Calcite Biomineralisation in the Caves of Nullarbor Plains, Australia

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In caves under the Nullarbor Plain, divers report extensive mantles of a biological material. The mantles are gelatinous and transparent and only can be seen because of the microcrystalline calcite associated with them. They cover the roof and walls of the partially and totally water filled passages to the limits of exploration.

The nature of these mantles was investigated using electron microscopy and DNA analysis. The microcrystalline calcite was examined using electron microscope techniques, X-ray diffraction, Fourier transform infrared spectroscopy, Raman spectroscopy and thermogravimetric analysis. The cave water was analysed for major ions and trace metals and modelled using geochemical modelling software.

Water analysis revealed high levels of sulfate and nitrate together with significant nitrite. The community structure showed a high proportion of novel phylotypes as well a high abundance of Nitrospira relatives. The unusual community, the nitrite in the water, and the apparent absence of aquatic macrofauna, means these microbial structures may represent biochemically novel, chemoautotrophic communities dependent on nitrite oxidation.

The waters were slightly saturated with respect to calcite, suggesting the microbial mantles play a role in calcite nucleation and crystal growth. The calcite crystals were predominately spindle shaped with curved {hk.0} faces lying parallel to the c-axis. Calcite precipitated under conditions designed to mimic the inorganic solution chemistry of the cave revealed a different morphology to that observed in the cave samples. These differences suggest that the formation and growth of the microcrystalline cave calcite is influenced by the microbial mantles.

The Nullarbor region of Australia is the worlds largest contiguous karst system (Figure 1), with an area in excess of 200 000 km^2. The area is an extensive plain of Eocene and Miocene limestones across which the annual rainfall ranges from 150 – 200 mm (GILLIESON & SPATE, 1992).

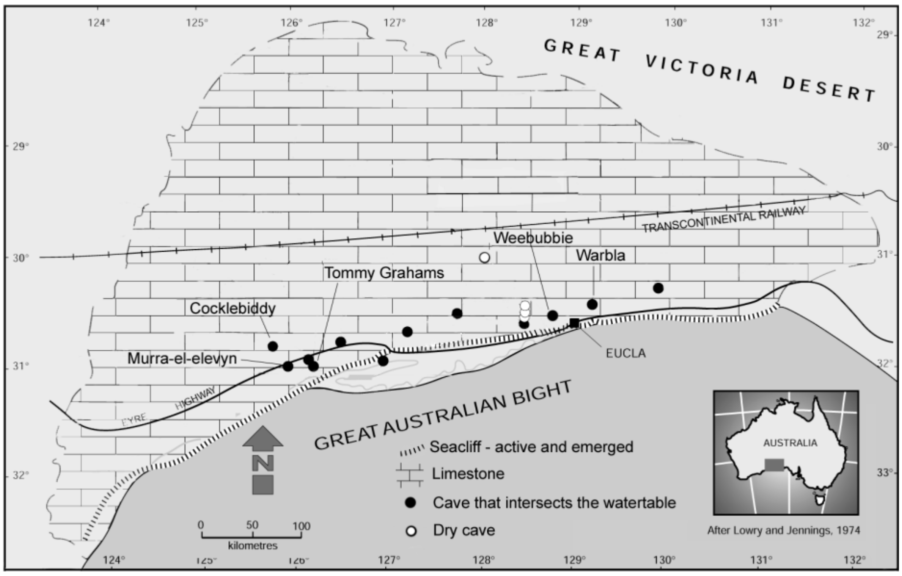

Figure 1 – The Nullarbor region showing cave locations

A number of extensive caves have been discovered in the area,including the largest chamber and longest underwater passage in Australia. The entrance to these caves is typically through dolines, formed as the result of collapse of a portion of the cave roof. A number of caves in this area intersect the Nullarbor aquifer, an extensive saline groundwater body that forms a watertable 100 m below the surface.

The length of the underwater cave passages (up to several kilometres) and the water clarity makes the region attractive to cave divers. Divers penetrating submerged passages of the caves have reported the widespread presence of dense 'mantles' of biological material associated with 'snowfields' of microcrystals. Morphologically similar microbial mantles have been reported from many Nullarbor caves and are generally found throughout the underwater sections, including remote chambers.

Samples were collected from Cocklebiddy (31º 57'S, 125º 54'E), Murra-el-Elevyn (32°02'S, 126°02'E), Tommy Grahams (32º 05'S, 126º 11'E) and Weebubbie (31°39'S, 128°46'E) Caves, Western Australia and Warbla Cave (31°31'S, 129°07'E), South Australia in May and June 1999 and May 2000.

Figure 2 - Phil Prust collecting water samples from the Nullarbor caves. The microbial mantles can be seen extending from the wall. (Photo Peter Rogers)

Microbial Mantles

The mantles take the form of hanging "sheets" or "tongues", with a mucoid consistency and up to 1 m in length (Figure 3). Suspended within the mantles are dense accumulations of crystals giving the mantles a semi-opaque white appearance. In near-entrance passages of the caves the curtains may have a red brown colour from dirt and dust knocked into the lakes. Limestone above the growths is usually irregularly pitted to depths of 3-5 mm. The floor beneath the curtains often comprises dense snowfields of material that apparently originates from the mantles above.

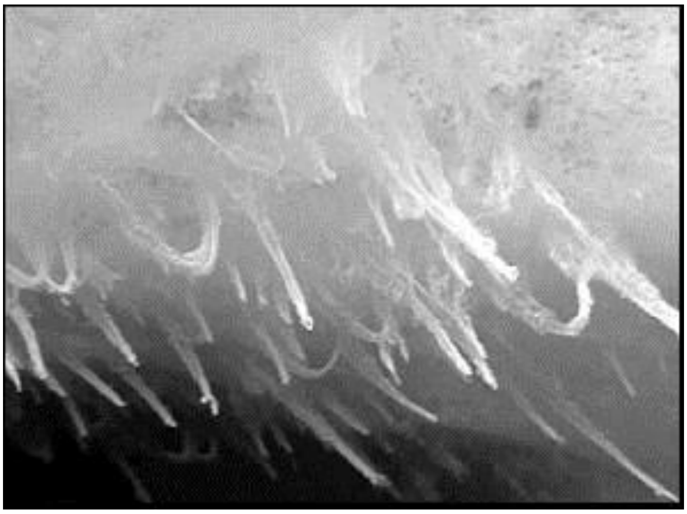

Figure 3 – The microbial mantles (photo P Bouler)

Observations under the light microscope show the biomass is primarily composed of densely packed, unbranched filaments together with spherical, rod and spiral shaped cells.

DNA analysis of the biomass revealed the phylotypic structure of the Weebubbie cave community had many features that suggest it represents a distinctive microbial community. These include a high relative abundance of *Nitrospira* relatives and a high proportion of phylogenetically novel sequences. Of the 36 phylotypes identified in Weebubbie Cave, 12 could not be identified to subdivision level. Furthermore, only two of the novel phylotypes showed any relationship to previously described environmental clones (HOLMES et al., 2001).

The relatively high proportion of novel phylotypes (subdivision or division) is strong evidence for the unusual nature of this community. Together with the absence, or low abundance, of typical terrestrial bacteria such as *Actinobacteridae*, *Acidobacteria*, α-*Proteobacteria*, and *Verrucomicrobia* this suggests the gene library represents a bacterial community which is remarkably different in structure from terrestrial communities. In this respect the community shows some parallels to the remarkable 'division-level' diversity reported for an American hot spring (HUGENHOLTZ et al., 1998).

The trophic structure of the Nullarbor cave community may also be distinctive. The Nullarbor caves contain a high standing microbial biomass, but little or no dissolved organic matter in the water. This implies that the community is primarily supported by chemoautotrophy. Previously reported chemoautotrophic cave ecosystems have been based on sulfur-oxidation and also support an enriched macrofauna (SARBU et al., 1996; AIROLDI et al., 1997; ANGERT et al., 1998; HOSE & PISAROWICZ, 1999).

On the basis of our phylogenetic survey nitrite oxidation is likely to play a significant role in the trophic structure of the Nullarbor cave community. Three phylotypes all show close relationship to one of the two described *Nitrospira* species. Although few *Nitrospira* cultures have been characterised, all known strains are obligate chemolithoautotrophs, which obtain their energy for growth from the oxidation of nitrite (EHRICH et al., 1995).

The remainder of the clones exhibited a high proportion of phylogenetically novel sequence types. The community structure, the presence of nitrite in the water, and the apparent absence of aquatic macrofauna, suggest these microbial structures may represent biochemically novel, chemoautotrophic communities dependent on nitrite oxidation.

Water Chemistry

The cave waters are saline, oxic and have a pH in the range of 7.5 – 7.85. Haloclines were observed in Cocklebiddy, Tommy Grahams and Warbla caves. Of note was the significant levels of nitrite 5-10 ppm and high nitrate levels (Table 1).

Table 1 Water chemistry of the caves investigated during this study

Cave	Tommy Grahams		Murra-el-elevyn	Cocklebiddy		Warbla		Weebubbie
Microbial community observed by divers	No		Yes	Above the halocline		Yes		Yes
Lake Temperature (oC)	23.1		23.7	18.8		22.4		18.9
Sample Depth	15 m	25 m	-5m	6 m	10 m	9 m	13 m	22 m
Conductivity mS/cm	17.2	37.7	14.3	14.2	21	n.d.	n.d.	23.3
SI_c	0.59	0.59	0.43	0.09	0.47	0.02	0.17	0.23
NO_3^-	150	170	180	900	210	30	30	100

n.d. = not determined

The saturation index for calcite (SIc) lies between 0 and 0.5 for the caves where the mantles have been observed. Under these conditions inorganic precipitation of calcite is unlikely due to activation barriers to nucleation and growth (WHITE, 1997). Microorganisms can alter their surrounding microenvironment and it is likely they are both acting as a nucleation source and influencing local supersaturation levels, resulting in calcite precipitation.

Calcite Biominerals

Crystals associated with these mantles were collected from the roof, walls and floor of the caves. Observations under the field emission scanning electron microscope showed the crystals were generally spindle-shaped with curved faces (X in Figure 4) defining the morphic form. Many of the crystals were morphologically polar; frequently one terminus was attached to the bacterial filament (T and F in Figure 4) whilst the other was truncated by three well-defined, symmetry-related faces. The crystals ranged in length from 1.5 microns to 11 microns. The crystals are calcite, confirmed by X-ray diffraction, thermogravimetric analysis, Fourier Transform infra-red spectroscopy and Raman spectroscopy (CONTOS et al., 2001).

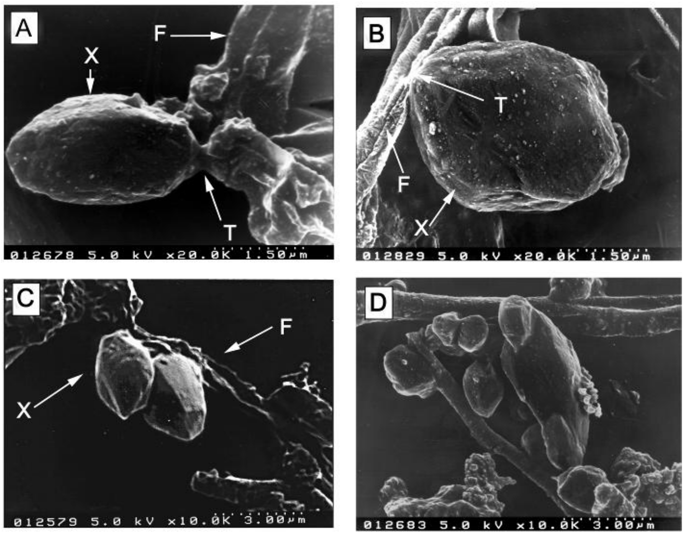

Figure 4 - SEM images of cave crystals

Inorganic Ions Influence on Calcite Morphology

To investigate the influence of the ions present in the caves waters on the calcite morphology a series of doped assays were carried out.

Calcium carbonate crystals were prepared in synthetic cave water. Carbon dioxide was bubbled through a continuously stirred mixture of AR grade calcium carbonate (2 g L^{-1}) and distilled deionised water for two hours. This solution, now saturated with respect to calcium carbonate, was filtered through a 0.22 micron filter to removed the undissolved calcium carbonate. The carbon dioxide was bubbled through the filtrate for a further half hour before the filtrated was poured into crystallisation dishes and left to stand (KITANO, 1962). Salts were added to the crystallisation dishes to mimic the cave water. The assays were repeated using 0.22 micron filtered cave water instead of the synthetic water. A control, with no salts added was prepared for each assay.

The cave shape is different to that expressed by synthetic calcite grown either with (Figure 6) or without (Figure 5) the influence of inorganic ions. In all the doped assays the synthetic calcite expressed negative {01.□} faces, which from a determination of the interfacial angles are most likely to be of {01.1} form (Figure 6).

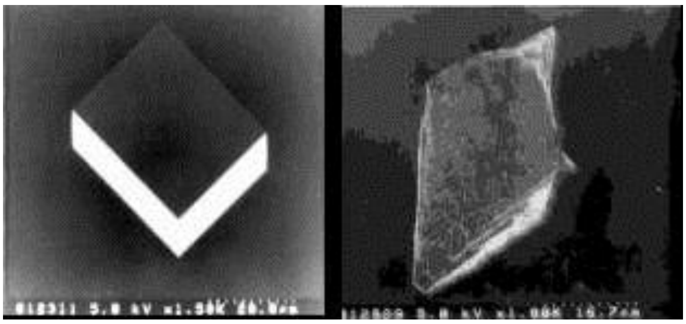

Figure 5 - SEM image of calcite grown without additives

Figure 6- SEM image of calcite grown under the influence of inorganic ions in cave water

Influence of Carboxyglutamic Acid

While the crystals collected from the caves did not bear a similarity to those gown under the influence of inorganic ions, these is a marked similarity between the morphology of the cave crystals and synthetic crystals grown in the presence of L-γ-carboxyglutamic acid and its chemical derivative, malonic acid (MANN et al., 1990). These crystals, also spindle shaped, expressed curved prismatic{hk.0} faces lying parallel to the c-axis and were truncated by smooth rhombohedral {10.4} faces (Figure 7).

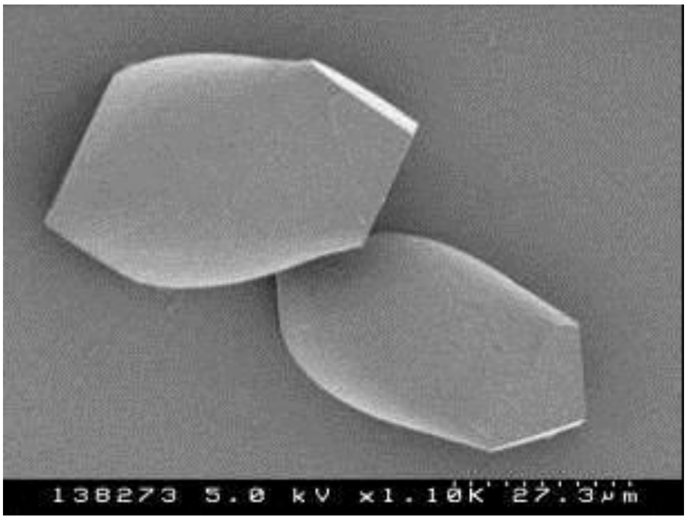

Figure 7 - SEM image of calcite grown under the influence of the α,ω-dicarboxylic acid, malonic acid

Growth perpendicular to the c-axis was inhibited by the adsorption of malonate onto the prismatic faces. A major characteristic of these faces is the orientation of the carbonate group with its C_{2v} axis perpendicular to the crystal surface. It is also noted that the distance between the carboxylate groups in the malonate is comparable with the distance between the carbonates in adjacent anion layers in the prismatic faces. Thus, it is suggested that these structural factors mediate the stereoselective adsorption of malonate onto the inorganic surface. The pseudo-curved appearance of the crystals suggests that adsorption is equal for all prismatic faces with the result that no one unique set is stabilised in the growth form.

The origin of the polarity in the growth form is less clear. One notes, however, that calcite crystals with a comparable crystallographic morphology are induced to form on ordered organic films *in vitro* (HEYWOOD & MANN, 1994)

Spindle-shaped calcite crystals, elongated along the c-axis, are a feature of the otoconia recovered from the inner-ear gravity devices of reptiles and mammals (ROSS & POTE 1989). These crystals are known to be formed in the presence of proteins containing γ-carboxyglutamic acid.

Conclusions

The microbial mantles found in the Nullarbor Caves have not been reported elsewhere. The microbial community shows remarkable division level diversity and a high proportion of phylogenetically novel sequence types. These microbial structures may represent biochemically novel chemoautotrophic communities.

With waters only slightly saturated with respect to calcite, the intimate association of the crystals with the bacterial filaments suggests inductive nucleation of the crystals on the cell walls of the bacterial filaments.

The cave crystals' morphology is unusual and differs from the form expressed solely under influence of the inorganic ions in the cave water. The morphology of the cave precipitates is likely to arise from the interaction of the microbial community with the evolving crystals. The habit of the crystals recovered from the cave is similar to that known to occur in a number of biominerals.

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