

## The biodiversity of subaerophytic phototrophic biofilms from Maltese hypogea

Gabrielle ZAMMIT<sup>1\*</sup>, Daniela BILLI<sup>2</sup>, Elliot SHUBERT<sup>3</sup>, Jan KAŠTOVSKÝ<sup>4</sup> & Patrizia ALBERTANO<sup>2</sup>

<sup>1</sup>Department of Physiology & Biochemistry, Faculty of Medicine & Surgery, University of Malta, Msida, Malta; \*corresponding author: gabrielle.zammit@gmail.com

<sup>2</sup>Department of Biology, Faculty of Science, University of Rome 'Tor Vergata', Rome, Italy

<sup>3</sup>Department of Botany, Natural History Museum, London, UK

<sup>4</sup>Department of Botany, Faculty of Science, University of South Bohemia, České Budějovice, Czech Republic

**Abstract:** The present study focuses on a description of the biodiversity of subaerial phototrophic biofilms occurring on archaeological surfaces in Maltese hypogean environments, namely St Paul's, St Agatha's and Abbatija tad-Dejr Catacombs, all situated in Rabat and the ancient Hal Saffieni Hypogeum at Paola, Malta. Direct observation of the biofilms, carried out using light (LM), epifluorescent and confocal laser scanning microscopy (CLSM), allowed the description of the major cyanobacterial and microalgal taxa, and also the associated heterotrophic microorganisms, mainly actinobacteria. Some biofilm microorganisms were able to grow in culture and this allowed the isolation of cyanobacterial, microalgal and chemoorganotrophic bacterial strains. Thylakoid arrangement and cell division were examined using transmission electron microscopy (TEM). The cytomorphology of isolated microorganisms was described. The undisputed phototrophic protagonists in these subaerial biofilms of Maltese hypogean environments were the non-heterocytous (Oscillatoriales) cyanobacteria. In order to increase the limited data available for *Leptolyngbya* spp. from aerophytic epilithic biofilms in catacombs, the 16S rRNA genes of isolated *Leptolyngbya* strains were sequenced and compared with those obtained for related strains. Phylogenetic trees of cyanobacterial 16S rRNA sequences were constructed using parsimony and Bayesian analyses. Microorganisms forming biofilms in Maltese hypogea were found to be similar, both cytomorphologically and genetically, to those colonising lithic surfaces of caves and catacombs in other Mediterranean countries.

**Key words;** catacombs, cyanobacteria, cyanoprokaryotes, hypogea, *Leptolyngbya*, microalgae, Oscillatoriales, phototrophic biofilms, Stigonematales, subaerophytic

### Introduction

The phototrophic microbial biofilms occurring in Maltese hypogea are multi-layered structures composed of physiologically different groups of microorganisms. The most important primary producers in this ecosystem are the filamentous cyanobacteria and Chlorococcal microalgae. These are accompanied by Chroococcalean cyanobacteria, filamentous microalgae, diatoms, moss protonema, actinobacteria, and gram-positive bacilli (ZAMMIT et al. 2009). The microorganisms are normally surrounded by thick extracellular polymeric substances (EPS), which glue the biofilm to the substratum.

The most comprehensive studies of subaerial

biofilms from catacombs have been carried out on the three catacomb sites dedicated to St. Domitilla, St. Callistus and Priscilla in Rome, Italy. Different species of terrestrial epilithic cyanobacteria belonging to the genera *Eucapsis* CLEMENTS et SHANTZ 1909, *Leptolyngbya* ANAGNOSTIDIS et KOMÁREK 1988, *Scytonema* AGARDH ex BORNET et FLAHAULT 1887, and *Fischerella* (BORNET et FLAHAULT) GOMONT 1895, were found to occur as dominant organisms in these phototrophic microbial communities. A few species of green algae and the diatom *Diademesmis gallica* W. SMITH 1857 occurred in minor amounts (ALBERTANO et al. 1989; ALBERTANO et al. 1995). The most frequently occurring actinobacteria were the streptomycetes; *Streptomyces* WAKSMAN et HENRICI 1943, *Nocardia* TREVISAN 1889 and *Micrococcus* COHN 1872.

Analysis of partial 16S rRNA sequences revealed that many isolates were of novel species. Other bacteria detected in the air samples were mostly Gram-positive chemoorganotrophs belonging to the genera *Staphylococcus* ROSENBAACH 1884, *Microbacterium* ORLA-JENSEN 1919 and *Bacillus* COHN 1872. Gram-negative bacteria were a minority among the isolated bacteria, and were species belonging to *Pseudomonas* MIGULA 1894, *Stenotrophomonas* PALLERONI et BRADBURY 1993, *Pectobacterium* WALDEE 1945 and *Acinetobacter* BRISOU et PRÉVOT 1954. Most of the anaerobically growing bacteria were Gram-positive cocci, mainly facultative anaerobic species of *Staphylococcus* ROSENBAACH 1884. Fungi, mainly white *Lecanicillium psalliotae* (TRESCHAW) ZARE et W. GAMS 2001, *L. Aranearum* (PETCH) ZARE et W. GAMS 2001, species of *Torrubiella* BOUDIER 1885 and *Beauveria alba* (LIMBER) SACCAS 1948 were isolated to a lesser extent than bacteria from both air and biofilm samples (SAARELA *et al.* 2004).

The structural relationships among phototrophic and chemoorganotrophic microorganisms forming subaerophytic biofilms inside catacombs environments in Rome have also been studied (ALBERTANO & URZI 1999). There is often an intimate association between actinobacteria, mainly *Streptomyces* spp., and filamentous cyanobacteria. It is however difficult to observe direct contact, and indeed direct cell wall association has only been reported between *Leptolyngbya* sp. and a filamentous bacterium, identified possibly as *Streptomyces* sp. (ALBERTANO *et al.* 1989). The EPS produced by cyanobacteria are able to mediate metabolite exchange and serve as an excellent growth substratum for a variety of heterotrophic bacteria (PAERL 1992).

Cyanobacterial diversity in nature (GARCIA-PICHEL *et al.* 1998; TATON *et al.* 2002) is substantially wider than is recognisable phenotypically, and for instance, only 5–10% of the real diversity particularly in tropical and extreme biotopes has been acknowledged (WATANABE 1999).

At present, both the cyanobacterial (KOMÁREK 2003) and microalgal (LOPEZ-BAUTISTA *et al.* 2007) taxonomic systems are in dire need of revision. Only a number of well-characterised and clearly distinct genera are easily defined (KOMÁREK *et al.* 2003; LOPEZ-BAUTISTA *et al.* 2007). Infra-generic criteria are still not clear (KOMÁREK 2002) and the

species concept, especially in cyanobacteria, is still being defined according to molecular criteria (KOMÁREK 2003).

In the present study, a polyphasic approach was applied (ANAGNOSTIDIS & KOMÁREK 1985a; WILMOTTE & GOLUBIĆ 1991) that combined morphological, ultrastructural as well as ecological and molecular criteria. In particular, the 16S rRNA gene was used to identify bacterial genera and 18S rRNA gene for eukaryotic groups. Apart from a general study of the biofilm diversity, this research was also concerned with the description of members of the genus *Leptolyngbya* in Maltese hypogean environments.

## Materials and Methods

**Sampling.** Subaerial phototrophic biofilms occurring as pigmented alterations on the archaeological surface of the Maltese palaeo-Christian catacombs dedicated to St Paul, St Agatha and Abbatija tad-Dejr, situated in Rabat and the ancient Hal Saffieni Hypogaeum at Paola were sampled during the period between May 2005 and November 2007. Sampling was conducted using non-invasive sampling methods by which only the biofilm was sampled and not the underlying substratum. Samples were removed using adhesive tape strips (URZI & DE LEO 2001) and damp sterile filter paper. Environmental variables such as the light intensity and photoperiod, relative humidity, substrate preference and the visible presence of water were recorded for each sample (ZAMMIT *et al.* 2008a, 2008b, 2009). Samples were stored at 4°C and subsequently investigated as described below.

**Cultures.** For the isolation of microorganisms, strips (2 × 2 cm) of adhesive tape and filter paper were streaked directly onto Petri dishes containing agarised culture media under sterile conditions. The nutrient media used were specific for the isolation of: heterotrophic bacteria (GYM after LERCH & ETLINGER 1972, without CaCO<sub>3</sub>); cyanobacteria (BG11, both enriched with nitrates and without, after Allen 1968); eukaryotic microalgae (BBM enriched with vitamins after Bischoff & Bold 1963); and fungi (DRBC after KING *et al.* 1979). During the preparation of all liquid and agarised media, the pH was adjusted to 7. Incubation for chemoorganotrophic organisms was carried out at 28°C for one month and investigations ensued as described in ZAMMIT *et al.* (2008a). Phototrophs were cultured at 18°C and a light intensity of 8 μmol.m<sup>-2</sup>.s<sup>-1</sup>.

The growth of phototrophic microorganisms in culture was initially very slow. Various approaches were followed in order to produce unicyanobacterial

or unialgal cultures. Continuous sub-culturing was carried out in the same medium which helped to obtain effective results. The mechanical separation by micromanipulation and repeated transfer of cells allowed successful isolation. The purity of the cultures was checked regularly under the light microscope. After one year of growth on agarised media, isolates were also transferred to liquid media, where better growth was obtained.

**Microscopy.** Direct observation of biofilms was carried out using light and fluorescence microscopy. Sections approximately 1x1cm were cut from adhesive tape samples (URZI & DE LEO 2001). These were labelled with the following fluorochromes; 3  $\mu\text{g.ml}^{-1}$  Acridine Orange (AO) for nucleic acids and polysaccharides, 50  $\mu\text{g.ml}^{-1}$  fluorescein isothiocyanate conjugated to Concanavalin A (FITC-conA) for  $\alpha$ -D-mannopyranosyl and  $\alpha$ -D-glucopyranosyl residues and 15  $\mu\text{g.ml}^{-1}$  4,6-diamidino-2-phenylindole (DAPI) for DNA localisation. Observations were carried out using an Olympus IX70 Delta Vision System (Applied Precision) equipped with a mercury lamp (103W/2 OSRAM 1XHBO) and 360–440 nm, 490–520 nm and 555–628 nm excitation filters and 457–470 nm, 528–538 nm and 617–673 nm emission filters.

Undamaged and intact biofilm fragments were transferred to cavity microscope slides. They were incubated in FITC-conA for 40 min in the dark. An Olympus IX81 CLSM was used. Wavelengths of the excitation lasers were in the blue (488 nm; Ar) green (543 nm; Ar/HeNe) and red (635nm; Ar/HeNe) regions. Images were acquired in the three channels simultaneously; red channel for pigment autofluorescence, green channel for labelled EPS and blue channel for calcite autofluorescence.

For TEM, micro-samples were fixed in 2.5% glutaraldehyde and post-fixed in a 1% osmium tetroxide solution, dehydrated in a graded ethanol series, and embedded in an epoxy 812 Resin Kit (Multilab Supplies, England). Thin sections were collected on copper grids for contrast staining with salts of uranium (uranyl acetate) and lead (lead citrate) (REYNOLDS et al. 1963) to reveal cell ultrastructure. Thin sections were also collected on gold grids for cytochemical staining with periodic acid for the detection of polysaccharides with unsubstituted vicinal OHs such as glycogen (THIERY 1967), and with phosphotungstic acid (PTA) in HCl for positively charged glycoproteins (ROLAND et al. 1972). Thin sections were observed using a HITACHI H-7100 operating at 100kV.

The cyanobacterial and microalgal isolates grown in culture were studied by means of LM and TEM. Identification was based on taxonomic monographs and reference books for terrestrial algae and cyanobacteria

(PRINTZ 1939, 1964; MIGULA 1907; GEITLER 1932; PRESCOTT et al. 1972; Ettl 1978, 1983; KOMÁREK & FOTT 1983; KRAMMER & LANGE-BERTALOT 1986, 1988, 1991a, b; Ettl & GÄRTNER 1988, 1995; KOMÁREK & ANAGNOSTIDIS 1998, 2005; RINDI et al. 2005; JOHN et al. 2002; WEHR & SHEATH 2003).

**Molecular analyses.** DNA was extracted from biofilms and isolates using the UltraClean Soil DNA Isolation Kit (Mo Bio Laboratories) according to the manufacturer's protocol.

The 16S rRNA gene was amplified using the cyanobacterial specific primers 2 (5'-GGG GGA TTT TCC GCAATG GG-3') and 3 (5'-CGCTCTACCAAC TGA GCT A-3') after BOYER and co-authors (2001). PCRs were carried out in 25  $\mu\text{l}$  aliquots containing approximately 100 ng DNA, a deoxynucleoside triphosphate mixture (0.2 mM each), buffer (1/2 volume of the supplied 2 $\times$  buffer) supplemented to give a final concentration of 2.5 mM  $\text{MgCl}_2$ , 1.25 U of *Taq* polymerase (all components supplied by Top-Bio, Czech Rep.), and 0.5 pmol of each primer. The profile used was 1 min 30 s at 95°C, 40 cycles at 95°C for 1 min, 52°C for 1 min, 72°C for 2 min, and a final elongation step of 7 min at 72°C. Amplifications were run in a Biometra T3000 Thermocycler or TC-XP Bioerg cycler.

The 18S rRNA genes were amplified using the primer pairs P73 (5'-AAT CAG TTA TAG TTT ATT TGR TGG TAC C-3') and P47 (5'-TCT CAG GCT CCC TCT CCG GA-3') (BERARD et al. 2005) and EukA (5'-AAC CTG GTT GAT CCT GCC AGT- 3') and EukB (5'- TGA TCC TTC TGC AGG TTC ACC TAC-3') (DIEZ et al. 2001). PCRs were carried out in 25  $\mu\text{l}$  aliquots containing approximately 100 ng DNA, a deoxynucleoside triphosphate mixture (0.2 mM each), buffer (1/10 volume of the supplied 10 $\times$  buffer) supplemented to give a final concentration of 3 mM  $\text{MgCl}_2$ , 1 U of *Taq* polymerase (Promega), and 0.5 pmol of each primer. Amplifications were run in a GeneAmp PCR system 2700 (Applied Biosystem) as follows. For the primer pair P73-P47, the profile used was; 1 cycle of 1 min at 94 °C; 37 cycles of 1 min at 92 °C, 50 s at 57 °C and 50 s at 72 °C and a final elongation step of 10 min at 72 °C. For the primer pair EukA-EukB, the profile used was; 1 cycle of 2 min at 95 °C; 35 cycles of 15 s at 92 °C, 50 s at 50 °C, and 2 min at 72 °C; and a final elongation step of 10 min at 72 °C.

PCR products were checked on 1.4% (w/v) agarose gel. Successful amplifications were purified using JetQuick PCR Purification Kit (Genomed) and cloned into pGEM-T Easy Vector System (Promega) 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  $\mu\text{l}$  of sterile water, heated for 5 min at 94°C and then 0.4  $\mu\text{l}$  were used as template in 10  $\mu\text{l}$  of PCR total volume. The PCR setup and cycling conditions were the same as for initial amplification.

After cloning, one strand of the 16S rRNA genes obtained from biofilms was sequenced by a commercial company (BMR genomics, Padova, Italy). The partial 16S and 18S gene sequences were compared with sequences available in the GenBank nucleotide database using the BLAST algorithm (ALTSCHUL et al. 1990) in order to obtain an overview of the biodiversity in each biofilm.

The cloned 16S rRNA genes obtained from isolates were sequenced using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on ABI 3730xl DNA Analyser (Applied Biosystems) in the Laboratory of Genomics at the Biology Centre ASCR and Faculty of Science, University of South Bohemia, České Budějovice. The amplification primers were used as the sequencing primers.

Nucleotide sequences of 16S rRNA genes, obtained from cyanobacterial isolates in this study and related sequences retrieved from GenBank, were aligned using CLUSTALX algorithm implemented in Bioedit 7.0.9.0 (HALL 1999), refined and used to generate a phylogenetic tree. Similar reliable sequences were selected from GenBank based on their length, in order to obtain a representative sample of the taxonomic variation. *Gloeobacter violaceus* PCC7421 was used as the outgroup. Phylogenetic trees were inferred by neighbor-joining (NJ) and maximum-parsimony (MP) methods in PAUP v. 4.0b10 (SWOFFORD 2002) and Bayesian inference (MB) in MrBayes\_3.1.1 (RONQUIST & HUELSENBECK 2003). MP analysis was carried out using the heuristic search with tree-bisection-reconnection algorithm (TBR) of ten replicates with random stepwise addition and the MulTrees option. For neighbour-joining, corrected distance were calculated with the HKY85 model (HASEGAWA et al. 1985). The robustness of branches was tested by bootstrap analysis on 1000 replicates.

For Bayesian analysis, two runs with four chains each were run simultaneously for 1,000,000 Markov Chain Monte Carlo (MCMC) generations starting with a random tree. The likelihood model was set to a general time reversible model (RODRÍGUEZ 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. The temperature of a hot chain was set empirically to 0.1 and trees were sampled every 100 generations. The standard deviation between the two MCMC runs was below 0.01, indicating convergence. The first 2500

trees were discarded as the burn-in phase and a 50% majority rule consensus tree was calculated including posterior probabilities.

## Results and Discussion

### The biofilms

The microbial consortium growing on archaeological surfaces formed thick, complex, multi-stratified biofilms in close proximity to the entrances, and thinner, more compact biofilms further inside the hypogea. The number of taxa within a particular subaerial biofilm was highly dependent on the micro-environmental parameters; more specifically the biodiversity increased with an increase in light intensity/ photoperiod (which never exceeded 10  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) and the degree of wetness of the surface. Environmental parameters were seasonally stable; the relative humidity always exceeded 90%, while the temperature ranged between 18 and 20 °C. The subaerophytic biofilms grew preferentially on calcite and gypsum substrata; the type of growth and biofilm morphology was dictated by the microenvironment (surface roughness, light availability and degree of wetness). The limited amount of photosynthetically active radiation provided an effective selection pressure and proved to be the principal determinant factor as to which taxa prevailed on the archaeological surface and as to whether a biofilm tended towards autotrophy (cyanobacteria and microalgae) or heterotrophy (bacteria).

The undisputed phototrophic protagonists in the subaerial biofilms of Maltese hypogean environments were filamentous cyanobacteria; both non-heterocytous (Oscillatorialean) (Fig. 1) and heterocytous (Stigonematalean, Nostocalean). These dominated at Saint Agatha's and Saint Paul's Catacombs. Associated with the cyanobacteria were microalgae; the prevailing types are Chlorococcalean (Chlorellalean) at St Paul's catacombs and the Hal Saflieni Hypogea, and filamentous (Trentepohlialean) at Abbatija tad-Dejr. Chroococcalean cyanobacteria and chemoorganotrophic bacteria were also present.

Micro-environmental parameters determined which microorganisms prevailed in a specific hypogea. Chlorococcalean microalgae dominated at the Hal Saflieni Hypogea where



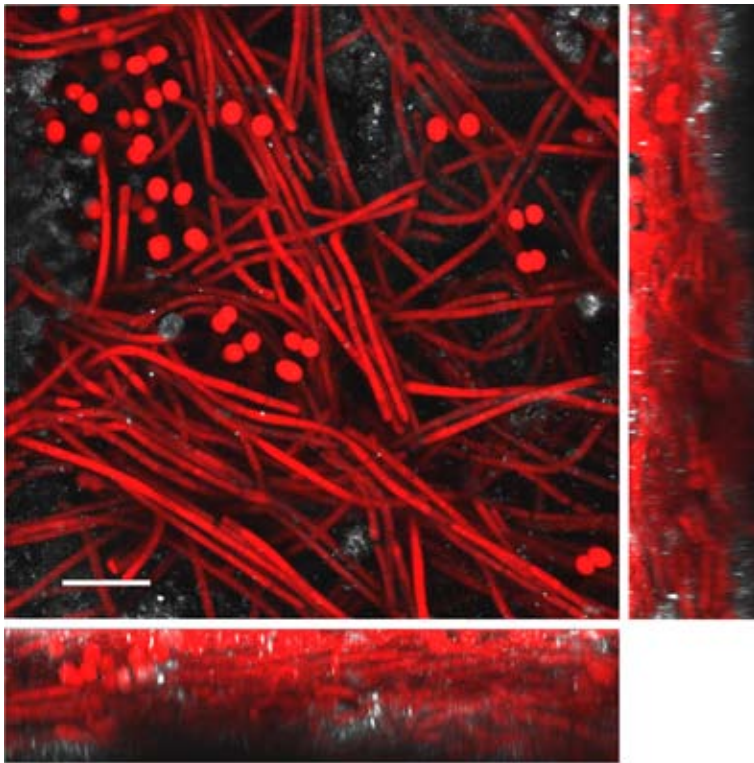


Fig. 1. 3-dimensional confocal laser scanning microscopy reconstruction of biofilm SA 38, composed predominantly of filaments of *Leptolyngbya* sp. together with coccal cyanobacteria. Red autofluorescence is of cyanobacterial phycobiliproteins, while the autofluorescence of calcite is white. Scale bar 15  $\mu$ m.

the internal environment has been under strict climatic control for the past twenty years. They were also abundant at Saint Paul's Catacombs, where they formed bright green biofilms over ochre inscriptions that were recently covered by a layer of plexiglass with an underlying light source. Filamentous microalgae prevailed at the Abbatija tad-Dejr Catacombs where the incidence of natural light was the highest.

A morphological and ultrastructural description of microorganisms belonging to the most common taxa present in these subaerophytic biofilms is given below. Table 1 lists the most common taxa forming the biofilms growing at the different hypogea. The cytomorphological and molecular characterisation of the filamentous cyanobacterial strains isolated from the same biofilms is discussed at the end.

## Biofilm organisms

### Cyanobacteria

Undoubtedly the most common filamentous cyanobacterial morphotype identified growing in biofilms from all four hypogean sites under study belonged to the genus *Leptolyngbya* (Oscillatoriaceae, Oscillatoriales) (Fig. 1). *Leptolyngbya* spp. from Maltese hypogea were

characterised by thin uniseriate trichomes ( $< 4 \mu$ m) surrounded by very thin, transparent, sometimes invisible, sheaths and a parietal arrangement of thylakoids. A number of strains were isolated in culture (results below) and their life cycle and cytomorphology are presently being examined in more detail. Members of this cosmopolitan form-genus *Leptolyngbya* are widely distributed in numerous ecosystems and have also been isolated from the catacombs of Rome (BRUNO et al. 2006, 2009)

and from cryptogamic crusts in desert soils across the western United States (EVANS & JOHANSEN 1999). The heterogeneity of *Leptolyngbya* has long been recognised (ALBERTANO & KOVÁČIK 1994) and its separation into different genera is expected according to molecular as well as ultrastructural and ecophysiological criteria (KOMÁREK 2007).

Filamentous cyanobacteria belonging to the genus *Pseudanabaena* LAUTERBORN 1915 were observed growing in the algal biofilm AD03 (Fig. 2b). These *Pseudanabaena* spp. (Pseudanabaenaceae, Oscillatoriales) were non-heterocytous cyanobacteria characterised by simple trichomes less than 4  $\mu$ m wide. The cells were longer than wide, they possessed parietal thylakoids and the cross walls were conspicuously constricted.

A number of filamentous heterocytous cyanobacterial species of *Nostoc* VAUCHER ex BORNET et FLAHAULT 1888 (Nostocaceae, Nostocales) were observed growing in biofilms at St Agatha's Catacombs and Abbatija tad-Dejr. *Nostoc* spp. formed microscopic green colonies on the surface of calcareous substrates. Phenotypically, the filaments were made of chains of vegetative cells, nitrogen-fixing heterocytes and spore-like akinetes. *Nostoc* is common in both terrestrial and aquatic habitats (DODDS et

al. 1995). The life style is photoautotrophic, diazotrophic and also facultatively heterotrophic, such as in *N. punctiforme* (MEEKS et al. 2001). The ability of *Nostoc* spp. to fix atmospheric  $N_2$  and to produce a glycan EPS (HELM et al. 2000) provided a considerable advantage for *Nostoc* spp. to grow on the hypogean surfaces being considered in this study.

As regards the branching heterocytous filamentous types, these morphotypes were easily observed in biofilms from the catacomb sites dedicated to St Agatha's and St Paul's. Filaments with the true V- type branching indicative of *Loriella* BORZI 1892 and the false branching of *Scytonema* were observed occasionally. However, strains belonging to these genera were not isolated in culture, and it is common, especially for the dichotomously branching taxa, to be rare in cultures and in phylogenetic analyses (GUGGER & HOFFMANN 2004). On the other hand, strains

of the *Fischerella* morphotype, with true T-type branches, were frequently observed to form biofilms colonising the archaeological surface in these two catacombs and were successfully isolated in culture. Cyanobacteria belonging to *Fischerella* spp. and *Scytonema* spp. have also been observed to form biofilms in the catacombs of Priscilla and San Callistus in Rome (ALBERTANO & BELLEZZA 2001) and members of *Loriella* sp. in Spanish caves (HERNÁNDEZ– MARINÉ et al. 1999).

The coccal (Chroococcales) types observed directly in the biofilms by LM and CLSM include *Chroococcidiopsis* spp. GEITLER 1932 growing in two biofilms: SA21 and SA27, at St Agatha's and three biofilms: SP01, SP02 and SP07, at St Paul's Catacombs. 16S rRNA sequences of uncultured *Chroococcidiopsis* sp. clones were obtained from biofilm SA27. *Chroococcidiopsis* cells observed growing in the biofilm produced abundant EPS, which may play a central role in desiccation

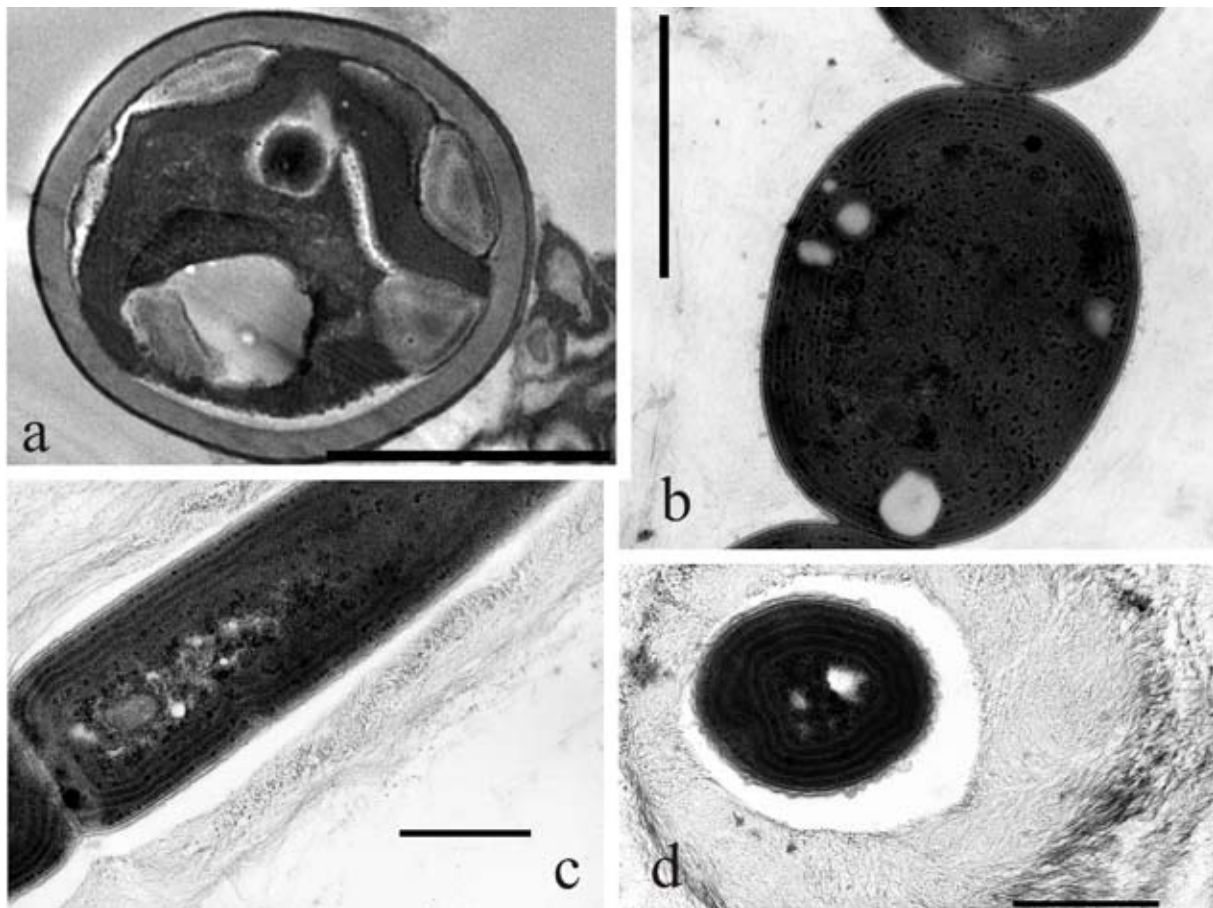


Fig. 2. TEM micrographs: (a) a microalgal cell from biofilm HYP714 with a thick sheath and a cup-shaped chloroplast; (b) *Pseudanabaena* cells with concentric arrangement of thylakoids; (c–d) *Leptolyngbya* sp. strain SP301 showing (c) parietal thylakoids and (d) a thick multilayered mucilaginous sheath made of distinct layers of fibrils. Scale bar 2  $\mu$ m (a, b), 1  $\mu$ m (c, d).



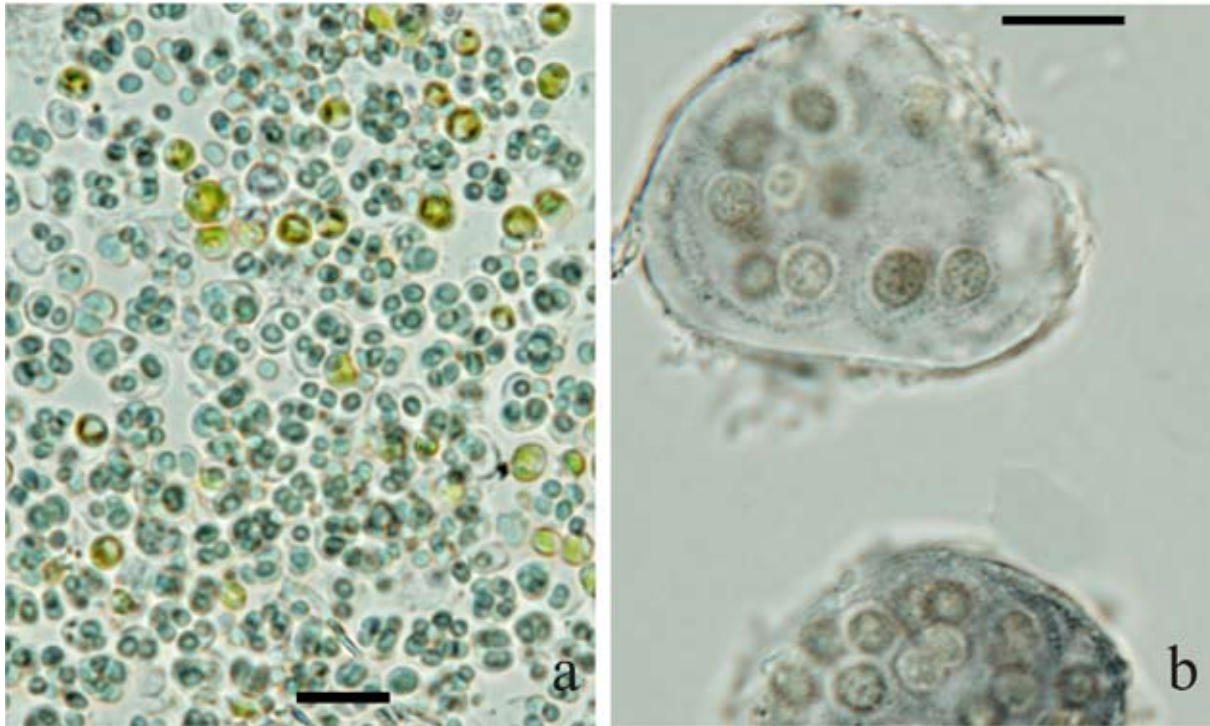


Fig. 3. Chroococcalean cyanobacterial morphotypes: (a) *Gleocapsa* cf. *aeruginosa* and coccal green algae growing in culture from biofilm HYP708, and (b) *Asterocapsa* cf. *divina* from biofilm SA36 growing in culture. Scale bar 10  $\mu$ m.

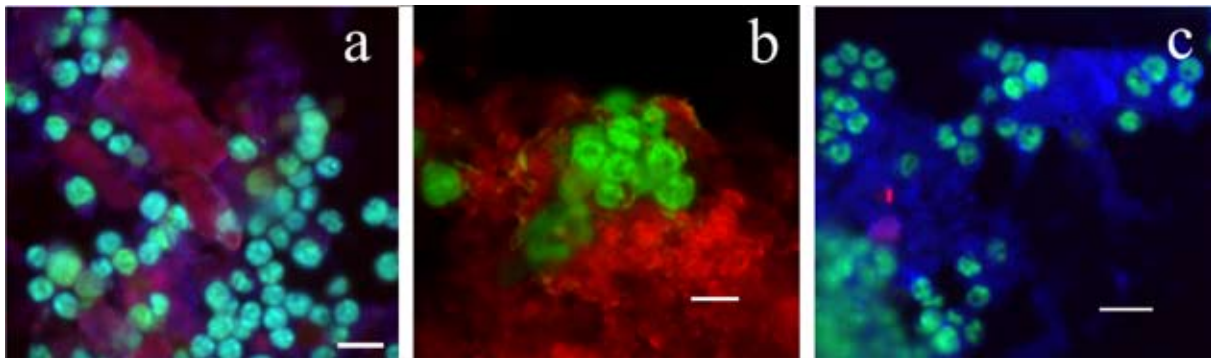


Fig. 4. Growth of coccal micro-algae on St Paul's artworks evidenced by epifluorescence microscopy: (c) aggregates grow inside calcite pore; (a) surrounded by thick polysaccharide matrix (blue); (b) surrounded by fiber calcite crystals. Red autofluorescence is of calcite. Scale bar 10  $\mu$ m.

tolerance by regulating the loss and uptake of water (BILLI & POTTS 2002).

Microscopic observation and enrichment culture usually allow only the recognition of *Chroococciopsis* morphotypes which are ubiquitous. However, community phylogenetic analysis has revealed considerable cyanobacterial diversity in the deserts of China (POINTING et al. 2007). Thus, the *Chroococciopsis* spp. identified in Maltese hypogea merit further study by community fingerprinting.

Chroococcal *Asterocapsa* cf. *divina* KOMÁREK

1993 and *Gleocapsa* cf. *aeruginosa* KÜTZING 1843 (Fig 3) were successfully grown in culture from biofilms sampled from St Agatha's Catacombs and the Hal Saffieni hypogea respectively. *Gleocapsa* spp. (ASENCIO & ABOAL 2000; ROLDAN & HERNÁNDEZ-MARINÉ 2009), *Chroococciopsis* spp. (ASENCIO & ABOAL 2000) and *Asterocapsa divina* (ABOAL et al. 2003) were also observed to occur in different caves in Spain. *Asterocapsa* sp. has also been reported from Leontari cave in Greece (LAMPRINOUS et al. 2009).

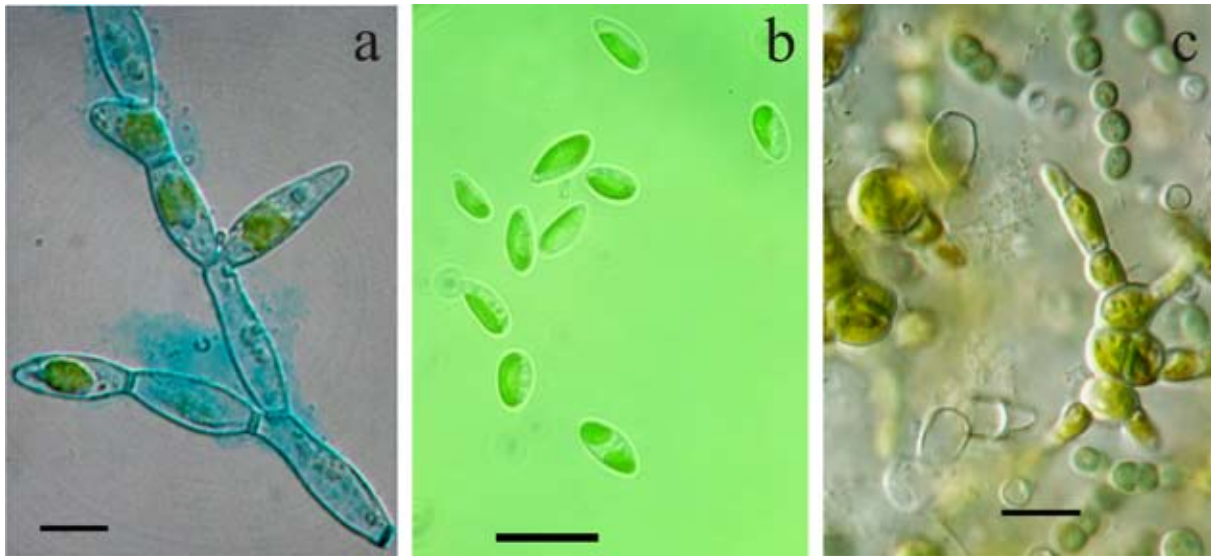


Fig. 5. Light micrographs of microalgae: (a) *Trentepohlia* morphotype; (b) *Pseudococcomyxa* sp.; (c) biofilm AD03 of *Trentepohlia* sp. and *Pseudanabaena* sp. growing in culture. Scale bar 10 µm.

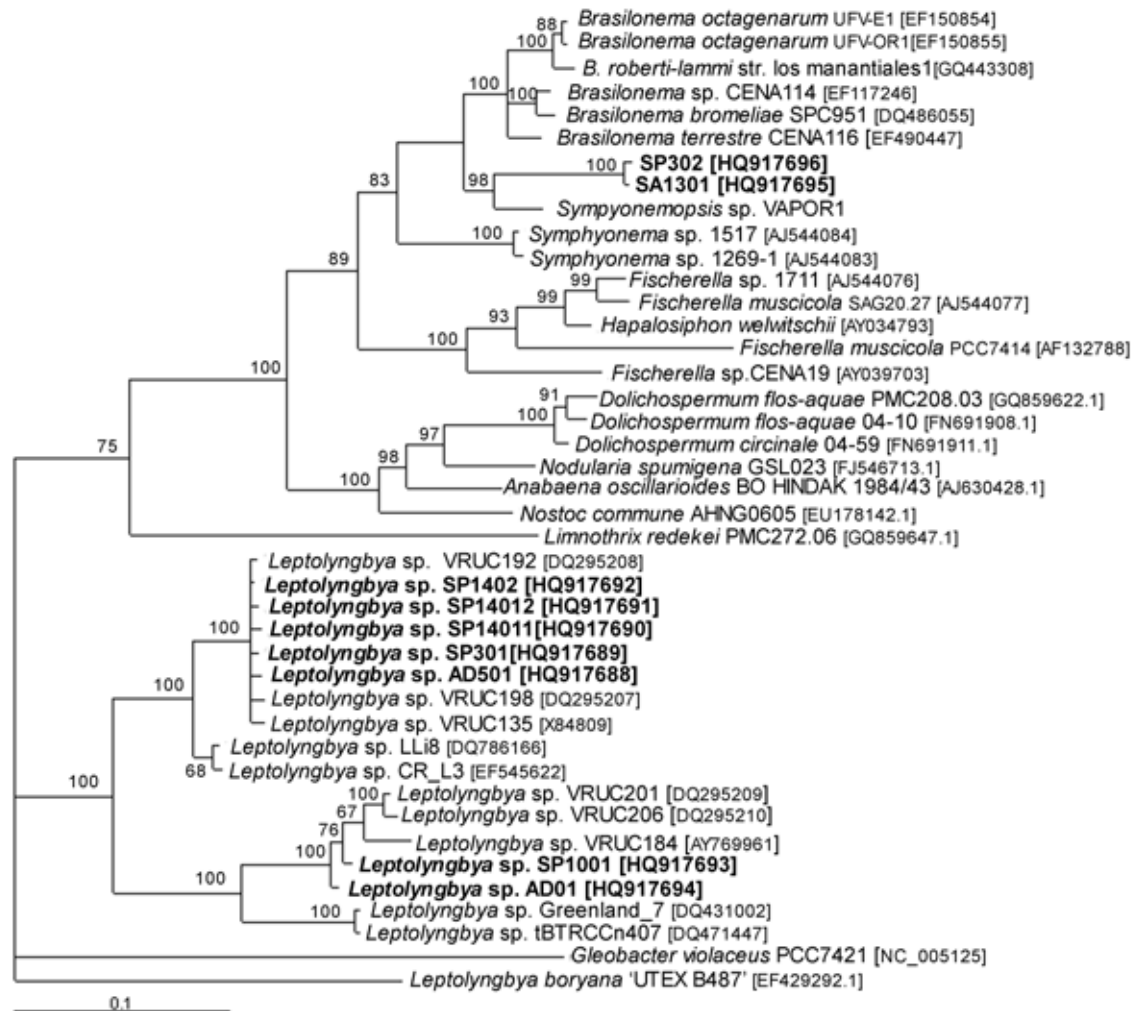


Fig. 6. Consensus tree from maximum parsimony and Bayesian analysis showing the phylogenetic relationships between Oscillatorialean and Stigonematalean cyanobacterial strains isolated from Maltese hypogea and similar strains based on the 16S rRNA gene sequences. *Gleobacter violaceus* PCC7421 was used as the outgroup. Strains isolated in this study are indicated in bold. Numbers near nodes indicate probability values obtained from Bayesian analysis. Scale represents number of substitutions per site.



### Green algae

Artworks of Maltese hypogea seem to support the growth of subaerial microalgae (Figs 4, 5) with relatively limited morphological types, which could be classified on the basis of morphology into two groups; the chlorococcalean *Chlorella*-like morphotype (Trebouxiophyceae, Chlorellales) and the filamentous *Trentepohlia*-like morphotype (Ulvophyceae, Trentepohliales) (Fig. 5a). These were generally observed growing in the biofilms in small populations as is a common occurrence with most subaerial algae (JOHN 1988). This fact, together with the small size of their cells, enabled accurate observation and examination only when grown in unialgal culture. However, even then, their proper classification was particularly problematic, due to the limited number of characters useful for a reliable morphological identification. Molecular methods were attempted, and while confirming the presence of those two taxa, the 18S rRNA data could not be resolved any further. Caution was exercised when comparing 18S *Trentepohlia* and *Chlorella* sequences to those deposited in GenBank, since it has long been recognised that many of these organisms are misidentified and indeed belong to other taxa (HUSS et al. 1999).

All the chlorococcalean algae observed had a thick, sometimes multilayered wall, whose surface appeared smooth or ribbed. Chlorococcalean algae of biofilm HYP 714 (Fig. 2a) had a parietal chloroplast with a pyrenoid and polysphosphate reserves. The number of autospores produced varied. Clonal cultures of these green algae are presently being studied further in terms of their ultrastructure, different stages in their life cycle, the presence of flagellated stages and molecular sequences based on a multi-gene approach.

Molecular data collected from microalgal groups in the last two decades has given rise to dramatic modifications at every level of classification, especially the genus level. In fact, several common genera of subaerial chlorophytes, such as *Chlorella*, have been shown to include species that in fact belong to different classes (HUSS et al. 1999; FRIEDL & O'KELLY 2002; KRIENITZ et al. 2003; LEWIS & McCOURT 2004). In the case of terrestrial green algae, a great deal of genetic diversity is often hidden behind identical or very similar morphologies (LOPEZ-BAUTISTA et al. 2007).

*Trentepohlia* spp. (Figs 5 a, c) were responsible for biofilm formation on archaeological surfaces. Members of the genus *Trentepohlia* are very difficult to classify since some aspects of their biology and their life cycle have not been completely elucidated (RINDI & GUIRY 2002). The morphology of the species is variable, and some are regarded as highly polymorphic organisms. Classification of species is based mainly on shape and size of vegetative cells, presence of setae, branching pattern, position and morphology of reproductive structures (PRINTZ 1939, 1964). However, some of these features are unstable and can vary in relation to ecological conditions. As has also been observed in another study (RINDI & GUIRY 2002), *Trentepohlia* strains from Maltese hypogea exhibited slow growth in culture and they rarely produced structures, such as zoosporangia, that were useful for species-level determination (UYENCO 1965). Remarkably, an ecological study carried out in western Ireland, pointed out that species of *Trentepohlia* differed in their substratum preference (RINDI & GUIRY 2002). All the *Trentepohlia* strains from Maltese hypogea were observed to grow preferentially on deteriorated, porous calcareous substrata.

One strain of *Pseudococcomyxa* cf. *simplex* (MAINX) FOTT 1981 (Fig. 5b) (Trebouxiophyceae, Chlorellales) was grown in culture from AD13, a grey green biofilm growing on a limestone scroll adorning a Baldacchino tomb. *Pseudococcomyxa* spp. frequently occur in soils and other aeroterrrestrial environments (ETTL & GÄRTNER 1995). Morphologically, this genus was distinguished from the similar genus *Coccomyxa* SCHMIDLE by differences in mucilage production. The cultured *Pseudococcomyxa* cells had elongated, irregularly-shaped elliptical vegetative cells and produced a mucilaginous cap on the apical part of the cells. Neustupa and Nemjova (2008) found a significant correlation between the shape, ecology and morphology of six *Pseudococcomyxa simplex* strains, however the morphological differences are often minute. Recent molecular data indicate that, even though members of these two genera form a single trebouxiophycean clade, they are not differentiated into genera according to the traditional mucilage-production criterion (FRIEDL et al. 2007). The taxonomy of *Coccomyxa*-like green algae is thus still confusing (KOSTIKOV et al. 2002) and the revision of this group will most certainly be based on molecular data (NEUSTUPA &

NEMJOVA 2008).

The occurrence of diatoms in subaerophytic biofilms from Maltese hypogea was not frequent. However, in two cases, bright green biofilms on the archaeological surface at St Paul's Catacombs in very close proximity to light sources were caused by growth of diatoms. Biofilms SP04 and SP16 were complex phototrophic biofilms whose bright green colouration was due to the dense growth of pennate diatoms belonging to *Diademsia* spp. KÜTZING 1844. The occurrence of *Diademsia* spp. in subaerial biofilms is rare, but has also been previously reported from Roman catacombs (ALBERTANO et al. 1995) and from cave environments (ROLDAN & HERNANDEZ-MARINE 2009).

### Chemoorganotrophs

Predominant bacterial strains obtained from qualitative cultural analysis were Gram-positive bacteria, mostly sporulating bacteria belonging to the genus *Bacillus*, (predominantly *B. subtilis*, *B. aquimaris* and *B. cereus*) and the filamentous actinobacteria *Streptomyces* spp., *Micrococcus* spp. COHN 1872, *Nocardia* spp. and *Isopterocola* spp. STACKEBRANDT et al. 2004. Ribosomal Intergenic Spacer Analysis (RISA) showed different electrophoretic patterns in agreement with the morphological groups, thus confirming the bacterial diversity causing whitish/greyish alterations on the archaeological surface (ZAMMIT et al. 2008a; ZAMMIT et al. 2009).

Fungi were rarely observed directly in the biofilm samples, but grew occasionally in culture. This is probably because ubiquitous spores of the genera *Aspergillus* MICHELI 1729, *Cladosporium* LINK 1791, *Penicillium* FRIES 1832 and *Mucor* FRESENIUS 1850, are present in the biofilm and germinated in culture. Only in two cases were biofilms caused by the growth of fungi; SA24, a black alteration on a replica resin statue was due to the occurrence of *Cladosporium* sp. hyphae and spores (ZAMMIT et al. 2009); and SP14, a reddish alteration on a deteriorated tomb ceiling was due to the growth of mycelia of *Serpula lacrymans*, (SCHUM. ex FR.) S.F. GRAY spores of which probably seeped through the soil with infiltration water from the overlying gardens. Both taxa are well known to be associated with the biodeterioration of cultural heritage artifacts.

### Isolated cyanobacterial strains

Strains of filamentous heterocytous (Stigonematalean) and non-heterocytous (Oscillatorialean) cyanobacteria isolated from subaerial biofilms were studied by microscopy and molecular techniques. Partial 16S rRNA gene sequences of the isolated cyanobacterial strains were used to construct the phylogenetic tree shown in Fig. 6.

The Stigonematalean strains (SP302 and SA1301 in Fig. 6) form part of a well supported main clade with other Stigonematalean and Nostocalean strains. Genetically and cytomorphologically they are similar to *Brasilonema* spp. and the latter were thus included in the phylogenetic analyses. It is interesting to note that the closest 16S rRNA sequence found in GenBank belongs to *Symphyonemopsis* sp. Vapor-1 isolated from the cave of El Vapor in Spain. Strains SP302 and SA1301 are described in more detail in ZAMMIT et al. 2010.

All the *Leptolyngbya* strains isolated in this study were phenotypically very simple. The filament diameter was approximately 1–4 µm. The sheath was transparent and in some strains thick and mucilaginous. Necridic cells and false branching were observed in strains SP1001 and SP1402. The strains could be divided into two phenotypes on the basis of pigmentation, as shown by CLSM analysis; strains SP301, SP1401, SP1402 and AD501 were red, due to relatively high phycoerythrin content; strain SP1001 was green due to a relatively high phycocyanin content when compared to other strains. However, by using morphological criteria (KOMÁREK & ANAGNOSTIDIS 2005) it was not possible to place these strains into one of the presently described species. The most similar descriptions to the strains isolated in this study are found in KOMÁREK & ANAGNOSTIDIS (2005); *Leptolyngbya* 'ALBERTANO/KOVACIK-red' 1997 for strains SP301, SP1401, SP1402 and AD501; and *Leptolyngbya* 'ALBERTANO/KOVACIK-green' 1992 for strain SP1001.

*Leptolyngbya* species normally possesses inflexible trichomes, nevertheless cell gliding of the trichomes was commonly observed for the strains isolated in this study. Moreover all the red *Leptolyngbya* strains isolated from the Maltese and Roman catacombs possess an 'orange spot' at the tip of the apical cell which resembles the eyespot (stigma) of carotenoid-rich lipid globules present

in phototactic flagellated algae (ALBERTANO et al. 2000). It has also been demonstrated that the red *Leptolyngbya* sp. strain VRUC 135, isolated from the Domus Aurea in Rome (also shown in the phylogenetic tree in Fig. 6) could turn towards a light source. The hypothesis is that *Leptolyngbya* strains possessing this ‘orange spot’ in the apical cell have ability to sense light is via a rhodopsin-like protein. In all the strains isolated in this study, the apical cell was rounded and in strains SP301, SP1401 and AD501 had a ‘brownish–yellow cap’.

Ultrastructurally, their cytoplasm was characterised by the presence of carboxysomes, cyanophycin, lipid globules and interthylakoidal glycogen granules. Ultrastructural differences included the structure and the thickness of the envelopes. However, sheath characteristics are often lost in culture, and thus this cannot be considered a reliable character. The thylakoid structure, which is not sensitive to varying environmental and culturing conditions, therefore provides a stable character that can be used to determine taxonomic relationships among thin–trichomed members of the Oscillatoriales. TEM sections showed that all the Oscillatorialean strains, isolated during this study had parietal thylakoids (Fig. 2 c, d).

Fig. 6 gives the topology and branch support observed in the consensus tree calculated from MB and MP analyses. Seven of the strains described as members of the genus *Leptolyngbya* are clustered into a highly supported clade. The six *Leptolyngbya* sp. strains isolated in this study form two clades with closely related *Leptolyngbya* sp. strains isolated from the catacombs in Rome. The top clade is the ‘red clade’ which contains strains high in PE; the second clade is the ‘green clade’ or strains with high PC content, as shown by CLSM analyses. A *Leptolyngbya* strain cloned directly from the biofilm AD01 also forms part of the green clade.

The green strains clustered into a group that was sister to a clade formed by two strains of *Leptolyngbya*, one benthic from Arctic hot springs; strain Greenland\_7 (GenBank accession no. DQ431002, ROESELERS et al. 2007), the other a filamentous strain from thermal springs in Jordan named tBTRCCn407 (GenBank accession no. DQ471447, IONESCU et al. 2007). The red strains were sister to a clade formed by two strains of

*Leptolyngbya* sp. from a hot volcanic stream in Costa Rica; strain LLi 18 (GenBank accession no. DQ786166, FINSINGER & HESS unpublished) and strain CR\_L3 (GenBank accession no. EF545622, MORALES et al. unpublished). It would be interesting to investigate further these close relationships between Oscillatorialean strains from hypogean and geothermal habitats. Since these Oscillatorian cyanobacteria are still undersampled from a variety of habitats, this might have created an artefact in the analysis that would be corrected by including Oscillatorialean sequences from different environments.

In order to compare sequence similarity of the strains used in this study, a distance matrix was constructed (Table 2). Strains within the ‘green’ and ‘red’ *Leptolyngbya* clade were found have 16S rRNA sequence similarity values above 95%. The similarity for the green strains is higher than 98%, for the red strains higher than 99%. The sequence similarity between the red and the green strains was about 92%. The high similarity between *Leptolyngbya* strains isolated from catacombs in Malta and Rome suggest that these might actually be morphologically variable ecophenes (phenotypic modifications produced within the same genotype due to environmental stimuli within the limits of the habitat under which the genotype is found in nature).

The cytomorphology and genetics of these hypogean strains are presently being studied further since their 16S rRNA clusters separately to the type species *Leptolyngbya boryana*, their ecology is different, and they possess specific characters which might be considered autapomorphic.

It can be concluded that the organisms forming biofilms in Maltese hypogea are similar morphologically to those colonising comparable habitats in the catacombs of Rome and those identified on lithic surfaces in various cave environments in Spain and Greece. Both cave and catacomb ecosystems can be classified as hypogean environments with rather stable conditions throughout the year, which seem to provide a refuge to these microorganisms. Specific hypogean environments may favour speciation, and various new cyanobacterial taxa have been described from this environment (LAMPRINO et al. 2009). The isolated Oscillatorialean and Stigonematalean strains were also shown to be closely related to strains isolated from other catacomb and cave environments.



The abundant EPS produced by the biofilm microorganisms as observed by microscopy of the investigated samples was crucial in their endurance on the archaeological surfaces by regulating the loss and uptake of water. The availability of liquid water as condensation droplets on the archaeological surface of these hypogean sites also played an important role in the activation of dormant or inactive cyanobacteria and microalgae (BOISON et al. 2004).

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Table 1. Predominant taxa observed in biofilms from St. Agatha's (SA), St. Paul's (SP), Abbatija tad-Dejr (AD), Hal-Saftieni (HYP); (F) filamentous, (C) coccal, (Pr) prokaryotic, (E) eukaryotic, (P) photosynthetic, (H) heterotrophic, (WP) wall painting, (R) rock.

Site	Name	Morphology	Substrate	Nutrition
SA	<i>Leptolyngbya</i> spp.	F, Pr	WP, R	P
	<i>Fischerella</i> sp.	F, Pr	WP, R	P
	<i>Streptomyces</i> spp.	F, Pr	WP, R	H
	<i>Nocardia</i> spp.	F, Pr	WP, R	H
	<i>Isoptericola</i> spp.	F, Pr	WP, R	H
	<i>Bacillus</i> spp.	Rods, Pr	WP, R	H
	<i>Chroococciopsis</i> spp.	C, Pr	WP, R	P
	<i>Nostoc</i> spp.	F, Pr	WP, R	P
	Moss protonemata	F, E	WP, R	P
	<i>Asterocapsa</i> spp.	C, Pr	WP, R	P
	<i>Cladosporium</i> spp.	spores	WP, R	H
	<i>Aspergillus</i> spp.	spores	WP, R	H
	<i>Fusarium</i> spp.	spores	WP, R	H
	<i>Penicillium</i> spp.	spores	WP, R	H
	<i>Pseudopleurococcus</i> sp.	F, E	WP, R	P
	<i>Trentepohlia</i> sp.	F, E	WP, R	P
	<i>Chlorella</i> spp.	C, E	WP, R	P
SP	<i>Serpula lacrymans</i>	F, E	R	H
	<i>Chlorella</i> spp.	C, E	WP, R	P
	<i>Trentepohlia</i> sp.	F, E	WP, R	P
	<i>Leptolyngbya</i> spp.	F, Pr	WP, R	P
	<i>Fischerella</i> sp.	F, Pr	WP, R	P
	<i>Diademsis</i> spp.	C, E	WP, R	P
	<i>Chroococciopsis</i> spp.	C, Pr	WP, R	P
	Actinobacteria	F, Pr	WP, R	P
Fungi	spores	WP, R	H	
AD	<i>Leptolyngbya</i> spp.	F, Pr	WP, R	P
	<i>Trentepohlia</i> sp.	F, E	WP, R	P
	<i>Pseudanabaena</i> sp.	F, Pr	WP, R	P
	<i>Nostoc</i> spp.	F, Pr	WP, R	P
	<i>Chlorella</i> spp.	C, E	WP, R	P
	<i>Pseudococcomyxa simplex</i>	C, E	R	P
	Moss protonema	C, E	WP, R	P
	Actinobacteria	F, Pr	WP, R	P
Fungi	spores	WP, R	H	
HYP	<i>Leptolyngbya</i> spp.	F, Pr	WP, R	P
	<i>Chlorella</i> spp.	C, E	WP, R	P
	<i>Gleocapsa</i> spp.	C, Pr	WP, R	P
	<i>Bacillus</i> spp.	Rods, Pr	WP, R	H
	<i>Streptomyces</i> spp.	F, Pr	WP, R	H
	<i>Micrococcus</i> spp.	C, Pr	WP, R	H
	<i>Aspergillus</i> spp.	spores	WP, R	H
Actinobacteria	F, Pr	WP, R	P	

Table 2: The 16S rRNA gene similarity of *Leptolyngbya*/ strains isolated from the catacombs in Rome and Malta.

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1 <i>Leptolyngbya</i> sp. AD01																
2 <i>Leptolyngbya</i> sp. SP1001	99.4															
3 <i>Leptolyngbya</i> sp. VRUC184	98.1	98.0														
4 <i>Leptolyngbya</i> sp. VRUC201	98.7	99.0	98.3													
5 <i>Leptolyngbya</i> sp. VRUC206	98.5	98.8	98.1	99.5												
6 <i>Leptolyngbya</i> sp. Green7	95.4	95.1	94.1	94.7	94.9											
7 <i>Leptolyngbya</i> sp. tBTRCCn407	95.3	01.0	94.0	94.6	94.8	99.8										
8 <i>Leptolyngbya</i> sp. VRUC192	92.9	92.4	91.4	92.0	92.2	92.3	92.2	92.2								
9 <i>Leptolyngbya</i> sp. VRUC135	92.6	92.1	91.1	91.7	91.9	92.0	91.9	99.4								
10 <i>Leptolyngbya</i> sp. VRUC198	92.7	92.2	91.2	91.8	92.0	92.1	92.0	99.5	99.2							
11 <i>Leptolyngbya</i> sp. AD501	92.8	92.3	91.3	91.9	92.1	92.2	92.1	99.6	99.3	99.4						
12 <i>Leptolyngbya</i> sp. SP301	92.9	92.4	91.4	92.0	92.2	92.3	92.2	99.7	99.4	99.5	99.6					
13 <i>Leptolyngbya</i> sp. SP14011	92.8	92.3	91.3	91.9	92.1	92.2	92.1	99.6	99.3	99.4	99.5	99.6				
14 <i>Leptolyngbya</i> sp. SP14012	92.9	92.4	91.4	92.0	92.2	92.3	92.2	99.7	99.4	99.5	99.6	99.7	99.6			
15 <i>Leptolyngbya</i> sp. LLi18	93.6	93.5	92.2	92.8	93.0	93.1	00.9	97.9	97.6	97.7	97.8	97.9	97.8	97.9		
16 <i>Leptolyngbya</i> sp. CR_L3	93.3	93.2	91.9	92.5	92.7	92.8	92.7	97.8	97.5	97.6	97.7	97.8	97.7	97.8	99.6	
17 <i>Leptolyngbya</i> sp. SP1402	93.0	92.5	91.5	92.1	92.3	92.4	92.3	99.8	99.5	99.6	99.7	99.8	99.7	99.8	98.0	97.9