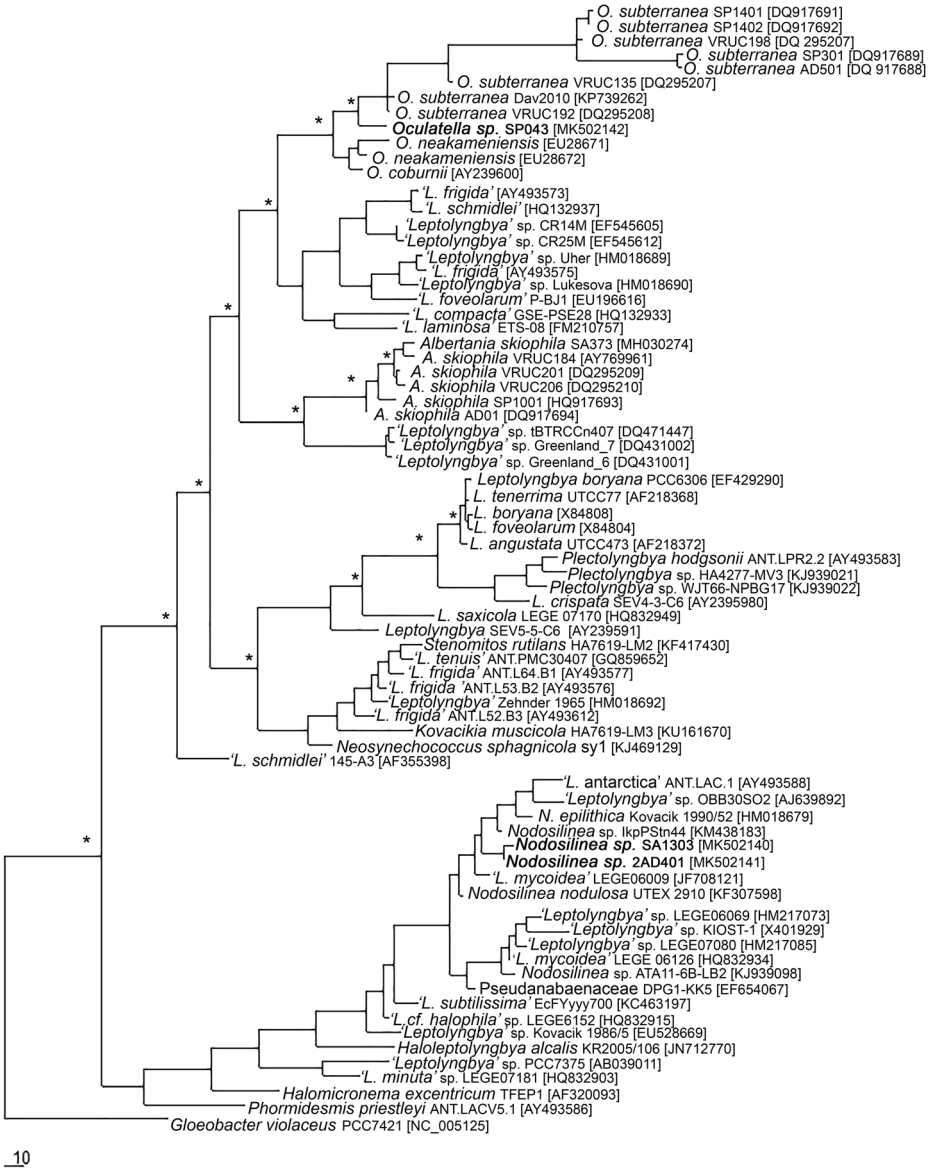

Fig. 9 Phylogenetic relationships inferred from Bayesian analysis of 16S rRNA gene sequences (1600 bp) of *Albertania skiophila*, *Oculatella subterranea* and *Nodosilinea* strains isolated from archaeological surfaces in Maltese hypogea. (Names of strains indicate the archaeological site from which these were sampled: SA, St. Agatha's Catacombs; SP, St. Paul's Catacombs; AD, Abbatija tad-Dejr). These are aligned and compared to sequences from other Leptolyngbyaceae. Trees with similar topology were recovered by NJ and MP analysis. Nodes marked by an * indicate support by both bootstrap and posterior probabilities (100% and 1.0 respectively). Sequences determined in the present study are indicated in bold. GenBank accession numbers follow in brackets. *Gloeobacter violaceus* PCC7421 was designated as the outgroup. Scale represents number of nucleotide substitutions per site

the low light intensity and low nutrient availability. In these, the carbon source is provided via CO_2 fixation through photosynthesis in cyanobacteria and microalgae, the simplest representatives of which were probably the pioneer microorganisms on the archaeological surface. Atmospheric N_2 is fixed via heterocytous cyanobacteria (Figs. 5 and 6) and is made available to the biofilm community. Heterotrophic bacteria contributed by recycling organic matter, especially from dead cells, within the biofilm itself. In turn, the biofilm community as a whole represented the base of the food chain in a more complex ecological setting. In fact, this biocoenosis also supported herbivore nutrition, as attested by the presence of slugs grazing on the biofilm (Fig. 1).

It was often difficult to obtain axenic cultures of phototrophic strains from the biofilm, as some of these formed intimate associations with heterotrophic bacteria (Figs. 2 and 3), indicating possible syntropy between bacteria and phototrophs (Bruno et al. 2006). However, on the other hand, it was also important to study mixed cultures in vitro in order to establish adaptation strategies of multispecies communities to growth in

biofilm structure. The state of conservation of the archaeological surface probably also contributed to biofilm growth, as it was weathered and aged, and thus pre-conditioned and receptive towards biofilm and biomat formation.

These cyanobacteria and microalgae fit a particular ecological niche and are equipped with a number of morphological features designed to exploit it. Among these are the facts that they possess cells that are smaller in size than those of the closest related taxa, as in trichome diameters of *Oculatella subterranea* (≤2 μm) compared to those of *Leptolyngbya* (0.5–3.5 μm) and cell diameters of *Jenufa perforata* (3.5–6.5 μm) compared to those of '*Chlorella*' strains (Safi et al. 2015). This happens due to the fact that small-sized cells with a large surface area to cell volume ratio probably have a greater competitive advantage to survival on the archaeological surface.

Most of these strains were found to belong to cryptic lineages due to a simple morphology. The life cycle was also simple, with cells or filaments having simplified modes of reproduction and dispersal when compared to planktonic cyanobacteria and microalgae. Propagules formed by fragmentation into short filaments or hormogonia in cyanobacteria (Figs. 2a and 5a), or repeated divisions to form small autospores in coccal green microalgae (Fig. 8). These became easily dislodged and dispersed to release small propagules and to facilitate the colonisation of new substrates, a process we know to be mediated through quorum sensing mechanisms (Trunk et al. 2018).

The cyanobacterial mode of life was predominantly filamentous, with the production of resistant akinetes to survive periods of nutrient depletion (Fig. 5a) and heterocytes for nitrogen fixation (Figs. 5b and 6) in different taxa, depending on the local contemporary microenvironment. Numerous large heterocytes, up to 8 μm in length, were often observed to be suspended in the EPS matrix (Fig. 5b). The presence of multilayered sheaths, capsules and thick EPS layers offered protection (Rossi and De Philippis 2015).

The cells of heterocytous filamentous cyanobacteria were observed to be more compact in prostrate filaments growing parallel to the archaeological surface, occupying less space, with frequent protruding filaments formed from T-type and Y-type branches and possessing more elongated cells growing towards the light (Fig. 6a, b).

Among the adaptations to growth in a low-light environment were the presence of the specialised pigments phycoerythrin, phycocyanin and phycoerythrocyanin in particular ratios that enabled absorption of light energy (Fig. 7). In fact, *O. subterranea* cells possessed a higher content of phycoerythrin than phycocyanin (Zammit et al. 2012) in their cells and appeared red, but these cells were also capable of chromatic adaptation at different light intensities. Moreover, *O. subterranea* filaments had a photosensitive apical cell containing a rhodopsin-like pigment (Zammit et al. 2012) enabling phototaxis. The pigment ratio in *A. skiophila* made the trichomes appear blue-green and gliding inside the sheath was frequently observed and enabled these cyanobacteria to reach the photic zone of the biofilm or biomat (Zammit 2018).

Thick sheaths (Fig. 2a) and EPS layers (Fig. 3) were observed in biofilms dominated by cyanobacteria, while thick capsules (Fig. 8) and copious quantities of secondary metabolites were produced and secreted by *Jenufa* strains (Agius 2018). In biofilm structure, the metabolic by-products of the cyanobacteria and microalgae served to support the growth of heterotrophic bacteria, while the adhesion of the pioneer species provided the EPS to allow the attachment of others (Flemming and Wingender 2010).

Biofilm formation provided a mechanism for attaching to an archaeological surface, concentrating cells in a localised microenvironment, accumulating nutrients in an EPS matrix that was rich in carbohydrate and protein (Agius 2018). High concentrations of released metabolites were retained in close proximity to the cells in biofilm structure, thus promoting the re-use and recycling of nutrients and conserving energy required for their production. The EPS enabled biofilm colonies to sequester and adsorb ions from the underlying archaeological surface (Zammit et al. 2011b).

Cells were found to be slow growing, but capable of sustained growth for an extended period of time with a minimal input of nutrients from outside sources (Agius 2018). Some studies have suggested that there are low levels of metabolic activity in microbial biofilms (Dornieden and Gorbushina 2000), so this too might be an adaptation to the biofilm mode of life. In fact, our study shows that *Jenufa* strains colonising the Hypogeum of Ħal-Saflieni remained viable and grew in culture even after a conservation program in which the light availability was drastically reduced or completely eliminated.

All the above characteristics indicate that these consortia of cyanobacteria, microalgae and bacteria in biofilms and biomats offered an improved chance of survival on the archaeological surface when compared to that of the constituent microorganisms. In fact, throughout this study, microscopic observations revealed well-developed subaerial phototrophic (both cyanobacterial and green microalgal) communities that were made of similar in composition to those forming biofilms on archaeological surfaces in other Mediterranean and temperate regions (Bruno et al. 2009; Christodoulou et al. 2015; Del Mondo et al. 2018; Vázquez-Martínez et al. 2018; Zammit et al. 2012; Zammit 2018). Such microbial consortia have also found to demonstrate high potential to adapt to more extreme environments such as desert habitats (Perera et al. 2018).

Archaeological surfaces elsewhere also supported the growth of biofilms formed by other morphologically

simple photoautotrophs, such as the filamentous *Timaviella circinata*, *T. karstica* and *Heteroleibleinia purpurascens* recently described from the Giant Cave, located near Trieste in Italy (Sciuto et al. 2017).

This low-light, highly humid archaeological cave environment was recently also found to harbour biofilms of a new species from the most primitive genus of cyanobacteria *Gloeobacter*, *G. kilaueensis* (Saw et al. 2013). Although *G. kilaueensis* grew on volcanic rock, the type species of this genus, *G. violaceus* is known to grow on calcareous rock. *Gloeobacter kilaueensis* formed purple colonies of obligately photoautotrophic, sessile, simple rod-shaped cells in which thylakoid membranes are absent. This fact supports the theory that this type of surface supports the growth of morphologically simple, biofilm-forming cyanobacteria.

Throughout this present study, phototrophic biofilms were monitored seasonally to study growth patterns. In the absence of major changes in the environment, no visible change occurred. When the internal environment was retained constant, detectable changes in the extent of biofilm growth only occurred after a 5-year period. However, at St. Agatha's Crypt and Catacombs, when local environmental conditions were altered, such as the wavelength of incident light, the surrounding biofilms were observed to change in colour and consistency in response to a change in the local microenvironment. Rapid re-colonisation of decorated archaeological surfaces via biofilms formed predominantly by Leptolyngbyaceae was also observed from June to October 2017 at St. Paul's Catacombs in Rabat, Malta, immediately after a cleaning and conservation project, during which a new lighting system was also installed. However, following biofilm establishment, the growth rate subsequently slowed down. The same phenomenon was also observed in culture where a temperature of 20 °C and a relative humidity above 95% were found to be sufficient for *Oculatella subterranea* biofilms to grow at a fast rate, in as little as 7 weeks (Del Mondo et al. 2018).

Archaeological surfaces in caves and hypogea can be considered under-examined environments as regards biodiversity. However, some recent descriptions of new taxa from cave environments are very similar to older genera and are probably synonyms of the latter. Examples include the genus *Spelaeonaias* (Lamprinou et al. 2016), which is probably synonymous with the genus *Spelaeopogon* (Borzí 1907, 1917), the type species of which, *S. sommieri,* was described from biofilms in calcareous caves on the Maltese islands (Sommier 1908). Also complicating the matter is the fact that the type species, *Spelaeonaias floccida*, is genetically characterised only by a 300-base sequence of the conserved 16S rRNA gene, which will presumably impede the description of morphologically cryptic species belonging to the same genus. The genus *Iphinoe* (Lamprinou et al. 2011) is another example since it is morphologically identical to *Geitleria calcarea* (Friedmann 1955) except for the production of heterocytes, and is described from the same environment. Challenges also lie with other species that have been recently described from archaeological surfaces in caves, such as *Nostoc cavernicola* (Miscoe et al. 2016), since no genetic sequences of strains belonging to this species are currently available on public databases. It is thus obvious that a further understanding of the morphology, life cycle and genetics of the microorganisms in these proposed taxa is thus required, with a more thorough understanding of their multiple adaptations to biofilm formation.

Conclusion

Heterogenous, structured phototrophic biofilms formed in response to the microenvironmental factors and stresses related to the growth of microorganisms on archaeological surfaces. Each biofilm had a particular morphology and 3-dimensional structure that depended on the respective microhabitat. The determining factors were light, nutrient and water availability, since temperature was very stable throughout the year. Biofilm formation provided a mechanism for attaching onto the archaeological surface, concentrating cells and nutrients in an extracellular matrix. The biofilm provided a protective barrier and thus an improved chance of survival in a low-nutrient environment via the presence of predominant phototrophic cyanobacteria and microalgae. Cells were small and possessed simple morphologies and life cycles. They were able to persist for extended periods of time, both in culture, as well as in situ on archaeological surfaces with minimal input of nutrients from outside sources.

Effective strategies to prevent and control the occurrence and growth of biofilms on archaeological surfaces should take into consideration the unique, persistent nature of these communities and their prevalent adaptation to this particular mode of life.

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Compliance with ethical standards

Conflict of interest The author declares that she has no conflict of interest.

Research involving human participants and/or animals No humans or animals were used in this work.

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