

MICROBIAL DIVERSITY OF SPELEOTHEMS IN TWO SOUTHEAST AUSTRALIAN LIMESTONE CAVE ARCHES

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Abstract

Peculiar cave structures, nicknamed lobsters, and shaped by drip water, wind, aeolian particles and microbial biofilms, are described from cave entrance arches at Jenolan and Wombeyan caves in southeast Australia. Subaerial biofilms on rock surfaces support complex microbial assemblages adapted to temperature, desiccation, and low irradiance stress. The community composition of active and inactive speleothems was elucidated by next generation sequencing. Active biofilms showed high abundances of cyanobacterial taxa, morphologically and phylogenetically belonging to the genera *Chroococcidiopsis* and *Gloeocapsa*, representing an endolithic lifestyle in desiccated and low light conditions. Significant differences were found between caves and between actively accreting and inactive and weathered structures. Functional taxa putatively occupying the same niches were found on active structures in both locations. A temporal succession is proposed, with dominance shifting from *Chroococcales* to *Actinomycetales* and highly desiccation-resistant and oligotrophic *Rubrobacterales* with decreasing water availability.

Introduction

Subaerial biofilms occupy the realm between air and rock surface, which is an ancient and hostile niche. Under favorable conditions, complex and less tolerant eukaryotic taxa, such as algae and fungi, dominate subaerial biofilms. However, under adverse conditions, prokaryotic taxa remain the only microorganisms capable of enduring the extreme environmental conditions characterized by inhospitable habitats like deserts and caves (Gorbushina, 2007). Colonization of a rock substrate is initiated by photosynthetic organisms that enrich the surface and provide a base for heterotrophic taxa (Crispim and Gaylarde, 2005). In subaerial habitats, a constant, close interplay between lithosphere, atmosphere, and colonizing microbes has a direct impact on the biogeochemistry within the biofilm and the underlying minerals (Warscheid and Braams, 2000; Buedel et al., 2004).

The organisms that inhabit subaerial biofilms present on cave structures, are influenced by a series of factors including light availability, the abundance and nature of mineral-enriched drip water, and the physical structure of the rock (Gorbushina, 2007). Substrate micro-topography influences niche availability (Golubic et al., 1981) and colonization speed, with smooth surfaces harder to colonize and favoring small coccoid organisms, such as cyanobacteria of the genus *Gloeocapsa*. Rougher surfaces with declivities, protecting organisms from wind, solar irradiation, or predation, are colonized faster and provide additional microniches (Miller et al., 2006) resulting in a greater abundance and diversity of organismal shape and metabolism (Gorbushina, 2007).

Cave entrances, as opposed to hypogeal environments, are strongly influenced by surface, physical parameters, including fluctuations in temperature, desiccation and light restriction. The extracellular polymeric substances (EPS) that envelop cell assemblages in microbial mats provide protection from these fluctuations (Kemmling et al., 2004) and govern mineral precipitation (Tourney and Ngwenya, 2009).

Lobster-type speleothems are unique, elongated, narrow structures present within cave entrances, which extend toward the main direction of wind in the cave arch systems (James et al., 1982; Cox et al., 1989b; Lundberg and McFarlane, 2011). Two cave arches in the Blue Mountains area of New South Wales, Australia, are the sites of the first description of this morphology, namely Nettle Cave in Jenolan Karst Conservation Reserve and Victoria Arch in Wombeyan Karst Conservation Reserve (Cox, 1984; James et al., 1982). Their nickname, lobsters or craybacks, originates from the crenulations and elevated ridges across the narrow axis that give the speleothems the appearance of a segmented, crustacean carapace (Cox et al., 1989a). Due to influx of allochthonous matter into the open caves, a plausible explanation for the formation of the lobster ridges is the introduction of particulate irregularities on the surface. Once a particle becomes attached to the surface, it creates a shaded area that phototrophic organisms will avoid, thus causing increased calcification and, ultimately, a ridge in the lighter part (Cox et al., 1989b). The width of the structures is at least partly determined by the height of the cave roof, whereby higher travel distance of the drops from above result in larger splash diameters and wider structures.

Cox and co-workers proposed that the growth of the lobsters is partially biogenic. They also confirmed the presence of *Gloeocapsa* spp. (Cox et al., 1989b), and *Chroococcales* have been shown to contribute to calcification in accreted

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structures, formed by the action of microbes, i.e. microbialites (Saghaï et al., 2015). Extremely little is known about the microbial communities of cave speleothems and there are only a handful of reports worldwide on the peculiar morphology of crayback or lobster stalagmites (Mulec et al., 2007; Lundberg and McFarlane, 2011). Purely physical, carbonate precipitation, driven by evaporation of the water film on the speleothem surface, usually leads to randomly structured precipitate (Hill et al., 1997). In contrast, cyanobacterial photosynthesis induces smooth, laminar precipitation. Seasonality contributes to layering within the structure, with solid or coralline layers deposited in wet seasons and allochthonous layers of dust, grains, and animal matter deposited in dry seasons (Cox et al., 1989b). Decreased, wind-driven evaporation of the scarce water conveys an advantage to organisms settling there (Lundberg and McFarlane, 2011).

Here, we characterize, for the first time, the microbial assemblage, in regard to composition and putative ecological roles, of lobster speleothems in cave arches. We address the extent of any heterogeneity that might occur between sites within a cave system, as well as between cave systems. Also, a sampling regime was enacted to shed light on the succession of microbial communities as speleothems transition from active to the inactive and weathered form over time.

Materials and Methods

Site description and sampling

Samples were collected from Nettle Cave (Jenolan Caves, Blue Mountains, NSW, 33.8206° S, 150.0214° E) in March 2013 and from Victoria Arch (Wombeyan Caves, NSW, 34.3167° S, 149.9833° E) in April 2013. Nettle Cave is the upper part of an 80 m high and 40 m wide tunnel called Devils Coachhouse that runs in a southwest-northeast direction. Almost all speleothems are located on this upper level, which receives light mainly from the smaller, narrow Nettle Cave exit, a roof hole, and to some extent, the large Devils Coachhouse opening (Cox et al., 1989a). Site 1 (sample J1) is an old, weathered and dry lobster (Fig. 1B) with white and flaking surface, and it is about 4 m away from the walkway to the right. This site, and all other sites, is located about 20 m from the Nettle cave exit. Site 2 (J2 and J3) is a small blue-green, extremely faint, ridged lobster at the edge of a crevice on the left side of the walkway. It is located directly beneath an old steel cable. Site 3 (Fig. 1A) is a well-ridged speleothem with blue-green surface (J4 and J5), as well as coarse, dry, green sides (J6 and J7). It is located 2 m behind Site 2. Site 4 (J8 and J9) is an elongate, only faintly blue-green speleothem underneath the walkway in the vicinity of sites 2 and 3. It was partially removed to allow construction of the walkway. Rock (J10) and soil (J11) samples were collected from between sites.

Victoria Arch at Wombeyan has roughly the same dimensions as Nettle Cave and runs in a north-south direction (Osborne, 1993). As opposed to Nettle Cave, it is at ground level. Site 1 (W1 and W2) is a conspicuous, large (1 m high) lobster with blue-green coloration 10 m from the entrance on the left side. Site 2 (W3–W5) is an elongate lobster 12 m further into the cave and directly to the right of the path. It is ridged and deep blue-green with light-green growth on the sides. Site 3 (W6 and W7) is a soft, deep grey-green flowstone, 1 m high and 3 m wide and about 10 m from the opposite cave opening. Site 4 (W8) is a weathered, chipped and white lobster close to site 2. Rock (W9) and soil (W10) samples are adjacent to site 4. Specimens were drilled to a depth of about 5 mm using a hand-held, battery-powered drill, equipped with a 20 mm diamond-coated, circular drill bit which was ethanol-sterilized between samples. Samples were kept at 4 °C for 1–2 days in sterile containers prior to DNA extraction. As of 2015, sampling core holes were still clearly visible.

Illuminance at the Jenolan lobster site was previously measured at low values of

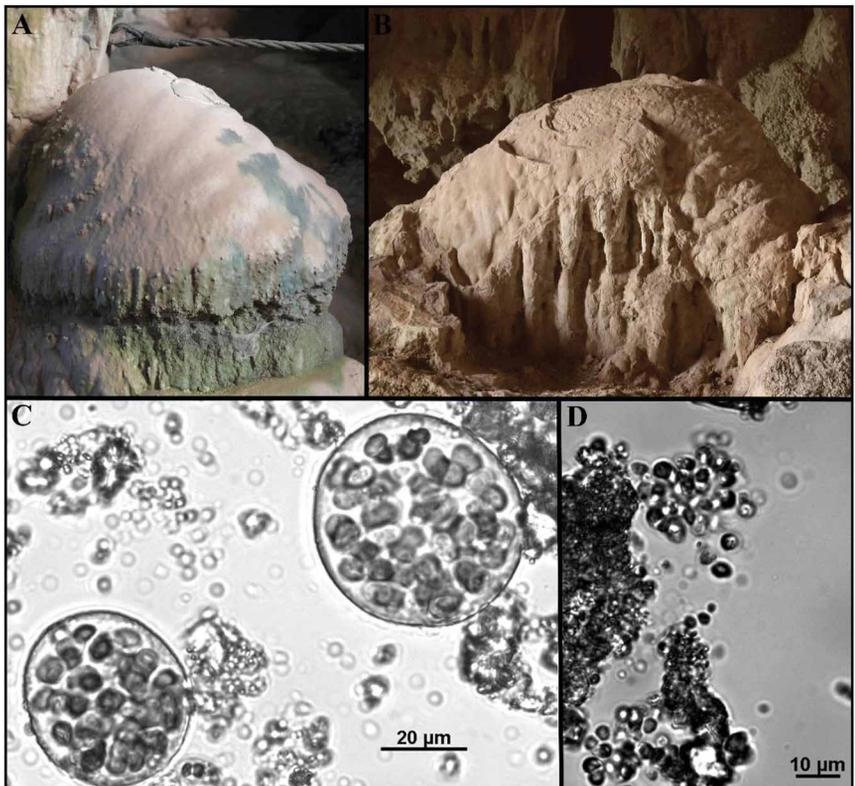


Figure 1. Representative active blue-green (A) and inactive weathered (B) Lobster speleothems from Jenolan Caves and light microscopy pictures of *Chroococcidiopsis-Gloeocapsa* morphologies with (C) and without (D) gelatinous envelopes.

50–100 lux on a summer midday, depending on exact location and positioning of the structure (Cox et al. 1989a). No measurements from the Wombeyan site are available, but due to the similarity in cave shape and speleothem positioning, a similar light intensity is presumed. Conditions at Jenolan Caves (792 m above sea level, station number 63036) were unusually wet in the weeks preceding, with February rainfall being registered at about three times the mean for that month (Bureau of Meteorology, 2013a). In contrast, conditions had been unusually dry at Wombeyan Caves (580 m above sea level, station no 63093) before sampling. Only 29.6 mm of rain fell in the month leading up to the sampling date and March and April were much drier than average (Bureau of Meteorology, 2013b). Both caves are open to the public and receive high numbers of visitors year-round, but most speleothem structures are not directly accessible. As this is an exploratory study covering large sampling areas, small-scale differences in environmental factors are likely, but these will need to be investigated using a narrower approach that includes more physical measurements. Wind, exposure to outside contamination and annual temperature fluctuations are comparable across all samples for the scope of this study. Average monthly temperatures are between 0.5 °C and 26 °C in both locations.

DNA extraction, amplification and sequencing

For each core, 400–500 mg of sample was ground coarsely and sample replicates from the same specimen and the same appearance were pooled. A FastDNA Spin Kit for Soil was used according to manufacturer's instructions (MP Bio). The final buffer was left on the column at 55 °C to enhance elution and re-centrifuged over the column once to increase yield. DNA was kept at –20 °C. DNA concentration and quality were measured using a NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific). DNA concentrations ranged from 10–40 ng μL^{-1} at 260/280-absorbance ratios between 1.59 and 2.02.

PCR for the V1–V3 region of the 16S rRNA gene was performed using oligonucleotides 27F and 519R, each featuring a unique sample specific 8 nt multiplex identifier (MID) tag (Caporaso et al., 2012). A 19.5 μL master mix containing 1× Tris-HCl buffer (Bioline), 1 mM MgCl_2 , 1 mM BSA, 1.5 mM dNTPs and 0.3 U BioTaq DNA Polymerase (Bioline), and 0.1 U *Pfu* proofreading DNA Polymerase (Promega) was pipetted into a sterile PCR tube along with 50 pmol forward primer, 50 pmol reverse primer, and 5–20 ng of sample DNA. PCR cycling was performed as follows: Initial denaturing for 5 min at 95 °C, 30 cycles of denaturation for 15 s at 95 °C, annealing for 30 s at 55 °C, elongation for 60 s at 68 °C, and a final elongation step for 7 min at 72 °C. Single-stranded DNA was digested at 37 °C for 15 min by the addition of 0.05 U Exonuclease I and 0.25 U Shrimp Alkaline Phosphatase (New England Biolabs). The enzymes were inactivated at 80 °C for 20 min. PCR products (~530 bp) were confirmed by gel electrophoresis on a 1 % agarose gel and then normalised and pooled using a SequelPrep Normalisation Plate (Thermo Fisher Scientific).

Concentration and quality of the pooled library were checked with Qubit and the library size confirmed using the Agilent 2200 TapeStation. The Agencourt AMPure XP bead clean-up kit (Beckman Coulter) was used on the pool to reduce primer dimers. The libraries were sequenced at the Ramaciotti Centre for Genomics, UNSW, Australia, on an Illumina MiSeq instrument using a MiSeq Reagent Kit v3 with a 2 × 300 bp run format and default run parameters including adaptor trimming.

Bioinformatic analysis

Only the 27F-derived sequences were used for taxonomic assignment, as it ensures comparable taxonomic assignment, while reducing an overestimation of operational taxonomic unit (OTU) richness due to progressively deteriorating sequencing quality (Caporaso et al., 2012). The following analysis was performed using the Mothur v. 1.36.0 package (Schloss et al., 2009). Sequence files were trimmed using a scanning window average (window size = 50, minimum quality score = 30). Sequences that were shorter than 270 bp in length, contained ambiguous bases, or homopolymers of length greater than 8 bp were removed. Unique sequences were aligned with the SILVA database (version 102). Sequences were pre-clustered allowing for a 1 bp mismatch per 100 bp and Uchime, as implemented in Mothur, was used to identify and remove chimeric sequences (Edgar et al., 2011).

Taxonomic annotation was first done with the RDP trainset9_032012 (Cole et al., 2013) reference and lineages, classified as mitochondria, Archaea, Eukarya, or unknown were removed. Sequences classified as chloroplast were retained to ensure that all cyanobacterial sequences were retained. The second taxonomic annotation was done using the GreenGenes 13_8_99 reference file with cut-off 0.8. Subsampling was performed at a level of 9351, consistent with the lowest, observed sampling depth, resulting in 196,371 sequences in total. Of these, 9,508 unique sequences were clustered into OTUs using average neighbor clustering and a distance threshold of 0.03. Taxonomy of each OTU was assigned using a consensus method and a cut-off of 0.8. A total of 1089 OTUs representing 17,136 sequences could not be classified beyond the level of kingdom.

Phylogenetic analysis

A phylogenetic tree was constructed by obtaining representatives for each OTU containing at least 10 sequences that annotated to the orders *Nostocales* or *Chroococcales*, in addition to any unclassified cyanobacterial sequences. For each OTU representative, the corresponding 519R sequence was recovered and the sequences assembled to form

~450 bp sequences. Reference sequences from NCBI GenBank nucleotide database (accessed June 2015) and OTU sequences were aligned in the BioEdit version 7.2.5. (Hall, 1999) with implementation of the Clustal W (Thompson et al., 1994) multiple alignment algorithm. Phylogenetic inference of the resulting alignment was made with PhyML (Guindon et al., 2010) using a gamma time-reversible model and 1,000 bootstrap replicates. The resulting tree was visualized with Dendroscope version 3.2.10 (Huson and Scornavacca, 2012).

Statistical analysis

Richness, diversity (inverse Simpson index) and evenness (log-based Shannon index) of the microbial community were calculated from the OTU matrix using Primer 6 (version 6.1.11, Primer-E Ltd., UK). Cluster analysis was performed on a Bray-Curtis similarity resemblance matrix derived from the square root transformed OTU matrix. PERMANOVA was performed using the Primer 6 PERMANOVA+ version 1.0.1 (<http://www.primer-e.com/permanova.htm>) add-on using location (Jenolan and Wombeyan caves) and group (Table 1) as fixed factors.

Indicator species analysis was performed to identify OTUs representative of each group using the Indicator Species package in R (de Cáceres et al., 2010). For inclusion in this analysis, OTUs were required to contribute at least 1 % to the total sequence number in a given sample. A correlation table was constructed featuring each OTU, the site (or sites) that it was an indicator of, the indicator value, and the observed p-value (see Supplementary Table 1). Visualisation of the relationships between indicator OTUs and the morphotypes as an indicator value, edge-weighted, spring-embedded network was performed using Cytoscape version 3.2.1 (<http://www.cytoscape.org/index.html>).

Results

Microbial composition of cave entrance subaerial biofilms

The most abundant taxa found on the active speleothems in Jenolan (groups 2 and 3) were *Cyanobacteria* (28.6 ± 21.3 %), *Actinobacteria* (25.5 ± 10.2 %), unclassified *Bacteria* (20.7 ± 9.7 %), *Alphaproteobacteria* (11.8 ± 9.8 %), and *Acidobacteria* (6.1 ± 2.3 %). The inactive speleothem in Jenolan (group 1) was dominated by *Actinobacteria* (69.3 ± 11.2 %), followed by unclassified *Bacteria* (10.2 ± 9.8 %) and *Alphaproteobacteria* (6.4 ± 2.8 %). The most abundant taxa in active Wombeyan biofilms (groups 6–8) were *Acidobacteria* (20.2 ± 11.4 %), *Actinobacteria* (21.3 ± 8.9 %), *Cyanobacteria* (19.1 ± 15.8 %), *Alphaproteobacteria* (7.3 ± 4 %), and unclassified *Bacteria* (5.5 ± 2 %). Weathered speleothems and rock controls (group 4) were dominated by *Actinobacteria* (79.9 ± 2.8 %), followed by *Cyanobacteria* (3.1 ± 4.4 %), most of which were identified as chloroplasts and *Alphaproteobacteria* (1.7 ± 0.9 %). Soil communities (group 5) contained *Actinobacteria* (44.3 ± 6.5 %), *Proteobacteria* (27.4 ± 1.2 %), unclassified *Bacteria* (3 ± 1.6 %), and *Cyanobacteria* (2 ± 2.1 %). Of the 14.1 ± 17.2 % of sequences annotated as belonging to the phylum *Cyanobacteria*, the major groups were *Chroococcales* (6 ± 9.4 %), unclassified *Cyanobacteria* (5 ± 9.5 %), and chloroplast sequences (2.1 ± 2.9 %).

Bray-Curtis similarities of square root transformed OTU abundances revealed several distinct groups of samples that are less than 30 % similar to one another (Fig. 2). These groups, consistent with the morphological appearance of the subaerial biofilms, are subsequently annotated as: blue-green mat (two per cave), green mat (Jenolan), grey-green flowstone (Wombeyan), soil controls, and a group containing old inactive speleothems and bare rock controls (shared by both caves, Table 1). Except for one instance, these groups coincided with the respective speleothem specimen from which they were sampled. The exception is sample J3 from the subsurface layer (ca 2–5 mm) of one active speleothem in Jenolan sharing about 40 % OTU similarity with the cores from site 4, a pale lobster with only faint hues of blue-green coloration.

Table 1. Groups from Jenolan and Wombeyan samples identified from cluster analysis and morphological description of sampling sites.

| Samples | Group | Description |
|-----------------|-------|---|
| J3, J8, J9 | 1 | Subsurface sample from blue-green speleothem (J3) and samples from inactive faint blue-green speleothem |
| J2, J4, J5 | 2 | Blue-green speleothem |
| J6, J7 | 3 | Dry green coarse mat on vertical side of group 2 speleothem |
| J1, J10, W8, W9 | 4 | Old weathered speleothems without any visible mat (J1, W8) and bare non-speleothem rock (J10, W9) |
| J11, W10 | 5 | Soil |
| W1, W2 | 6 | Blue-green speleothem at cave entrance |
| W3, W4, W5 | 7 | Blue-green speleothem with some dry green growth |
| W6, W7 | 8 | Green-grey wet soft flowstone |

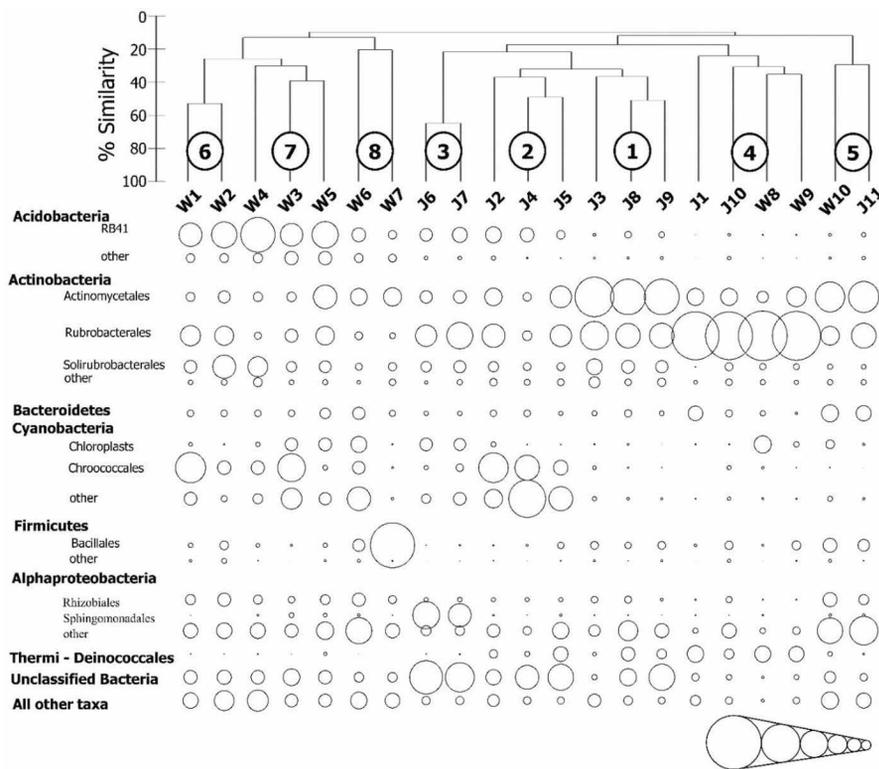


Figure 2. Abundances of the major taxa (contributing >2 % of total sequences) and clustering of Jenolan and Wombeyan samples into 8 distinct groups. Scale represents 60-40-20-10-5-2.5 % of total abundance. For samples and groups see Table 1.

ty-defined groupings ($p < 0.0001$, $F = 18.23$) and, to a lesser extent, morphotype ($p = 0.0281$, $F = 3.604$) on the richness was observed. A significant effect on evenness was observed for Bray-Curtis groupings ($p = 0.0008$, $F = 7.867$), but not morphotype ($p = 0.0559$, $F = 2.896$). Pair-wise, the richness of microbial communities associated with subaerial biofilms in the Jenolan Cave entrance was not significantly different from bare rock and inactive speleothems in both caves. The richness of subaerial biofilms in the Wombeyan Cave arch was significantly different from that of the soil control for two of the three structures, namely the two blue-green speleothems, but not the green flowstone. No clear pattern was observed from the differences in evenness within or between caves.

The blue-green and green microbial mats of both Jenolan and Wombeyan caves were distinct from the rock and soil control samples by the presence of abundant cyanobacterial sequences (Fig. 2), consistent with microscopic analysis that confirmed an abundance of *Gloeocapsa* and *Chroococciopsis* morphotypes. Phylogenetic analysis (Fig. 3) of dominant cyanobacterial 16S rRNA gene sequences indicated the presence of a number of diverse OTUs associated with cultured *Chroococciopsis* and *Nostoc* species. In addition, a subset of dominant cyanobacterial OTUs were identified that lacked phylogenetically-related, cultured representatives. Cyanobacterial OTUs were shown to vary in their relative contribution to the total sequence count, both within a cave and, more notably, between caves (Fig. 3).

Indicator species analysis of OTUs that contribute at least 1% to any given samples identified a number of species indicative of the distinct sites, and groups of sites, within each cave system (Fig. 4). A total of 95 indicator OTUs were identified (Supplementary Table 1). Active speleothems were characterized by a range of cyanobacterial OTUs, phylogenetically similar to *Chroococciopsis* and *Gloeocapsa*, as well as a few chloroplast, *Sphingomonas*, *Rhizobiales*, *Gemmatimonadetes*, and unclassified bacterial OTUs. Acidobacterial OTUs were only indicative of the Wombeyan active structures. One OTU of *Cyanobacteria* and *Solirubrobacterales* were indicative of all active structures in both caves. The cyanobacterial genus *Acaryochloris* was indicative of the coarse, green mat sampled on the side of a Jenolan microbialite. No cyanobacterial indicator was found to be exclusive to rock or soil control sites. Indicator OTUs for the soil samples were *Actinomycetales* and *Gammaproteobacteria*.

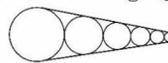
Discussion

This study represents the first description of the microbial community composition of subaerial biofilms associated with a unique speleothem structure in cave arches (James et al., 1982; Cox et al., 1989b). Speleothems provide a unique opportunity to study how microbial, meteorological and geographical processes interact to introduce heterogeneity into

Microbial heterogeneity between geographically isolated speleothems

Soil and rock controls from Wombeyan or Jenolan caves were not found to be substantially different, based on the Bray-Curtis similarities, although the lack of replicate samples did not permit this to be demonstrated statistically. PERMANOVA supported a distinction between Jenolan and Wombeyan active speleothem OTU communities ($p = 0.001$, Pseudo- $F = 4.1555$) and a distinction amongst groups ($p = 0.0001$, Pseudo- $F = 3.1621$). Differences between the groups were investigated by post-hoc, pairwise t-test. Significant differences were identified between rock controls and Site 1 ($t = 0.038$) and Site 2 ($t = 0.039$) from Jenolan Cave and Site 7 ($t = 0.023$) from Wombeyan Cave entrances.

There was a significant difference ($t = 0.0334$) in richness of subaerial biofilm communities between Jenolan and Wombeyan caves, but not of evenness ($t = 0.0638$) or diversity ($t = 0.0950$). A significant effect of Bray-Curtis similarity-



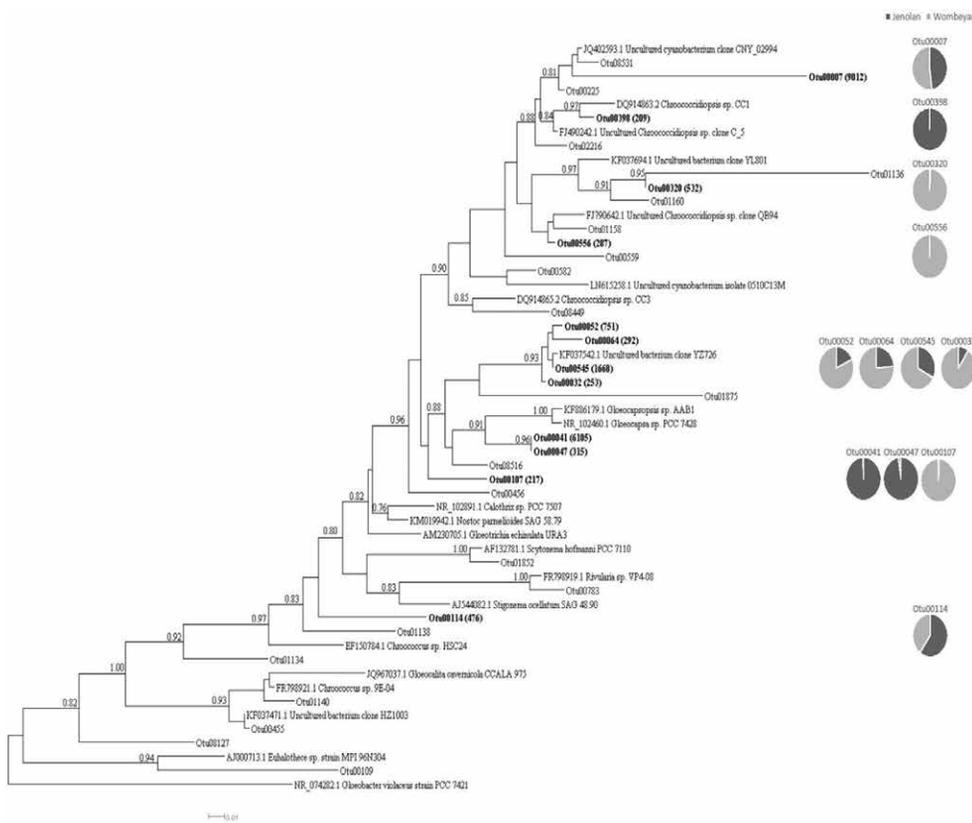


Figure 3. *Chroococcales*, *Nostocales* and unclassified cyanobacterial sequences from Jenolan and Wombeyan caves. Only OTUs with more than 10 sequences are included and relative shares of the most abundant OTUs (>200 sequences) are shown as pie charts and absolute sequence numbers are given in parentheses. Scale bar represents 0.01 substitutions per site.

landscapes. We sought to gain an understanding of the nature of microorganisms persisting in these biofilms, given the restricted availability of nutrients via drip water and dust. A focus has been placed on cyanobacteria as the main producers of organic matter within these systems, particularly with regards to the specific restrictions placed on this taxon by desiccation and low light.

Differences between and within caves

Despite significant differences between Jenolan and Wombeyan OTU community composition, no apparent differences in abundance of the major taxa were observed, except for the much higher abundance of *Acidobacteria* at Wombeyan sites (Fig. 2), suggesting that higher taxa and their respective metabolic and functional niches are represented by similar organisms. In both locations, cyanobacteria of the morphotypes *Chroococcidiopsis*/*Gloeocapsa*

were the dominant primary producers and either share OTUs between caves or feature one representative of a putative, functional taxon (Fig. 3). This reflects an extent of functional redundancy to ecological roles and niche-specific adaptations (Yin et al., 2000). Given that the mechanisms of speleothem formation are likely the same in both caves, the key species in initial colonization influences subsequent biofilm growth. No clear pattern of richness, evenness and diversity was found between the caves, indicating that they experience similar environmental conditions and the differences in rainfall before sampling did not result in significant proliferation or decline of certain microbial clades, with the possible exception of an effect on *Acidobacteria*, discussed below.

Within caves, active blue-green mats were found to host a distinct microbial community compared to the weathered speleothems, bare rock or soil. Indicator OTU analysis showed that *Cyanobacteria* are indicators of active speleothems only, while *Actinobacteria* are mainly indicators of inactive structures and bare rock and soil (Fig. 4, Supplementary Table 1). Exceptionally abundant actinobacterial OTUs were distributed throughout the samples and are indicators for several sites, while multiple, less-abundant actinobacterial OTUs are associated with the aforementioned structures and controls. Therefore, a clear distinction in the importance of this taxon exists between active and inactive locations. Cyanobacterial indicators were closely related to the *Chroococcales* and uncultured species from extreme environments (Pointing et al., 2009; Wong et al., 2010). Jenolan active-site indicators were all cyanobacterial, whereas Wombeyan indicators are phylogenetically more diverse and include *Acidobacteria* and *Sphingomonas* OTUs. These taxa are also important constituents of soil and are able to degrade complex organic compounds (Juhász and Naidu, 2000; Ward et al., 2009). A *Gemmatimonadete* OTU, indicative of all Wombeyan active mats, while poorly known, is well-adapted to desiccation (DeBruyn et al., 2011).

Low-light, desiccation tolerant Cyanobacteria represent a critical component of speleothem subaerial biofilms

Cyanobacteria (including chloroplasts) accounted for, on average, 18.6 % of the microbial community of active, speleothem-associated biofilms (Fig. 2). Consistent with their vibrant coloration, this value is likely to underestimate the cyanobacterial contribution to the total biomass in these systems, due to their large cell size. The microscopically- and phylogenetically-identified order *Chroococcales*, which includes the low-light adapted *Chroococcidiopsis* spp. and

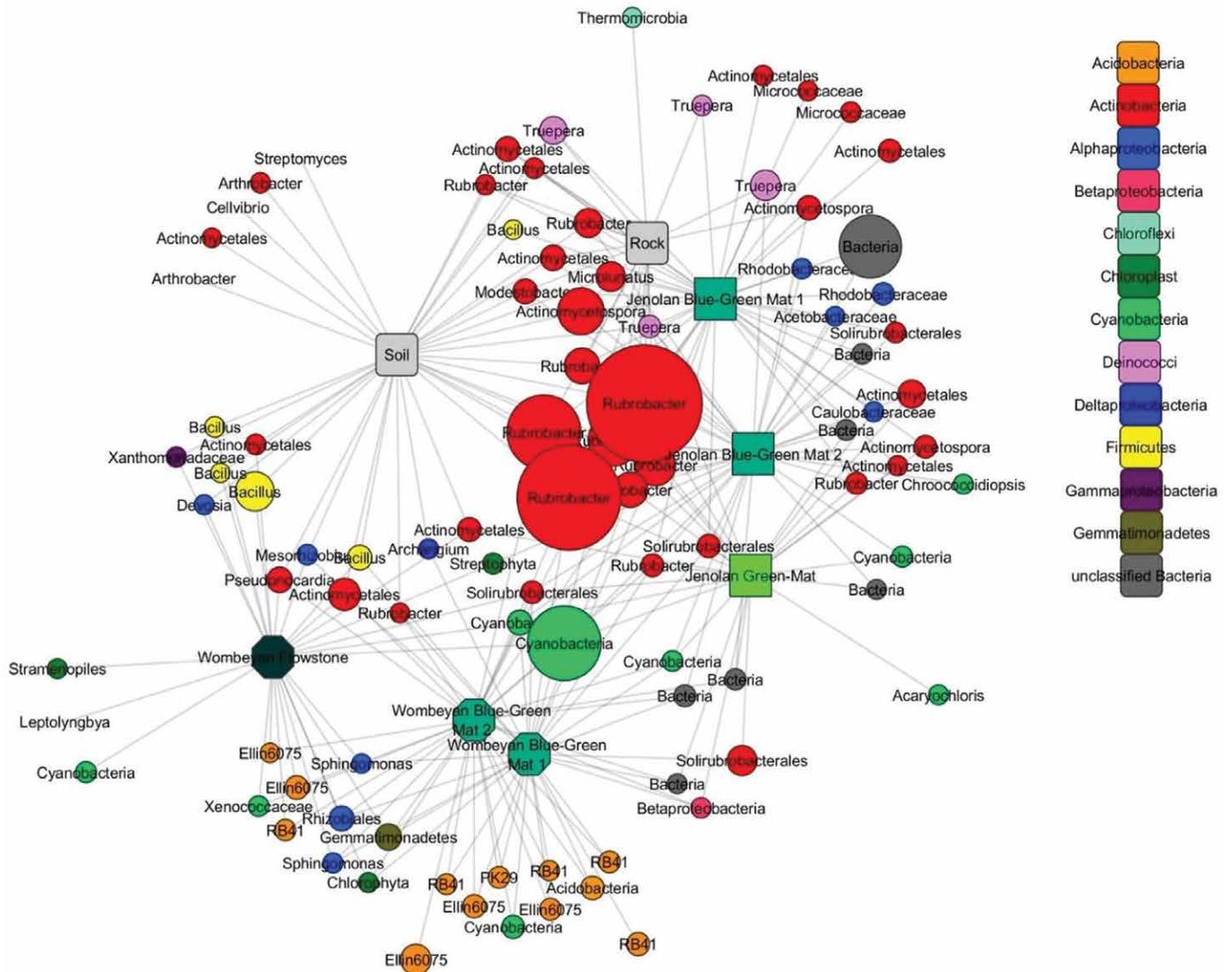


Figure 4. Indicator spring-embedded OTU network of sampled cave sites. Jenolan active and combined control sites in squares, Wombeyan active sites in octagons, OTUs in circles. Circle diameter corresponds to sequence abundance (not the same scale as Fig 2). Soil and rock denote bare cave soil and bare non-speleothem cave rock, respectively.

Gloeocapsa spp., contributed 8.3% of the total microbial community and were indicators (Fig. 4, Supplementary Table 1) of active biofilms. These findings are consistent with previous reports of biofilms on stone surfaces (Macedo et al., 2009) and caves (Asencio and Aboal, 2000; Lamprinou et al., 2009; Martinez and Asencio, 2010; Popović et al., 2015; Cox, 1984) and the premise that a biofilm established under low-light conditions would be dominated by cyanobacteria (Mulec et al., 2008). The morphotypes found in the cave arches possess thick sheaths (Baque et al., 2013) and are prolific producers of EPS, which provide labile organic carbon for heterotrophs and offer protection from desiccation (Caiola et al., 1996) and freezing (Knowles and Castenholz, 2008). Production of EPS has also been proposed to support growth of low-light adapted photoautotrophs by facilitating the penetration of photons into deeper layers and minimizing shading of mineral grains and allochthonous particles in caves (Decho et al., 2003).

Low-light tolerance of the *Chroococcales* enables an endolithic lifestyle that mitigates desiccation stress (Potts, 1999), which is supported by very efficient DNA repair mechanisms conveyed by radiation resistance (Billi et al., 2000). Carbonate, which constitutes the cave speleothems, has high moisture retention and is more translucent than other common rock types and allows for colonization of *Chroococcidiopsis* (Smith et al., 2014). Retreat of endolithic cyanobacteria into the porous substrate ensures that the organisms receive sufficient water and light, while avoiding desiccation, temperature extremes, competition for space, and predation (Gorbushina, 2007).

It has been proposed that these cyanobacterial species contribute to the formation of cave structures (Cox et al., 1989a), leading to embedding of the biofilm. Several endolithic cyanobacteria have been found to precipitate intracellu-

lar, carbonate granules, thereby slowing down biomineralization (Benzerara et al., 2014). Decreased burial rate benefits both the primary producers and heterotrophic constituents of the subaerial biofilm. Büedel et al. (2002) proposed a three-story *Cyanobacterial* biofilm based on endolithic *Chroococcidiopsis* and *Gloeocapsa* colonizing crevices and a medial layer of epiliths. The necessity to retain water efficiently makes a similar scaffold in cave biofilms likely, with the majority of the taxa building the understory.

An abundant cyanobacterial OTU related to cultured representatives of the genera *Acaryochloris* and *Loriellopsis* was observed in a shaded, vertical section of the Jenolan specimens. *Acaryochloris* inhabits deep-green, shaded limestone surfaces at Jenolan Caves and the genus is known to possess a red-shifted chlorophyll to facilitate growth at low-light (Behrendt et al., 2015). The branching filamentous *Loriellopsis cavernicola* has also been described from cave walls (Lamprinou et al., 2011).

Speleothem subaerial biofilms support the growth of oligotrophic microorganisms

Despite a high abundance of cyanobacteria within the speleothem biofilms, many of the heterotrophic groups resembled oligotrophic, rather than copiotrophic, taxa. Actinobacterial OTUs, similar to those identified from environments such as Roman catacombs (Saarela et al., 2004), lava caves (Hathaway et al., 2014), and granite walls (Laiz et al., 2009) represented the largest proportion of sequences present on both active and inactive speleothems, in accordance with previous reports on stone surfaces (Urzi et al., 2001). The next most common taxa were *Acidobacteria* and *Alphaproteobacteria*, both known to be abundant in caves (Schabereiter-Gurtner et al., 2004).

Filamentous *Actinobacteria* encompassed the family *Pseudonocardiaceae*, members that have been found to form net-like mycelia on weathered limestone (Cao et al., 2017), providing an advantage for the establishment of biofilms (Scheerer et al., 2009), and the family *Geodermatophilaceae*, described from a variety of stone surfaces (Urzi et al., 2001). *Pseudonocardia* is recognized for its capacity to degrade a variety of complex substrates (Reichert et al., 1998). The dominance of *Actinomycetes* on inactive structures is supported by their growth on recalcitrant organic matter and detrital particles. *Geodermatophilaceae* have been described from stone surfaces in various climates and are major colonizers of calcareous stone in the Mediterranean (Urzi, 2004). Heavy colonization by *Geodermatophilaceae* was associated with deterioration of stone surfaces (Urzi et al., 2001) and is potentially the cause for biodegradation at the weathered cave sites.

The presence of dominant OTUs, closely related to the gamma-radiation tolerant *Rubrobacter radiotolerans* (Yoshinaka et al., 1973), is consistent with the notion that radiation resistance in actinomycetes also conveys a level of desiccation tolerance via an efficient DNA repair mechanism. Tolerance to desiccation results in this group dominating and being indicators of the oldest, and therefore driest, cave structures (Fig. 2, Fig. 4 and Supplementary Table 1). Several OTUs also exhibited relatedness to *Rubrobacter bracaensis* that was isolated from biodeteriorated granite walls (47).

Most acidobacterial sequences recovered from the cave communities were associated with the taxon *RB41* (Fig. 2). Similar sequences have been recovered from lava caves (unpublished), rock surfaces (Yunnan stone forest, China, unpublished), and Antarctic soils (Yergeau et al., 2012). Abundance of this subgroup in soil is negatively correlated to pH (Jones et al., 2009), but the neutral pH of drip water in Wombeyan caves (McDonald et al., 2007) does not seem to support this finding and nutrient limitation might be the critical factor. *Acidobacteria* feature inconsistent nutrient utilization and preference patterns even within a subgroup (Naether et al., 2012). Nutrient-limited conditions were found to select for them, signifying their ability to degrade complex carbon sources in an oligotrophic environment (Rasche et al., 2011). Despite *Acidobacteria* often representing up to 20% of soil bacterial communities (Janssen, 2006), the origin of this taxon in the Wombeyan Cave structures does not seem to be aeolian. Sequence numbers are very low in the soil and rock controls, which indicates an enrichment on the Wombeyan speleothems rather than passive transport.

Various acidobacterial OTUs were indicators for the blue-green mats and flowstone sites in Wombeyan, but not in Jenolan (Fig. 4, Supplementary Table 1) caves. *Acidobacteria* have previously been reported in a comparative study of the walls of two caves, where visitor load might explain the differences in abundance (Schabereiter-Gurtner et al., 2004). In the caves, a difference in drip water chemistry is more likely to deter them from one site, but little is known about the physiology and ecology of *Acidobacteria* to explain this pattern, and without environmental data, it is difficult to understand it.

Most *Rhizobiales* sequences found were very similar (99-100%) to sequences of desiccation-tolerant *Dichotomicrobium thermohalophilum* isolated from a hypersaline lake (Hirsch and Hoffmann, 1989), and the families *Bradyrhizobium*, *Mesorhizobium*, and *Devosia*, from root nodules of various plants (Laranjo et al., 2014). *Bradyrhizobium* is a major nitrogen fixer and, in symbiosis with algae, provides a valuable service to microbial communities on oligotrophic substrates. Even though *Bradyrhizobium* was found in all but one cave sample, the relatively high biomass of the microbialite surfaces makes a contaminant origin unlikely.

For most chloroplast and a third of unclassified bacterial sequences, the closest homologue (>91%) is *Prasiolopsis* (*Trebouxiophyceae*), a widely distributed group and a common colonizer of stone surfaces in temperate regions (Rindi

et al., 2007; Hallmann et al., 2013). Almost all *Prasiolopsis*-like sequences were accounted for by the dry, green mat (group 3). This taxon forms lichens with fungi, which can stabilize a forming biofilm. It has also been found in dry valleys of Antarctica (de Wever et al., 2009) and on coastal rocks fertilized by bird guano (Heesch et al., 2012). This ability to grow in dry, oligotrophic conditions, where nutrient input is mainly via aerial transport and bird or bat droppings, might explain why this taxon might be prevalent, even though the low similarity to cultured organisms prevents detailed predictions.

Putative successional stages

The distinct groups observed in the caves are in line with the finding that, as opposed to algae and fungi, no significant spatial structure at the centimeter scale for bacteria can be found (Cutler et al., 2014). Cave locations were sampled at a centimeter to decimeter scale. While active sites have low numbers and roughly equal proportions of *Actinomycetales* and *Rubrobacterales*, this proportion shifts drastically in the inactive and weathered speleothems (groups 1 and 4), with almost no cyanobacterial sequences found. The proportion of *Actinomycetales* to *Rubrobacterales* shifts from about 2:1 in group 1 to about 1:4 in group 4.

A long-term temporal succession, most likely governed by water availability, may explain this pattern. On active, blue-green structures, where an intact, three-dimensional biofilm is maintained, water can be retained, and extremely desiccation-tolerant species are at much less of an advantage. The roofs of cave arches change over time, when crevices on the surface become clogged and new ones are created. Water availability for speleothems below fluctuates, leading to a shift from *Cyanobacteria*-driven primary production to a drought-induced collapse of cyanobacterial populations and retreat of coccoid genera such as *Chroococcidiopsis* and *Gloeocapsa* into endolithic niches. Oligotrophic *Actinobacteria* persevere on recalcitrant and complex organic molecules, dead cells, and aeolian particles. When desiccation stress becomes more intense, such as in the weathered speleothems and bare rock samples, only the highly desiccation-resistant *Rubrobacterales* prevail.

Conclusions

Significant differences between the microbial communities of speleothem biofilms of two cave arches were found. While rock and soil controls were especially similar between caves and dominated by *Actinobacteria*, active blue-green biofilms on speleothems were indicated by endolithic *Chroococcidiopsis* and *Gloeocapsa*-like cyanobacteria. Biofilms from both caves harbored similar cyanobacterial representatives, potentially fulfilling the same functions. As a consequence of desiccation-driven progression from active mats to inactive ones and to weathered speleothems, it is proposed that endolithic phototrophs retreat below the surface while oligotrophic *Actinomycetes* and, later, highly desiccation-resistant *Rubrobacterales*, dominate the speleothem surface. Inclusion of environmental long-term data would shed light on the dynamics through time, and reasons for distinct community compositions between sites, such as differences in the abundance of *Acidobacteria*.

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