# CARBONATE PRESERVATION OF DINOSAUR EGGS IN THE UPPER CRETACEOUS ANACLETO FORMATION AT AUCA MAHUEVO, NEUQUÈN BASIN, ARGENTINA

by

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A thesis submitted in partial
of the requirements for the degree

of

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in

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January 2011

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# ABSTRACT

Preservation of dinosaur eggs and footprints by precipitation of calcium carbonate in the Upper Cretaceous Anacleto Formation at Auca Mahuevo, Argentina represents a relatively unusual occcurence in the fossil record. Under normal condition, eggs are readily destroyed in sediments shortly after burial by physical, chemical, and biological processes. This study attempts to determine a preservational model for carbonate eggs by characterizing their mineralogical composition and microstructures using a variety of analytical instruments including petrographic microscope, cathodoluminesce (CL) microscope, X-ray powder diffraction (XRD) and field-emission scanning electron microscope (FEM) to characterize the composition and fabric of the fossilized eggs. Several textutal features have been observed in the carbonate eggs, including membrane, embryonic skin, spherulites, ooids, peloids, *Microcodium*, calcified filaments, and micrite. Microbial actvity is likely responsible for the formation of these microfabric features, facilitating calcium carbonate precipitation leading to exceptional preservation of eggs. Although microbial influence in the carbonate egg preservation has not been clearly elucidated, laboratory experiments by other workers provide an argument for the role of microbes in the precipitation of calcium carbonate. The rare preservation of egg contents in the Anacleto Formation have been linked to biological-mediated processes. This preservation also provides evidence for penecontemporaneous carbonate precipitation under subaerial conditions before significant burial.

# CHAPTER 1

## INTRODUCTION

Biomineralization is the processes by which a mineral is produced by an organism (Weiner and Dove, 2003). This mineralized product consists of both inorganic and organic materials. Calcium carbonate minerals are the most abundant biogenic minerals in terms of their quantities and widespread distribution (Lowenstam and Weiner, 1989). Understanding the mechanism by which biological systems precipitate certain carbonate minerals is one of the most intriguing challenges in the biomineralization field. The primary evidence of biomineralization may be obscured by subsequent diagenesis, resulting in biases in the fossil record. Some workers have attempted to gain better understanding of biomineralization processes by reproducing them in laboratory experiments (Sagemann et al., 1998; Kolo et al., 2006; Kandianis et al., 2007).

Fossil assemblages containing preserved three-dimensional structures and soft tissue are relatively rare in the fossil record (Seilacher et al., 1985; Allison, 1988a). Rapid biomineralization processes occuring prior to significant alteration or destruction of soft-part morphology commonly produce exceptional preservation. Early diagenetic mineral growth under favorable conditions may also lead to exceptional preservation (Allison, 1988 a, b). In addition, there is the intriguing possibility that microbial activity not only promotes decay, but also plays an essential role in the fossilization process as a result of rapid authigenic mineralization (Seilacher et al., 1985).

Preservation of dinosaur eggs containing soft tissues and embryonic remains morphology is relatively uncommon (e.g. Hirsch et al., 1990; Kohring, 1999b; Grellet-Tinner, 2005a). Under normal conditions, egg contents are readily destroyed in sediments shortly after burial as a result of scavenging, microbial decay, and any physical disturbances during or immediately after burial.

Whether preservation of egg contents is a result of inorganic or organic processes remains enigmatic. Nevertheless, many laboratory experiments (e.g. Briggs and Kear, 1994; Sagemann et al., 1998; Kolo, et al., 2007, and Raff et al., 2008) have demonstrated that exceptional preservation of soft tissue is driven by microbial activity. It also has been suggested that there are distinctive carbonate crystal morphology formed by extracellular polymeric substance (EPS) of the bacteria. The EPS controls the mineralogy and morphology of the carbonate crystals by changing their composition and concentration (Zammarreño et al., 2009); therefore, study of mineralogy and microfabric of carbonate eggs may provide insight into biomineralization processes.

In the Upper Creataceous (Campanian) Anacleto Formation of Argentina, Chiappe et al. (1998) documented preservation by micritic carbonate of the first known sauropod embryonic remains (skin and bone) within megaloothid eggs. The enclosing eggshell was originally composed of inorganic calcite ($CaCO_3$) and organic material components that are potentially susceptible to microbial diagenesis. Investigation of the mode of preservation of sauropod egg contents can provide information relevant to improving our understanding of fossilization, nesting site paleoecology, and dinosaur reproductive biology (Chiappe et al., 2003; Jackson et al., 2004).

This study focuses on sauropod eggs from the Auca Mahuevo locality containing embryonic material preserved within calcium carbonate. The objectives of this study are to: 1) determine the mineralogical composition of the egg contents, 2) describe their microfabric, and 3) test the hypothesis that microbial activity influences the preservation of soft-tissue morphology. This study employs a combination of mineralogical and geochemical analyses, and morphological characterizations to unravel the mode of calcium carbonate precipitation leading to exceptional preservation of sauropod egg contents. The specific questions to be addressed in this study are:

1. What processes lead to the mineral precipitation in the dinosaur eggs that preserved embryonic contents? Are these processes penecontemporaneous, pedogenic, or eogenetic?
2. Is there evidence to suggest that microbial activity played a role in preservation of the dinosaur embryonic remains?

In this study, the term *soft tissue preservation* refers to replication of the morphology of the original soft tissues by authigenic mineralization (permineralization). It does not imply preservation of primary endogeneous organic molecules. *Authigenic mineralization* is defined as rapid in situ mineral growth. Authigenic mineralization involves infiltration of ion-bearing pore water (permineralization); however, the mineral not only permeates the organism, but it also replicates the morphology of the organism (Briggs, 2003).

# CHAPTER 2

## GEOLOGIC SETTING

The Neuquén basin is located on the east side of the Andes Mountains in western Argentina. This basin has a triangular shape and two main regions are commonly recognized: the Neuquén Andes to the west and Neuquén embayment to the east and southeast. The majority of the basin's hydrocarbon fields are located in the Neuquén embayment where most Mesozoic sedimentary strata are relatively undeformed. Conversely, Mesozoic strata in the Andean region have been deformed by development of Late Cretaceous – Cenozoic deformation resulting in a series of N-S oriented fold-and-thrust belt structures (Howell et al., 2005).

The triangular Neuquén basin is bounded on its northeast and southern margins by wide cratonic areas of the Sierra Pintada Massif and North Patagonian Massif. The western margin of the basin is the Andean magmatic arc on the western margin of the Gondwana – South American Plate. The Neuquén basin has experienced three stages of evolution and basin development:

1. Late Triassic – Early Jurassic

   This stage includes prior subduction on its western margin that is characterized by large transcurrent fault systems. This led to extensional tectonics within the Neuquén basin and evolution of a series of narrow, isolated depocenters (Francese and Spalletti, 2001).

2. Early Jurassic - Early Cretaceous

This phase is characterized by the development of a steeply dipping, active subduction zone and the associated evolution of a magmatic arc along the western margin of Gondwana that led to back-arc subsidence within the Neuquén basin (Vergani et al., 1995).

3. Late Cretaceous – Cenozoic

This phase marks transition to a shallow dipping subduction zone, resulting in compression and flexural subsidence associated with crustal shortening and uplift of the foreland thrust belt (Introcaso et al., 1992).

## Lithostratigraphy of the Upper Cretaceous Anacleto Formation

The Anacleto Formation exposed at Auca Mahuevo is composed primarily of fluvial sandstone, siltstone, and mudstone. This formation contains one of the most important Late Cretaceous vertebrate faunas from South America (Dingus et al., 2000).

During the late Campanian, an inversion of the regional slope occurred in the Neuquén basin as a consequence of subsidence related to flexural loading (Zambrano, 1981; Uliana and Biddle, 1988). This event triggered the Atlantic transgression into the Neuquén basin, which is recorded at Auca Mahuevo by the Allen, Jagüel, and Roca Formations of the Malargüe Group that overlie the Anacleto Formation (Dingus et al., 2000; 2009).

The Anacleto Formation is the uppermost unit of the Neuquén Group and consists primarily of reddish brown sandstone, siltstone and mudstone that were deposited in

fluvial settings. Dinosaur eggs were found in the finer grained units of siltstone and mudstone, especially in levee and overbank facies of a meandering fluvial environment (Dingus et al., 2000; 2009). In some egg-bearing intervals, thin channel and crevasse-splay sandstone interfingers with mudstone. Egg beds are preserved on various substrates ranging from sandstone to mudstone (Chiappe et al., 2004).

At least four titanosaurian egg-bearing layers (Figure 2.1) occur in the floodplain facies in siltstone and mudstone units, where rare flooding events covered the areas extensively, burying exposed eggs, and drowning the incubating embryos under a thick layer of mud (Dingus et al., 2000). The egg contents, including embryonic skin and bone, were preserved by micritic carbonate.

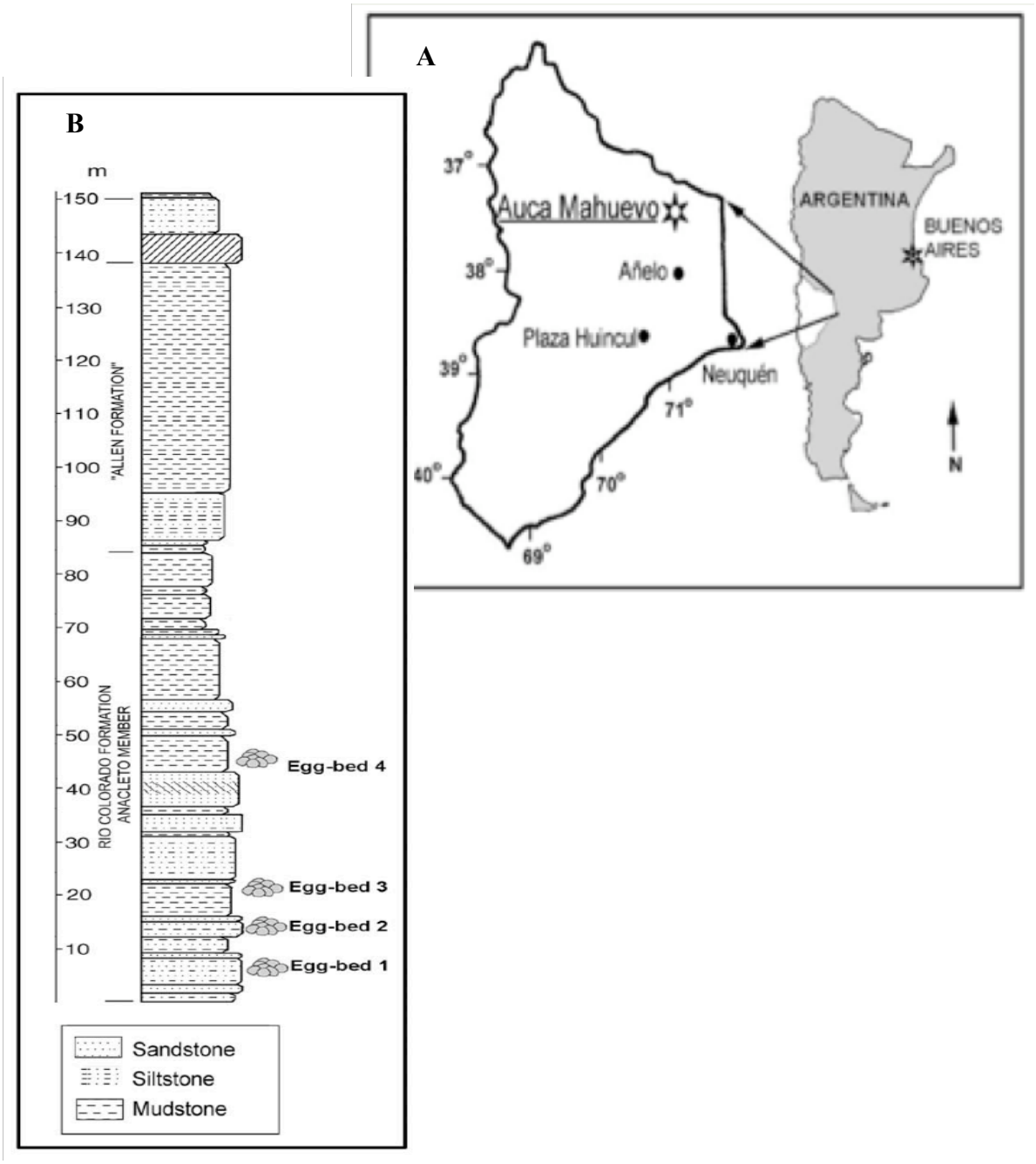



Figure 2.1. Regional map and stratigraphic sections of the Auca Mahuevo study are. A. Regional map. B. Stratigraphic sections showing position of egg layers. (From Chiappe et al., 2004)

# CHAPTER 3

# METHODS

This study uses a variety of analytical methods, including petrographic microscopy, cathodoluminesce (CL) microscopy, X-ray powder diffraction (XRD) and field-emission scanning electron microscopy (FEM) to characterize the composition and fabric of the fossilized egg contents. Petrographic microscope and cathodoluminescence (CL) microscope observations were conducted in Department of Earth Sciences at Montana State University, whereas X-ray powder diffraction (XRD) and field-emission scanning electron microscope (FEM) analyses were done in the Imaging and Chemical Analysis Laboratory (ICAL) at Montana State University.

1. Petrographic microscopy

   Thirty-five standard thin sections of carbonate eggs without cover slip and impregnated with clear epoxy were examined. Most of the egg samples were taken from egg-bearing layer 3, which was quarried extensively (Chiappe et al., 2000), and a few from egg-bearing layer 1. Standard petrographic microscopy was used to describe mineralogical composition of the eggs and their microfabric characteristics. Petrographic microscopy is an essential step for characterizing diagenetic components before geochemical analysis. Photomicrographs were taken using a Nikon Eclipse LV100 POL microscope with an attached camera.

2. Optical cathodoluminescence (Optical CL) microscopy

Cathodoluminescence (CL) analysis was conducted on polished thin sections of carbonate eggs, using a Luminoscope mounted on a standard petrographic microscope equipped with a digital camera. Optical cathodoluminescence was used to observe textures, deformation features, compositional variation, and cement generation that could not be seen using standard polarized light microscopy. The luminescence in carbonate is primarily caused by the presence of transition metals, such as $Mn^{2+}$ as an activator and $Fe^{2+}$ as quencher (Have and Heijnen, 1985). Operating conditions of the Luminoscope were 100 mTorr for the vacuum pressure and 8.8 Kv for the voltage.

3. Field-emission scanning electron microscopy (FEM)

Fifteen egg fragments and five polished thin sections of carbonate eggs were analyzed using the field-emission scanning electron microscope (FEM). Fragment samples were mounted on aluminium stubs. All polished thin sections were coated with 150 Å of carbon in a vacuum sputter coater to a thickness about 30 nm prior to FEM examination in order to do elemental analysis. A carbon coat prevents charging of the sample while providing sufficient conductivity. The instrument used was a Zeiss Supra 55VP in the Imaging and Chemical Analysis Laboratory (ICAL) at Montana State University. The variable pressure mode at high pressure (20-40 Pa) was used to compensate charging of the samples. The field-emission scanning electron microscope (FEM) provided high-resolution images using a secondary electron imaging (SEI) detector and allowed insulating samples to be analyzed

without coating at low voltages. The attached energy-dispersive X-ray (EDX - Spirit) detector on the FEM allowed qualitative elemental analysis of the samples. Phase discrimination was also done using backscattered electron (BSE) imaging.

4. X-ray powder diffraction (XRD)

Five egg samples were finely grounded into powder using a mortar and pestle, and passed trough a 210-mesh (63 μm) sieve. The samples were analyzed using the Scintag X-1 diffractometer with CuKα radiation ($\lambda$= 1.5418 $^0$A) and acceleration voltage of 45 kV. The samples were collected at 2θ ranging from $15^0$ to $75^0$ at a rate of $2.4^0$ /minutes. The X-ray diffractograms were used to identify mineral phases present in the egg samples.

# CHAPTER 4

# OBSERVATIONS

## Eggshells

### Hand Sample

The external shape of the eggs is elongate and approximately 16 cm long and 9 cm wide (Figure 4.1). Some of the outer shell surface of the eggs has been weathered. The egg contents were highly mineralized with calcium carbonate ($CaCO_3$).

### Thin Section



Figure 4.1. Preservation of a carbonate-filled egg in hand sample from egg-bearing layer 3.

Eggshells observed using the petrographic microscope have thickness ranging from 0.7 to 1.3 mm (Figure 4.2 A, B). The outer surface of the eggshells shows nodular

ornamentation (Figure 4.2 C), whereas the inner shell reveals nucleation sites with radiating spherulites calcite that form the shell units (Figure 4.2 D). The egg contents display bright red-orange luminescence under CL microscope (Figure 4.3 A, B). In contrast, unaltered calcite eggshell shows non-luminescence (Figure 4.3 A).

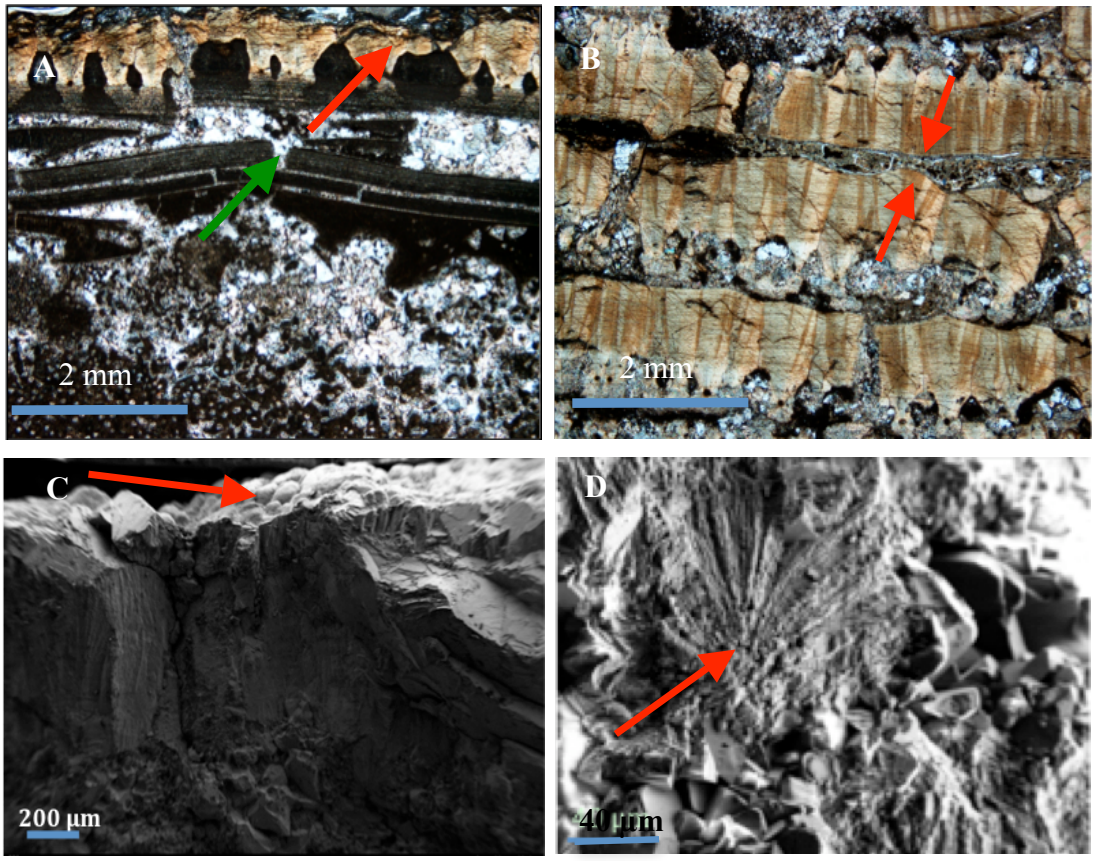



Figure 4.2. Photomicrograph (A-B) and FEM images (C-D) of eggshells. A. Eggshell (red arrow) and fractured membrane (green arrow). B. Eggshells facing different directions (red arrows) indicate a compressed egg resulting from burial processes. C. Outer surface with nodular ornamentation. D. Inner surface showing radiating spherulites at the base of the shell unit (arrow). Note the outer shell surface is at the top of the images.

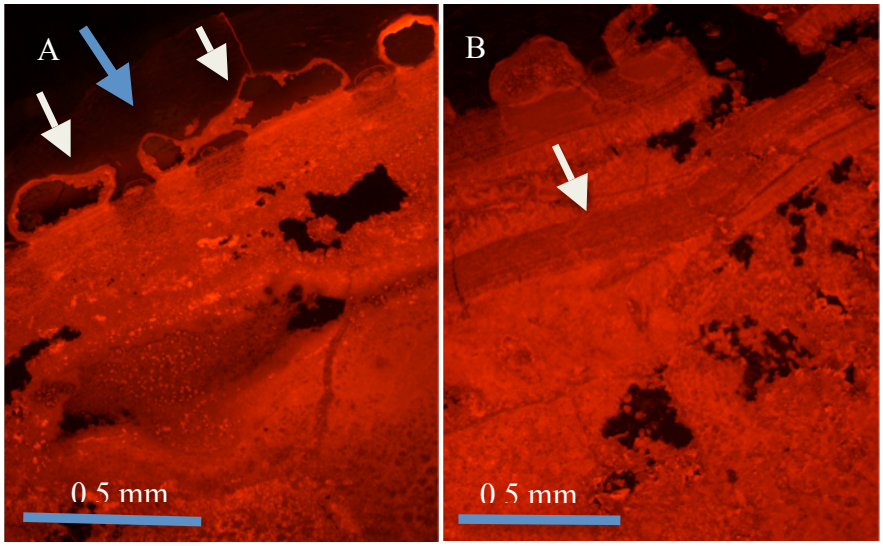



Figure 4.3. CL photomicrograph of carbonate eggs. A. The eggshell (blue arrow) does not luminescence, whereas the dissolution cavities between nucleation centers are filled with manganese calcite (white arrow) that luminesces bright orange. B. The membrane below of the eggshell is replaced by manganese calcite causing bright orange luminescence (white arrow).

## Membrane

Thin dark gray layers inside the eggs and below the inner eggshell surface were observed under plane-polarized light that represent eggshell membrane (Figure 4.4). Some of these membranes were fractured because of compaction during burial. The membrane observed in this study consists of parallel laminae, which are mineralized by calcium carbonate, as confirmed by CL that showing bright orange luminescence.

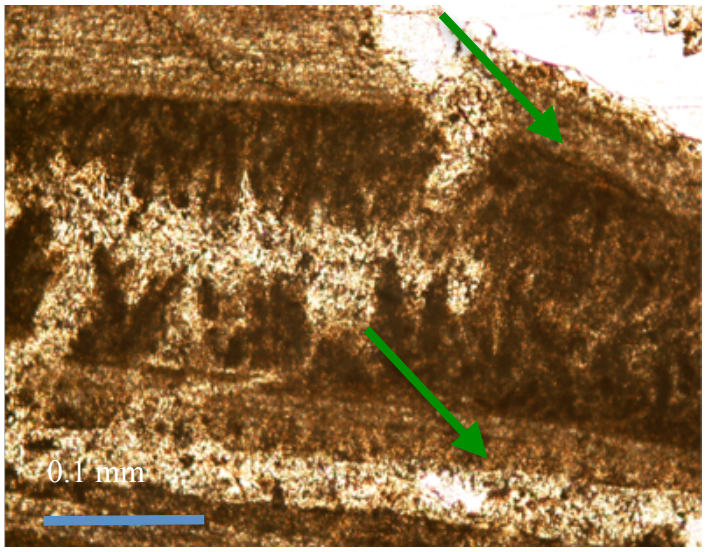



Figure 4.4. Photomicrograph of membrane (green arrows) from egg layer 1 (sample number: F1 – 3b).

## Embryonic Skin

In cross sectional view, the skin thin sections display uniform distribution of densely arranged subrounded structures, some of which are infilled by calcite (Figure 4.5 A). Coria and Chiappe (2007; Figure 4.5 B) reported similar morphology and uniform distribution of tubercles on embryonic skin from the Auca Mahuevo locality. Unfortunately, investigation of embryonic skin in cross section using FEM/SEM failed to reveal detail morphology of the skin. Identification of embryonic skins in thin section is problematic because the fragments are small and there are no documented features of dinosaur embryonic skin for comparison. At Auca Mahuevo, embryonic skin appears very thin (about 400 microns).

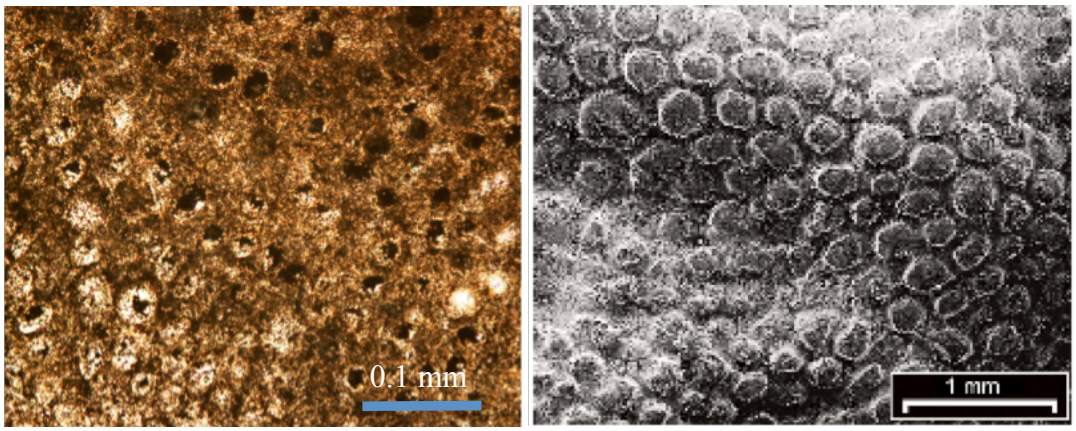



Figure 4.5 Photomicrograph of embryonic skin (Sample F1-3f). A. Interpreted epidermis in transverse view. B. Ground tubercles pattern of embryonic skin from Auca Mahuevo (from Coria and Chiappe, 2007; from surface view).

## Spherulites

Field Emission Microscope observations reveal subround spherulitic structures inside of the eggs. Their diameters range from about 50 μm to more than 200 μm (Fig 4.6 A, B). The morphology of these structures is somewhat similar to biological spherulites that occur at the base of the sauropod eggshell. However, the biological spherulites radiate outward from nucleation sites and form individual shell units which comprise the shell. These shell units exhibit bright yellow color under plane polarized light. In contrast, the subrounded spherulitic structures are dark brown in color.

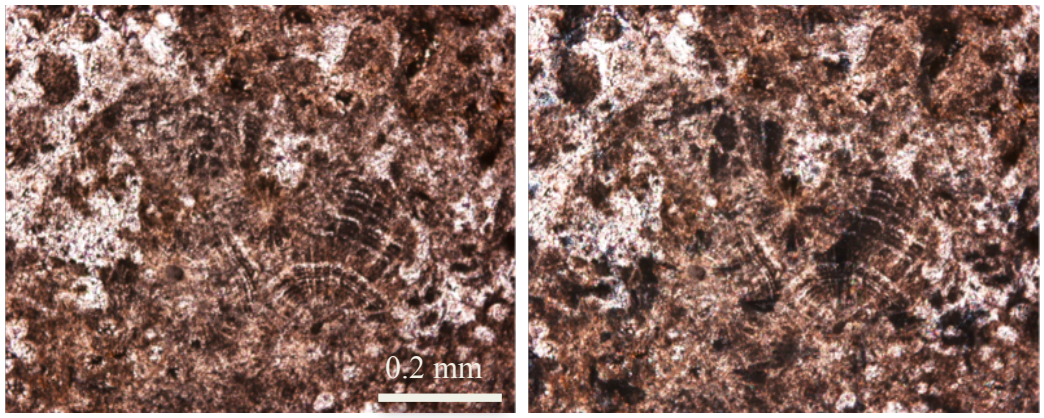



Figure 4.6. Spherulitic structures present in Auca Mahuevo sauropod eggs (Sample F1-3b). A. Plane-polarized light (PPL) and B. same structure under cross-polarized light (XPL).

## Ooids

Thin section observations show abundant ooids inside of the eggs (Figure 4.7). The ooid distribution in the samples is both scattered and concentrated. The shape of the ooids is spherical with tangential crystal orientation and some nuclei show radial orientation. Their diameter varies between 50 μm to about 100 μm. Micrite fills the pores among ooids. These ooids have well-preserved fabrics, including concentric layering, brown color, and combination of both tangential and radial fabrics.

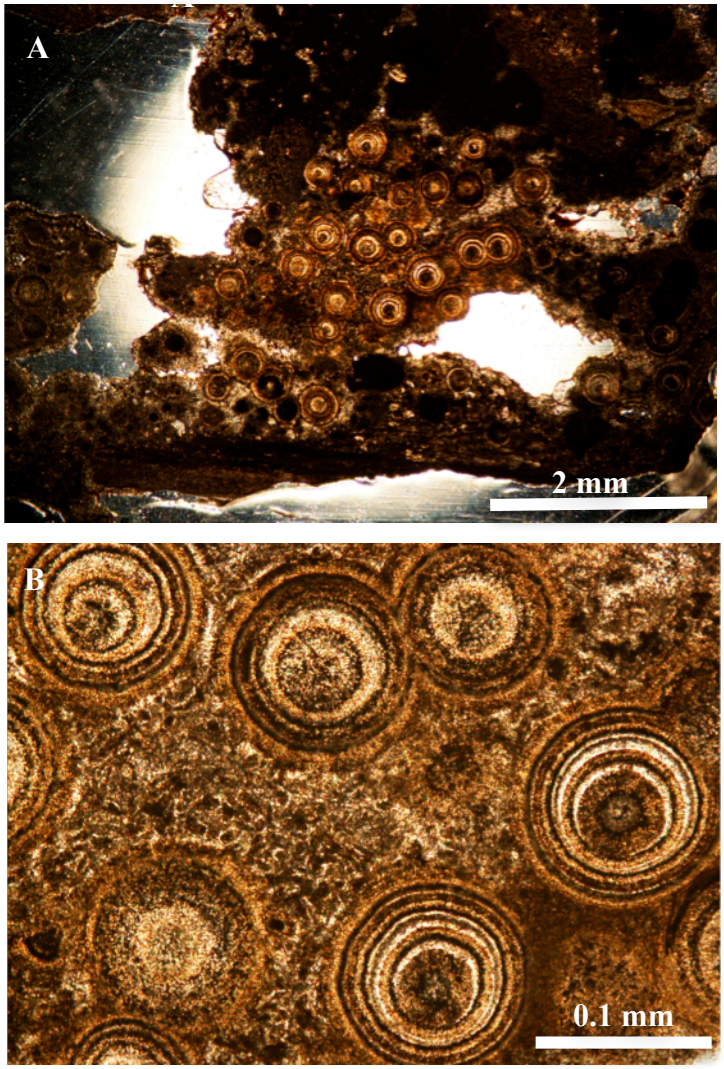



Figure 4.7. Ooids features found inside of the eggs in thin sections from egg layer 1 (F1-3g). A. View in lower magnification. Note the clustered distribution. B. Same sample at higher magnification illustrating combination of both radial fabric on the center of the structure and tangential fabric on the cortex.

## Pellets and Peloids

The peloids are spherical to ovoid-shaped grains from 0.01 to 0.1 mm in diameter, whereas pellets are rod-shaped with typical grain size from 0.04 to 0.08 mm long. Pellets

and peloids distribution in the eggs occurs in clusters. Peloids are diverse in size and shape (Figure 4.8 A), whereas pellets are uniform in both size and shape (Figure 4.8 B). In addition, pellets and peloids are composed of micrite.

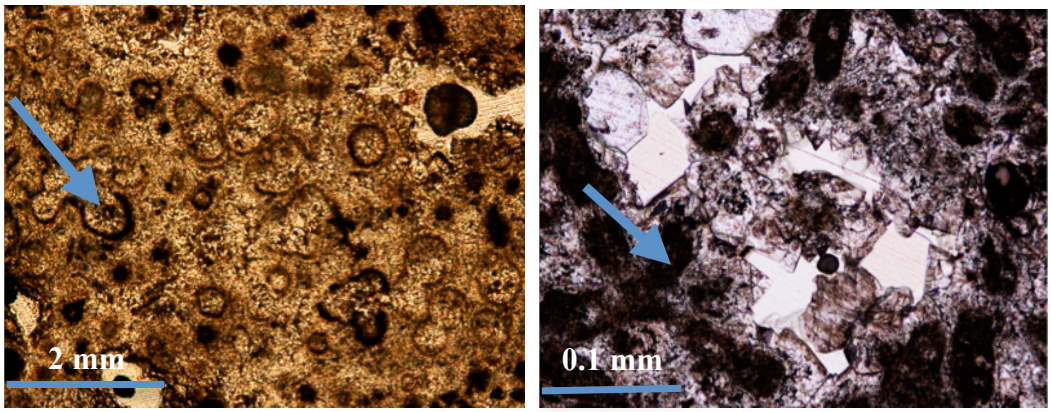



Figure 4.8. Photomicrograph of pellets and peloids. A. Peloids in sample F1-3j. B. Pellets in sample F1-3g. Arrows indicate structures in A and B.

*Microcodium*

*Microcodium* feature has a circular structure with bladed calcite crystals radiating from the center. These structures range in size from about 50 µm to more than 100 µm. Single and aggregate *Microcodium* exhibit curved faces with a dark nucleus. These rosette-like structures are most easily identified in thin sections.

There are two major forms of *Microcodium* (Kosˇir, 2004): 1) *Microcodium* in corn-cob aggregates (Figure 4.9 A), and 2) spheroids (rosettes) with polyhedral crystals radiating from hollow central and lamellar colonies (Figure 4.9 B).

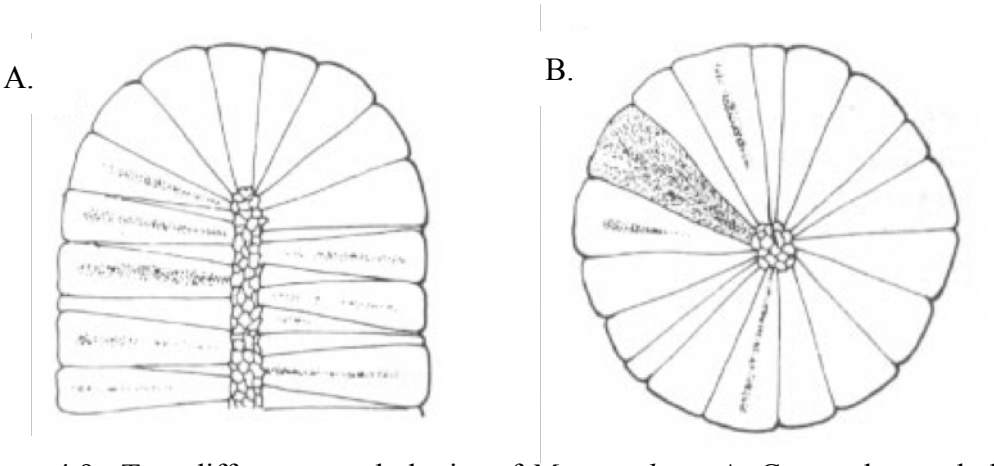



Figure 4.9. Two different morphologies of *Microcodium*. A. Corn-cob morphology, B. rosette-like morphology (From Kosˇir, 2004).

At Auca Mahuevo, *Microcodium* are present inside eggs that are enclosed with clear microsparite cements (Figure 4.10 A). The *Microcodium* colonies display rosette-like structure in transverse sections. Martin and Meyer (2006) reported similar *Microcodium* morphology in their study of carbonate palustrine deposits near the K-T boundary in the southeastern Pyerenean foreland of southwestern France (Figure 4.10 B).

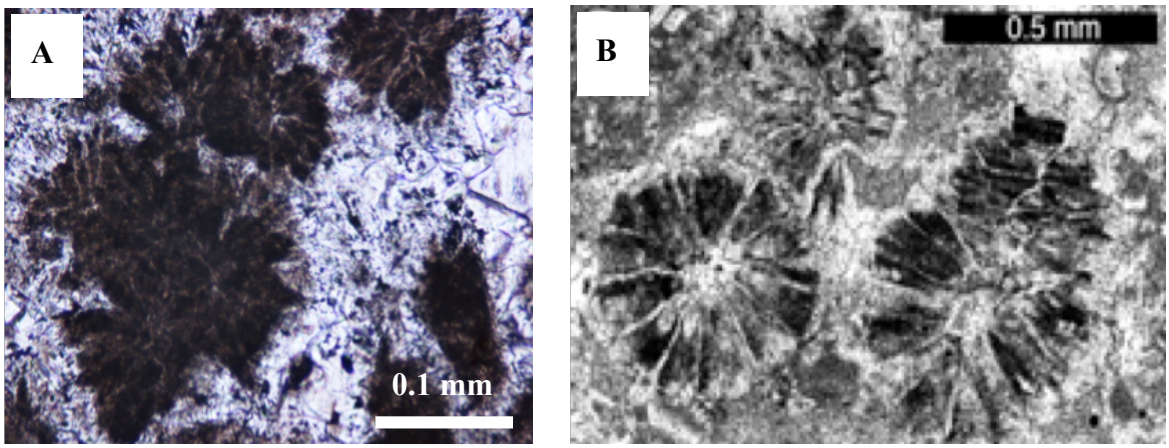



Figure 4.10. A. Micritic carbonate of sauropod eggs with *Microcodium* in a microsparite cement in the Anacleto Formation from egg layer 1 (F1-3b). B. Mudstone with *Microcodium* colonies in a micritic matrix (Marty and Meyer, 2006).

## Filaments

Field Emission Microscope analysis revealed calcified filaments inside of the eggs up to 35 μm long and 3 μm wide. These calcified filaments are common in voids. Some filaments are tubular in shape (Figure 4.11 A), whereas others are branching (Figure 4.11 B).

Based on the dimension and branching morphology, these filaments may be fungal in origin. Typical fungal hyphae are about 4 μm in diameter (Alexander, 1977). Alternatively, these filaments may represent the organic matrices of the eggs that have similar morphology to fungi (Jackson et al., 2004).

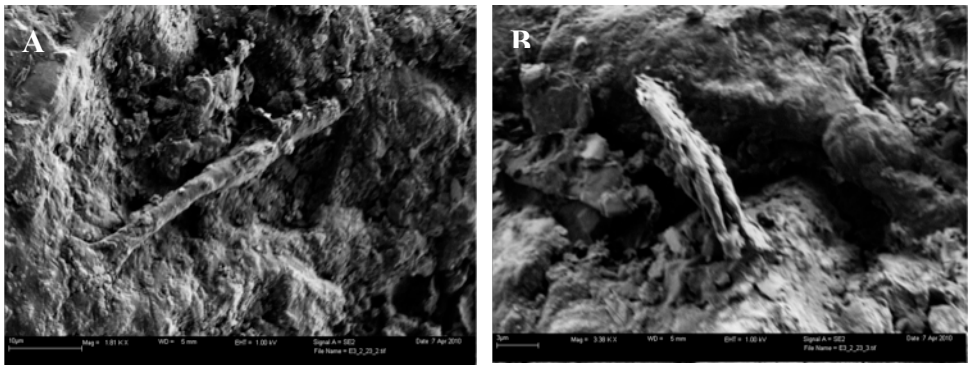



Figure 4.11 FEM figures of calcified filaments found inside eggs. A. Straight form of calcified filament. B. Branching form (toward the reader) of calcified filament.

## Micrite and Sparite

Thin section and Field Emission Microscope analyses displayed the presence of micrite and sparite with scattered distribution in the eggs. Some of these micritic materials were recrystallized into microsparite and/ or sparite (Figure 4.12).

Micrite has a grain-size limit <4 μm (Folk, 1959). Micrite, together with sparite cement has preserved many features in the eggs, including *Microcodium*, ooids, pellets and peloids, and spherulites.

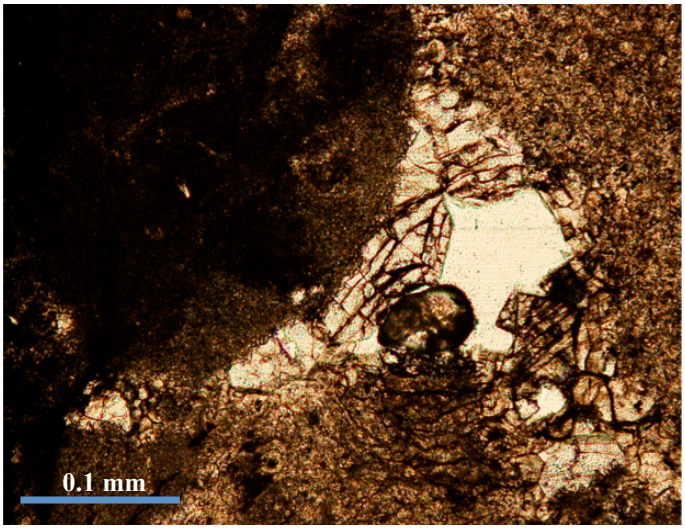



Figure 4.12. Photomicrograph of transition from micrite (left) to sparite (right) from egg layer 1 (F1-3L). Note the center of the image contains a hole.

## Carbonate Component

Six egg samples were characterized using X-ray diffraction for analyzing bulk mineralogy. Four X-ray diffraction patterns of these egg samples are shown in Figure 4.13. The diffraction patterns are similar for most eggs samples. X-ray diffraction analysis revealed that the carbonate-filled eggs are composed mainly by low-Mg calcite.

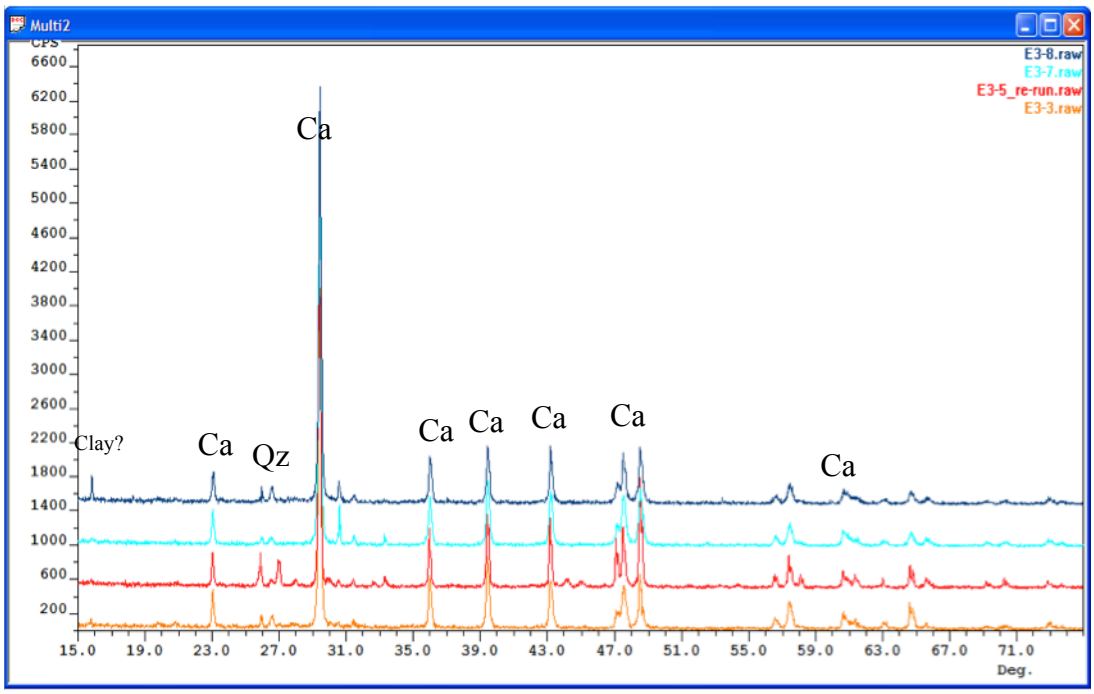



Figure 4.13. XRD diagram of bulk carbonate egg samples from egg-bearing layer 3. Ca: calcite; Qz: quartz

## Siliciclastic Components

### Quartz

X-ray diffraction (Figure 4.13) and thin sections (Figure 4.14) and) show the presence of quartz in some egg samples as a minor component, including the samples from E3 – b4, E3 – b7, and E3 – b10. These quartz grains are subangular to rounded and silt-sized. Detrital quartz possibly originated from sand in the surrounding area that was transported to the egg's locality by running water and/or eolian dust by wind.

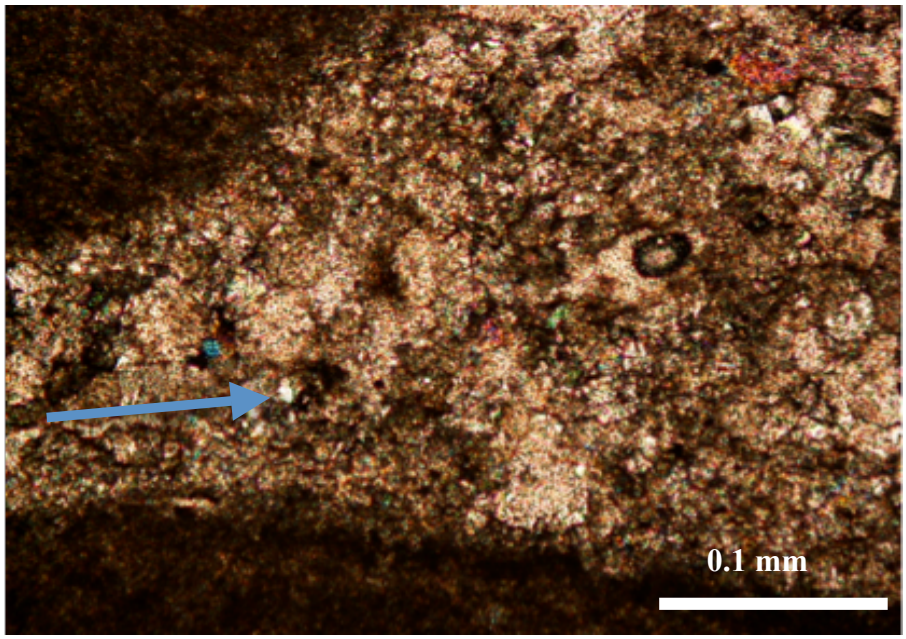



Figure 4.14. Photomicrograph of detrital quartz (blue arrow) in the egg from layer 3 (E3-b10).

Gypsum

The eggs also contain gypsum (Figure 4.15) in addition to quartz, which could be observed by FEM imaging and EDX analysis. Individual crystals are euhedral to sub-hedral in form.

Gypsum crystals were possibly re-precipitated from gypsum in the Allen Formation, which is comprised of marine mudstone in the lower part and gypsum in the upper part of the formation (Dingus et al., 2000). Gypsum crystals from Allen Formation may have dissolved, producing sulfate-rich water that moved through fractures into the underlying Anacleto Formation.

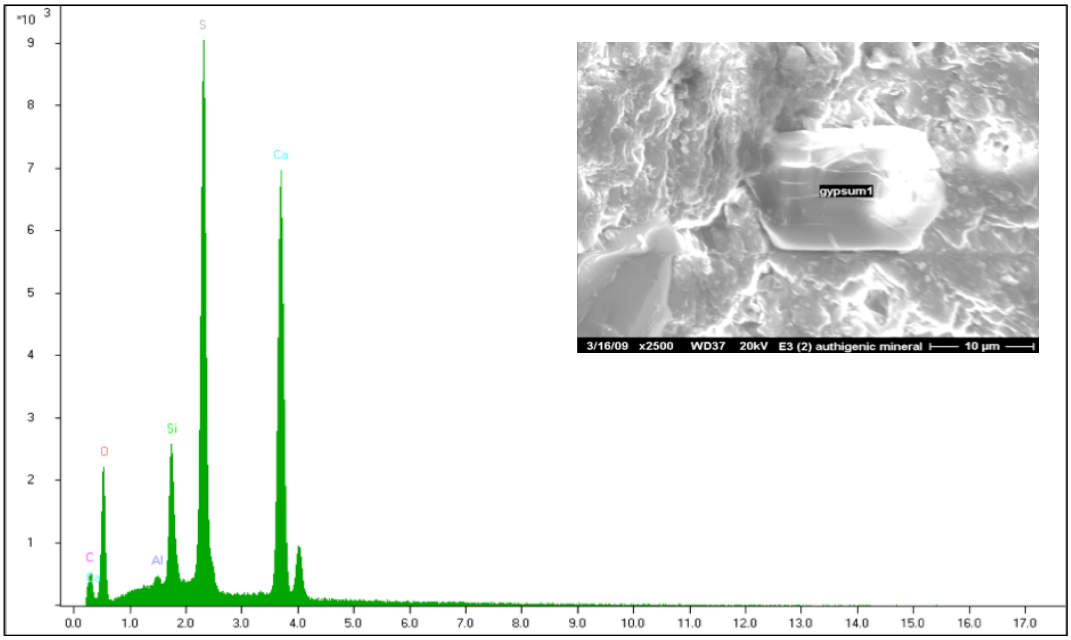



Figure 4.15. EDX patterns and FEM image of gypsum from egg-bearing layer 3

Feldspar

Minor amounts of plagioclase feldspar are also present in the eggs (Figure 4.16). Most of feldspar grains are finely crystalline and difficult to see under petrographic microscope; however, they are clearly visible by FEM imaging.

Feldspar grains in the eggs are likely derived from sediment in the surrounding area. This detrital component can enter the eggs through fracturing of the shell.

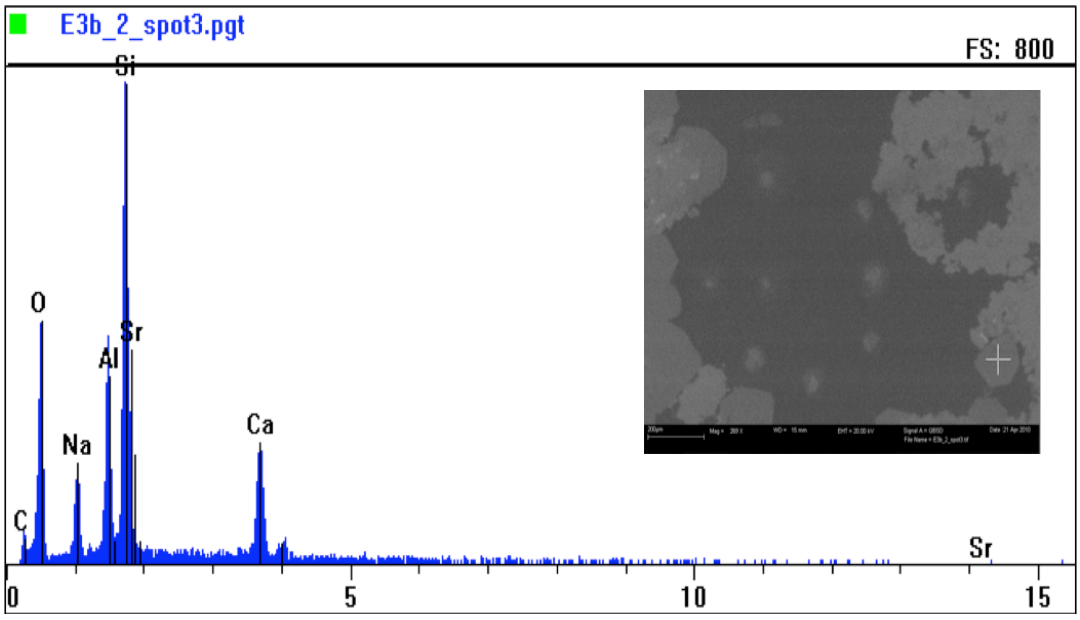



Figure 4.16. EDX patterns and FEM image of detrital plagioclase feldspar from egg samples from layer 3.

Non-Carbonate Authigenic Mineral

Field Emission Microscope imaging and Energy Dispersive X-ray analysis revealed minor amount of analcime (Figure 4.17) in the inner eggshell. This authigenic zeolite mineral filled the space between the eggshell and membrane in a sample from egg-bearing layer 3 (e.g. E3 – 11).

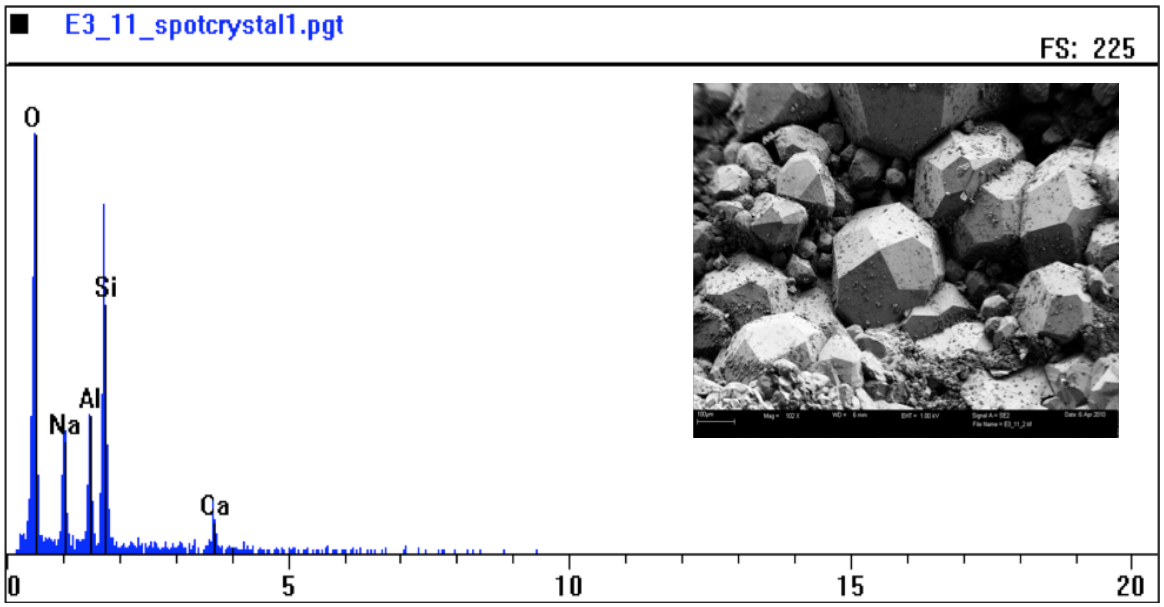



Figure 4. 17. EDX patterns and FEM image of analcime.

Hay and Shepard (2001) suggest that analcime is formed from alteration of zeolite. Nevertheless, there is no evidence of zeolite in the Anacleto Formation (Schmitt, personal communication); therefore, it is probable that analcime formed as a result of authigenic precipitation. Although there is altered volcanic ash in the Allen Formation at this locality (Dingus et al., 2009), analcime is only identified in the carbonate eggs in the Anacleto Formation. Based on the textural evidence, which there is no replacement from precursor zeolite, indicates that analcime was not formed by alteration of volcaniclastic materials. Analcime at Auca Mahuevo shows euhedral crystals, which is in contrast to analcime derived from volcaniclastic materials that commonly form subhedral crystals (Campo et al., 2007). Analcime may come from the reaction of feldspar of biote-rich sandstone. It has been suggested that analcime could be formed by bacterial activity (Castanier et al., 1999) as it forms in Soda Lake, Kenya. There, the analcime occurs as a result of passive precipitation by bacteria when the $Ca^{2+}$ in the medium is low and the pH

increases. There are other examples of analcime that formed without association with volcaniclastic materials, such as in Lake Natron, Tanzania, where the analcime was formed by direct precipitation at the sediment-water interface in the bottom mud of the lake (Hay, 1966), and in Lake Lewis, Australia, analcime formed as a result of the reaction of Na-rich brines interstitial fluids below the water table (English, 2001). Considering these cases that analcime may occur as a result of reaction with their environment or direct precipitation; therefore, interpretation about the origin of analcime needs further investigation, including the possibility of analcime component in the mud substrate and/or surrounding environment where the eggs deposited.

# CHAPTER 5

# DISCUSSION

## Evidence of Microbial Activity

At Auca Mahuevo, the role of microorganisms in the preservation of eggs and soft tissues inside of the eggs is indicated by the presence of microbial features such as spherulites, *Microcodium*, and ooids. While none of these features alone are indicative of microbial mediation of carbonate mineral precipitation, taken collectively they provide strong circumstantial evidence that microbially mediated precipitation is permissible.

### Spherulites

Spherulites are commonly observed in carbonate soils and laminar crusts (Verrecchia, 1995). Lee and Golubic (1999) found spherulitic structures in the Mesoproterozoic Gaoyuzhuang Formation, China, and interpreted them to be as a result of microbial-mediated processes. Carbonate spherulitic bioliths are commonly precipitated by different types of bacteria in natural environments and in artificial culture media, and are frequently formed by calcium and magnesium carbonate minerals (Sánchez-Navaz et al, 2009).

Braissant et al. (2003) compared calcium carbonate crystals precipitated in bacterial cultures with those precipitated abiotically by conducting an *in vitro* experiment. They point to the influence of extracellular polymeric substances (EPS) and amino acids in the mineralogy and morphology of calcium carbonate produced by bacteria. Treatment

using bacterial cultures produced two polymorphs of calcium carbonate, vaterite and calcite. Vaterite was mainly produced by *Xanthobacter autotrophicus*, and calcite was produced by *Ralstonia eutropha*. The morphology of individual crystal ranges from fan shape to needle shape. The crystals radiate from a central point to form a sphere. There are two main forms that have been observed from the bacterium *Xanthobacter autotrophicus*: 'fried egg' shape that formed at the surface of the bacterial colony, and the flipped 'fried egg'.

Similar morphology of calcite crystals occurring as spherulites is also present inside the eggs at Auca Mahuevo locality. This evidence suggests that calcium carbonate precipitation processes forming the subrounded spherulites at Auca Mahuevo could be similar to the biogenic precipitation described by Braissant et al. (2003) in their laboratory experiment. Furthermore, calcium carbonate produced by abiotic precipitation processes shows different morphology than that produced in bacteria cultural experiments. In the abiotically mediated experiment, the crystal morphology produced is a dendritic shape (Figure 5.1) and euhedral rhombohedra shape (Braissant et al., 2003). Both of these abiotic shapes of calcite crystal were observed inside of the eggs at Auca Mahuevo. The result of this experiment suggests that spherulitic calcite and/or vaterite crystal may indicate the presence of mucilaginous bacteria or biofilms at the time of precipitation of calcium carbonate. Moreover, the possible role of other microorganisms in the formation of calcium carbonate, such as cyanobacteria or fungi, is not excluded. Indeed, Verrecchia et al. (1995) reported that carbonate spherulite formation could also

be linked to cyanobacterial activity and photosynthetic uptake of $HCO_3^-$ leading to $CaCO_3$ precipitation, the bicarbonate causing release of $OH^-$ in the mucilaginous sheath.

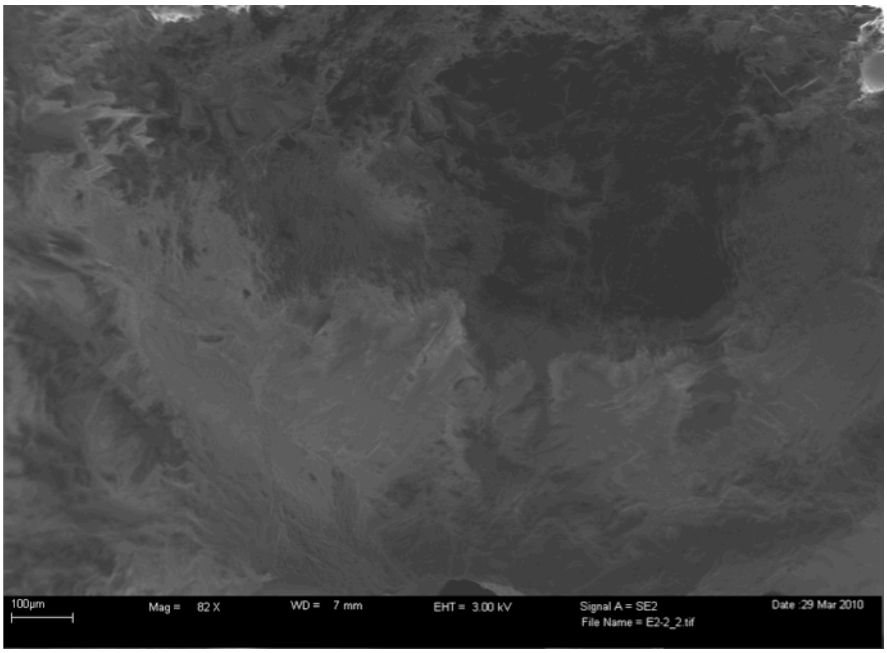



Figure 5.1. FEM images of calcite crystals in the Anacleto Formation. Dendrite-like calcite crystal shapes suggest an abiotic origin.

*Microcodium*

The origin of *Microcodium* has long been debated. The term *Microcodium* was first introduced by Glück (1912), and a root origin of *Microcodium* was proposed by Klappa (1978) as a calcification product of the myccorhizal (fungal and plant root) association.

Freytet and Plaziat (1982) argued that multilayer cell arrangement of modern calcified roots differ from the *Microcodium* aggregates that are composed of a single

layer. Kabanov et al. (2008) suggests *Microcodium* is a kind of structure produced by actinobacterial or fungal substrate mycelia associated with other bacteria that are capable of consuming acidic metabolites. Bodergat (1974) proposed two microbial organisms possibly involved in *Microcodium* formation: 1) saprotrophic fungi, which serves as a corroding microorganism on carbonate substrates, producing oxalic acid ($C_2O_4H_2$) and then reprecipitating $Ca^{2+}$ from the carbonate substrate into calcium oxalate, with breakdown of oxalate and acidic acid, together with a supply of $Ca^{2+}$ from the microenvironment, inducing calcium carbonate precipitation, and 2) actinobacteria, which constructs the *Microcodium* aggregate.

Morphologic and carbon istope ($\delta^{13}C$) studies by Kabanov (2008) suggest that *Microcodium* are not formed by plants, algae, roots, or root-associated mycorrhiza. Rather, the thin curved radiating monocrystalline prisms with occasionally hyphae-like morphology and thin hyphae-like canals suggest that these hyphae belong to actinobacteria. However, fungal hyphae may also be involved in the formation of *Microcodium* structure.

Ooids

Ooids as a component of the rock record are commonly found in broad range of depositional environments from marine to non-marine settings. Ooids are spherical grains that consist of a detrital or fossil nucleus and cortex, and are smaller than 2 mm. The internal structure of the cortex may be tangential, or radial, or a combination of both. In this study of Auca Mahuevo eggs, ooids have been observed having tangential cortex with a radial nucleus.

There has long been controversy as to whether ooids are formed by inorganic chemical precipitation (Scoffin, 1987) or are microbial in origin (Folk and Lynch, 2001). Recent investigation by Plee et al. (2007) attempted to demonstrate the mechanism of ooid formation by conducting both laboratory experiments and field observations. They did *in situ* experiment in modern freshwater Lake Geneva, Switzerland to bridge the gap between field observations and laboratory experiment. Their microscopic observations of freshly retrieved samples show a patchy pattern development of biofilm containing extracellular polymeric substance (EPS) and numerous microorganisms, with low-Mg calcite as the dominant carbonate polymorph. Carbonate aggregates produced during *in situ* experiments display microbial imprints, either as cyanobacterial filaments within biofilm and/or the imprint of former filaments. Their field observation data indicate that ooids are formed during quiet periods, with biological factors controlling the ooid cortex formation. Further laboratory experiments demonstrated that low-Mg calcite only precipitated in the presence of biofilms.

## Precipitation of Calcium Carbonate

Modern eggshells are composed primarily of calcium carbonate and other organic and inorganic compounds. Bacteria under anaerobic conditions could transform these organic compounds into energy sources and increase alkalinity of the environments leading to the precipitation of calcium carbonate. Microbes are commonly present in sediments if there is source of energy (Nealson, 1997). Zammarreño et al. (2009) examined the ability of bacteria to produce calcium carbonate in limestone monuments

and concrete, confirming that carbonate crystals in their samples were produced by bacteria through biocalcification processes. This biocalcification process includes entrapping of extracellular polymeric substances (EPS) produced by bacteria, then using the EPS as a nucleation site for carbonate precipitation. From this laboratory experiment with freshwater, it has been found that bacterial species, particularly *Myxococcus xanthus* precipitated calcite and vaterite as the main $CaCO_3$ polymorph with a spheroidal shape. *Myxococcus xanthus* metabolic activity produces $NH_3$ and $CO_2$ that can increase alkalinity and $CO_3^{2-}$ concentration in the environment (Zammarreño et al., 2009). If $Ca^{2+}$ ions are available from the environment, precipitation of calcium carbonate can occur. *Myxococcus xanthus* is a group of soil bacteria that have the capability to produce minerals with a large variety of compositions and morphology. Intense bacterial metabolic activity leads to supersaturation of $CaCO_3$ that increases calcification of bacterial cells. Amorphous or hydrated calcium carbonate will form at the initial stage, and subsequently be transformed into stable calcite There are four distinctive calcite morphology formed in the presence of bacteria that could be observed from their experiment: 1) hemispherical aggregates with diameters of 50 to 100 μm, which are composed of worm-like calcite crystals, 2) calcite spherulites with a diameter of 30 μm, 3) disphenoid and scarce dipyramid-like calcite crystals with micron-size rhombohedra (up to 40 μm in size), and 4) scarce calcite rhombohedra. Abiotic calcite crystals in this experiment display a distinctive dipyramid-like morphology, and the presence of micron-size triangular-shaped aggregates. The EPS play an important role in the formation of

calcium carbonate by providing nucleation sites and attaching small crystals to one another (Chekroun et al., 2004).

Moreover, the nature of the organic matrix of organisms also determines which ion is preferentially adsorbed and which mineral phase is formed. Dolomite will be formed if the bacteria preferentially adsorbed $Mg^{2+}$, whereas calcite will be precipitated if $Ca^{2+}$ is preferentially adsorbed (Van Lith et al., 2003). The biocalcifying bacteria tend to occupy pore spaces. Biocalcification, which is carbonate precipitation by microorganisms, is a complex process that involves metabolic pathways, ion exchange, nitrogen and sulfur cycles, and photosynthesis (Castanier et al., 1999). There are four factors that control precipitation of calcium carbonate: 1) the $Ca^{2+}$ ion concentration, 2) the concentration of bicarbonate ion, 3) pH, and 4) the availability of nucleation sites (Castanier et al., 1999).

## Microbial Mineral Precipitation

Exceptional preservation of soft tissue requires very rapid mineralization before significant burial, anoxia, and low energy conditions (Allison, 1988b). The eggshell is an important source of calcium that allows local supersaturation of $Ca^{2+}$, with the availability of $CO_3^{2-}$, calcium carbonate ($CaCO_3$) can be rapidly precipitated. When the water level rose and drowned the eggs, microbes likely invaded the surface of the eggs and initiated biomineralization reactions. Microorganisms within biofilms are capable of controlling the microenvironment at surface interfaces, thus producing minerals and mineral replacement reactions that are not predicted by thermodynamic arguments (Little

at al., 1997). After the embryos were dead, microbial biofilms assembled rapidly and formed pseudomorphs. Biofilm formation starts with adsorption of macromolecules, such as protein, polysaccharides, and humic acids, and smaller molecules, such as fatty acids and lipids, at interfaces (Little et al., 1997). The molecule adsorption promotes conditioning films that change the physico-chemical properties of the interface. Microbial colonization is initiated with transport of microorganisms to the interface by three mechanisms: 1) diffusive transport, 2) convective transport due to fluid flow, and 3) active movement of bacteria near the interface. Convective transport is the most common transport mechanism (van Loosdrecht et al. 1990). Microbial cells that were transported later by flowing water likely accumulated and interacted with the film. Microbes start to produce extracellular polymeric substances (EPS) immediately after attachment. EPS that facilitates diagenetic mineralization possibly form as a result of the liberation of adsorbed cations during degradation (Little et al., 1997). Initial colonization by microbes promotes further biofilm formation, as biofilm accumulation is an autocatalytic process (Little et al., 1997). The formation of biofilm on the surface of egg contents might provide a protection from shear forces and act to help preserve soft tissues and embryonic remains inside the eggs.

Mineral precipitation requires an oversaturated solution in either biogenic or abiotic mineral precipitation. Nucleation as an initial stage of mineral precipitation takes place in two ways: 1) homogenous nucleation, in which nuclei form as a result of random collisions between ions, and 2) heterogenous nucleation, in which nuclei develop on the surface of a solid that enhances nucleation (Stumm, 1992). Bacteria play a role in mineral

precipitation directly as a catalyst of chemical reactions and indirectly as reactive solids (Stumm, 1992). Bacteria act as a catalyst because bacterial metabolic activity can cause changes in solution chemistry leading to local oversaturation. Indirect mineral precipitation occurs as a result of changing geochemical conditions such as $CO_2$ degassing associated with growth of mineral on the outer surface of bacterial cells (Fortin et al., 1997). In natural settings, direct and indirect mineral precipitation processes may occur at the same time, with the resulting products difficult to distinguish.

As mentioned above, calcium carbonate precipitation may occur naturally as a result of either an abiotic chemical precipitation or biogenic products. Abiotic chemical precipitation from saturated solutions might be produced by evaporation, reduction in $CO_2$ pressure, and temperature increase. Biogenic products include minerals produced by animals and plants, precipitated by photosynthesis, fungal carbonates (Callot et al., 1985; Verrecchia and Loisy, 1997) and bacterial carbonate (Castanier et al., 1999).

In addition to precipitating in natural settings (continental and marine) (e.g. Chafetz and Folk, 1984; Chafetz, 1986; Vasconcelos and McKenzie, 1997), calcium carbonate is often precipitated in laboratory experiments using various bacteria (e.g., Krumbein, 1973; Buczynski and Chafetz, 1991; Vasconcelos et al., 1995). Bacteria mediate calcium carbonate precipitation by changing their local environment through different chemical pathways, including autotrophic and heterotrophic pathways (Castanier et al., 1999).

Autotrophy incorporates three metabolic pathways (Figure 5.2): non-methylotrophic methanogenesis (Marty, 1983), anoxygenic photosynthesis and oxygenic photosynthesis. All of these pathways use $CO_2$ as a carbon source; thus, if there are $Ca^{2+}$

ions present in the medium, calcium carbonate will be precipitated.

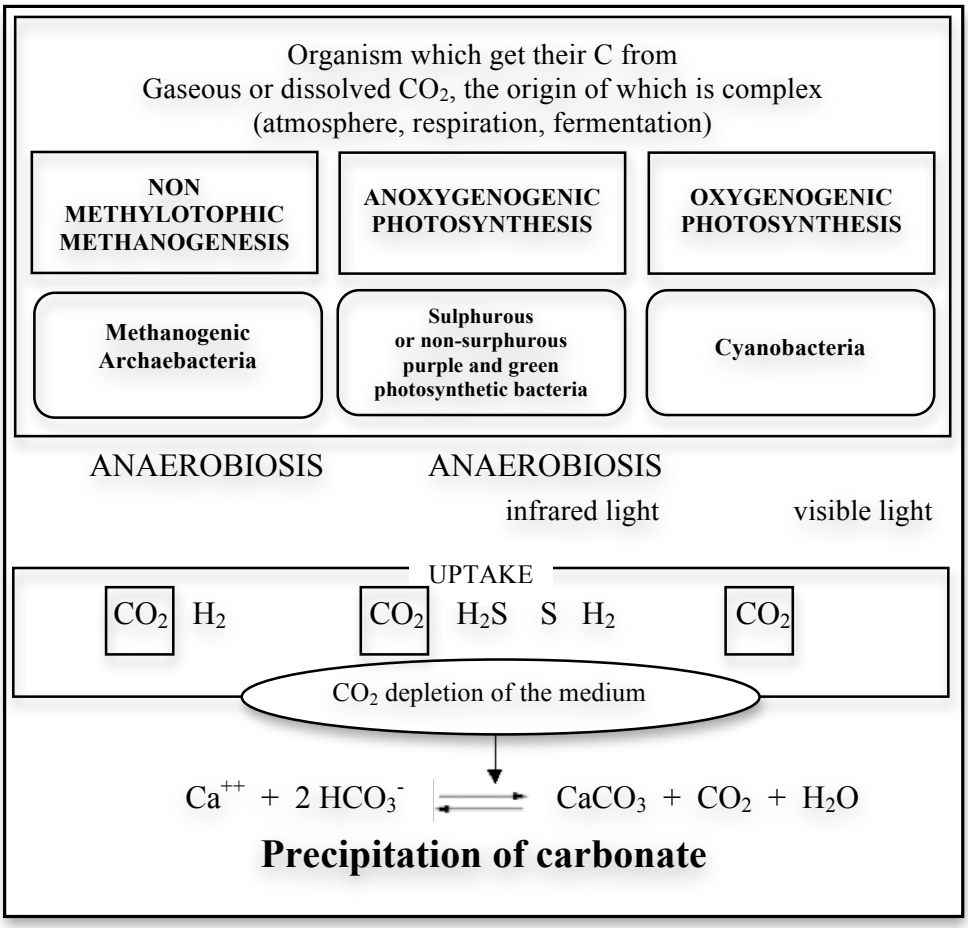



Figure 5.2. Autotrophic pathways of carbonate bacteria (From Castanier et al., 1999).

Heterotrophic pathways include two bacterial processes: passive and active precipitation. In passive precipitation, bacteria produce carbonate and bicarbonate ions that lead to calcium carbonate precipitation. When $Ca^{2+}$ ions are present, calcium carbonate precipitation will take place. However, if there are low amounts of $Ca^{2+}$ in the medium, carbonate and bicarbonate ions will accumulate. If the pH increases, zeolite might be formed by bacterial activity, as for example in Soda Lakes, Kenya (Castanier et al., 1993).

In active precipitation, the carbonate particles are produced by ionic exchange as the heterotrophic bacterial enrich in organic matter. Thus, carbonate and hydrogenocarbonate will be accumulated in the medium, followed by pH increase that is conducive for carbonate precipitation. Generally, active precipitation occurs first, and is followed by passive precipitation.

Zilberbrand (2003) also documented that carbonate precipitation is common as a result of weathering and biological activity. He reported that calcite precipitation occurs based on the following reaction:

$$Ca^{2+} + 2HCO_3^- \leftrightarrow CaCO_3\downarrow + H_2O + CO_2(aq)$$

$$\leftrightarrow CaCO_3\downarrow + H_2O + CO_2$$

$CO_2$ consumption may be associated with cyanobacteria activity (Krumbein and Giele, 1979), and water loss may occur as a product of evapotranspiration and water vapor movement. Additionally, pore-water degassing and subsequent removal of $CO_2$ also play an important role in carbonate precipitation. Gas diffusion possibly triggered by fractures and animal burrows may cause such removal.

Rapid mineralization of soft tissues by calcium carbonate and its association with bacterial activity have been demonstrated in the laboratory by Martin et al. (2003). In this experiment, conditions became anaerobic within 1 day and precipitation of calcium carbonate occurred within 15 days. The mineralized funiculus (attachment stalk) enclosed the surface of lobster eggs, preserving their shape. This mineralized coating is made of amorphous calcium carbonate and some bacterial filaments and spheres. This experiment confirms the preservation style under laboratory conditions, produced features similar to

those observed in the Auca Mahuevo fossil sauropod eggs, permitting an interpretation involving bacterially mediated mineralization of calcium carbonate at the Auca Mahuevo locality.

Fossilization processes involving the eggs may occur in three steps as suggested in the laboratory experiment by Raff et al. (2008). First, because self-destruction or autolysis takes place under aerobic conditions by lytic enzymes, preservation of soft tissue requires anaerobic conditions that will block autolysis on death of the embryo. The key factor in preservation is the bacterial activity that acts as an essential mediator of taphonomic processes other than autolysis. Second, colonization of bacteria then consumes the embryo. In this step a biofilm is formed during bacterial colonization, maintaining the embryo form by making a pseudomorph. Third, bacteria change the embryonic chemistry and promote biomineralization. Raff et al. (2008) demonstrated that embryonic structure provides a template for bacterial biofilms to form. The biofilms replace, replicate, and stabilize the morphology of the consumed embryo in detail. The bacterial pseudomorph retains the embryonic soft tissue structure. Bacterial biofilms may form under aerobic and anerobic conditions, but they will have different bacterial constituents. Raff et al. (2008) found that long filamentous bacteria only formed under anaerobic conditions. The result of their experiment suggests that fossils may reflect their original structure, but in fact they may be fossilized bacterial pseudomorphs of that original structure. This experiment provides direct evidence of microbial biofilms and identification of bacteria involved in decay, pseudomorphing biological structure, and biomineralization that can lead to exceptional preservation.

In the Auca Mahuevo samples, some filaments inside of the eggs are similar morphologically to those produced in the experiment by Raff et al. (2008). These long filaments possibly formed when eggs drowned under anaerobic condition with an inoculum of anaerobic mud. However, in the study of Auca Mahuevo eggs, it is not clear whether the 35 μm long filaments and 3 μm wide and threads belong to microorganisms or represent organic matrices from the eggs, or both.

Taking into account the laboratory experiments discussed above, similar processes could be applicable to the preservation of embryonic skin and membrane inside the Auca Mahuevo eggs. These processes may have included the initial stage of colonization by microbes (e.g. fungi and bacteria) when the eggs drowned by flooding under anaerobic conditions. These microbes would consume the embryo and soft tissue, then forming a biofilm that maintained the form of the embryo and soft tissue by creating a pseudomorph of the biological structure. Finally, a microbially-mediated change in the chemistry of the eggs would promote biomineralization, leading to preservation of soft tissue and embryonic remains.

Evidence of soft tissue preservation, such as embryonic skin and membrane, indicate rapid replication by calcium carbonate mineralization. These rapid carbonate mineralization processes were likely driven by microbial activity along steep geochemical gradients (Briggs, 2003). These gradients are produced by diffusion of specific ions into the eggs from decaying soft parts of eggs and embryonic remains inside of the eggs.

Furthermore, calcium carbonate formed by abiotic or biogenic processes is very difficult to differentiate based only on the fossil record. Warren et al (2001) distinguished abiotic versus microbially-mediated processes in the laboratory by setting up an experimental system under three conditions: abiotic control (media and contaminant), bacterial control (bacteria and media), and bacterial treatment (bacteria, media, and contaminant). They found that pH values were significantly higher in the bacterial treatment compared to abiotic controls. The final pH of the abiotic system is about 7.9, whereas the bacterial control has the maximum value 9.2. In the abiotic system, although the conditions were supersaturated with $Ca^{2+}$, no carbonate precipitation occured. These results suggest that the presence of bacteria accelerates the precipitation processes. The only observed calcium carbonate morphologies in their experiment resulting from bacterial control include dumbbell shapes, rods, calcified filaments, and globular shapes. Their study suggests that very rapid calcium carbonate precipitation may only occur in the presence of microbes as a catalyst for the precipitation process.

# CHAPTER 6

# CONCLUSION

Microbial activity may play an important role in the precipitation of calcium carbonate in sauropod eggs at Auca Mahuevo in which low-Mg calcite is the dominant calcium carbonate polymorph. Microbes could be responsible for the formation of the carbonate microfabric found in the eggs, including filaments, spherulites, *Microcodium*, peloids and ooids.

Although calcium carbonate precipitation by microbial-mediated processes is not clearly elucidated, the similarities of carbonate morphologies associated with microbial activity in both the Auca Mahuevo fossils and laboratory experiments provides an argument for the permissable role of microbes in the precipitation process. However, microbes may not have fully controlled the precipitation processes, as indicated by the presence of such abiotic morphology as dendritic calcite. Microbial carbonate precipitation could not have proceeded if the saturation state of ambient water did not favor $CaCO_3$ precipitation. During microbial carbonate precipitation, the microbial activity provides a stimulus for $CaCO_3$ precipitation through metabolic processes that raise the alkalinity of microenvironments, while environmental conditions influence the oversaturation level (Riding, 1997). Consequently, the preservation of eggs, embryonic skin, and the microbe-related morphologies observed in this study likely resulted from some combination of microbial and abiotic factors, attaesting to the possibility of both

microbial and abiotic influences on precipitation of the calcium carbonate that preserved eggs contents at the Auca Mahuevo locality.

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