

Note

## Occurrence of mineralization disturbances in nacreous layers of cultivated pearls produced by *Pinctada margaritifera* var. *cumingi* from French Polynesia. Comparison with reported shell alterations

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*Déficiences de la biominéralisation nacréée dans les perles produites par Pinctada margaritifera var. cumingi en Polynésie française. Comparaison avec les données relatives aux altérations des coquilles.*

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### INTRODUCTION

Considerably increased during the last decade, production of "black pearls" is now the most important source of income in French Polynesian Territory. This industry is based on cultivation and grafting of the "black lip" pearl oysters, *Pinctada margaritifera* var. *cumingi*, which is used in the entire area: from Gambiers archipelago, in south-eastern part of Polynesia, to Society Islands (îles Sous-le-Vent), in the western part, the most important production centres being concentrated in Tuamotu.

In Polynesia, the first pearl-grafting experiments were carried out during 1962 (Rambaud, 1990), and since recognition of the purely natural character of various colours that occur in the resulting pearls (these colours were originally thought to be artificial), the Polynesian products have acquired a remarkable position on the international pearl market. In addition to the global quality of the officially commercialized products (shapes, sizes, thickness of nacreous layer due to a long cultivation time), interest for the rare and very attractive nuances that can occur allows some specimens to reach the first rank among high priced jewellery products. The number of pearl-farming settlements therefore rapidly increased, mostly

based on the activity of sometimes only one family. A few of them, however, have reached industrial size and organization.

First occurrence of mass mortality generally associated with perturbation of shell formation in cultivated oysters were reported about a decade ago, in parallel with similar observations made in Australia (Dybdahl and Pass, 1985). Facing the continuous spreading of this pathology and its increasing intensity, coordinated research projects were organized. During this research programme, observations were carried out on microstructure and biochemical composition of disturbed parts of the *P. margaritifera* shells (Dauphin and Denis, 1987; Dauphin and Cuif, 1990; Marin and Dauphin, 1992).

### METHODS

Microstructural characteristics of normal and pathological shell structures have been studied on radial section and internal growth surfaces of the shells, using a Philips 505 SEM, water cleaned (eventually moderately sonicated) without any chemical etching. Air-dried specimens were mounted on aluminium stubs with a carbon-containing adhesive.

The preparation were then coated with gold-platinum in a diode sputtering device cooled by the Peltier effect.

The soluble organic matrices have been isolated from prisms and nacreous layers by the following preparative process.

- Thoroughly cleaned pieces of selected shell layers were sliced, crushed and finely powdered in a ball-crushing mortar (Reesch).

- Resulting powder was decalcified with acetic acid (pH 4). Soluble and insoluble organic compounds were separated by low-speed centrifugation. Soluble organic matter was desalted by low pressure chromatography (Sephadex G 25) and desalted again (FDC G25 Sephadex Sperfine) after concentration.

- Amino acid quantitation was performed on High-performance liquid chromatography (HPLC) reverse-phase system (Beckman) using PITC precolumn derivatization with methanol gradient.

## RESULTS

### Setting the reference: microstructural and biochemical features of nacreous layer formation in *P. margaritifera* in healthy and diseased conditions

As members of the Pteriomorphid Pelecypods, *Pinctada* produces a two-layered shell (plate 1: 1). The external layer is built of mosaic prismatic calcitic units which are perpendicular to the shell surface (plate 1: 2). Each prism is sheathed with a thick organic envelope (plate 1: 3).

Formation of the "aragonitic line" (plate 1: 1 and 4) is a consequence of the progressive extension of the internal part of the mantle epithelium, which stops the longitudinal growth of calcitic prisms. A new set of biomineralizing conditions arises, resulting in a complete change in mineralogy, shape, size and growth modalities of biocrystals. But to understand the structural and chemical features of the diseased parts of the shells, it is important to note that, despite the sharp-edged aspect of the separation between calcitic and aragonitic domains, the settlement of true nacreous layer is very progressive.

Firstly, the internal surface of the outer shell layer (calcitic prisms) is covered by a continuous organic sheet: thus prismatic shell layer and internal sector of the shell (aragonitic domain) are completely separated: there is no direct contact between the two different

mineralogical components of the shell (plate 1: 4, fig. 1). In the first step of aragonitic production, aragonite appears with a fibrous habitus, fibre bundles radiating from nucleation points on the organic layer that isolate the aragonitic compartment from the previously produced calcite (plate 1: 5).

But looking closely at these aragonitic fibre bundles, progressive occurrence of a new microstructural component can be observed (plate 1: 6), demonstrating that mantle metabolism is turning to production of true nacreous biocrystals. The initially continuous aragonitic fibres become separated in distinct growth steps by organic sheets that are parallel to the general growth surfaces (perpendicular to fibre growth direction). In the first step of production, these organic sheets are very limited in extension (plate 1: 6), but rapidly they become produced by the whole surface of the aragonitic zone. With these repeatedly produced (every 0.5 to 0.8  $\mu\text{m}$ ), perfectly synchronous and parallel organic layers (in fact rather complex in ultrastructure – see TEM pictures by Bevelander and Nakahara, 1969, 1980; Nakahara, 1981, 1991), the production of the typical nacreous structure is achieved (plate 1: 7).

Observed on the internal surface of the shells, each level of the nacreous layer is initiated by small units which appear as parallel lines of nucleating points and has become contiguous through rapid lateral growth (plate 1: 8).

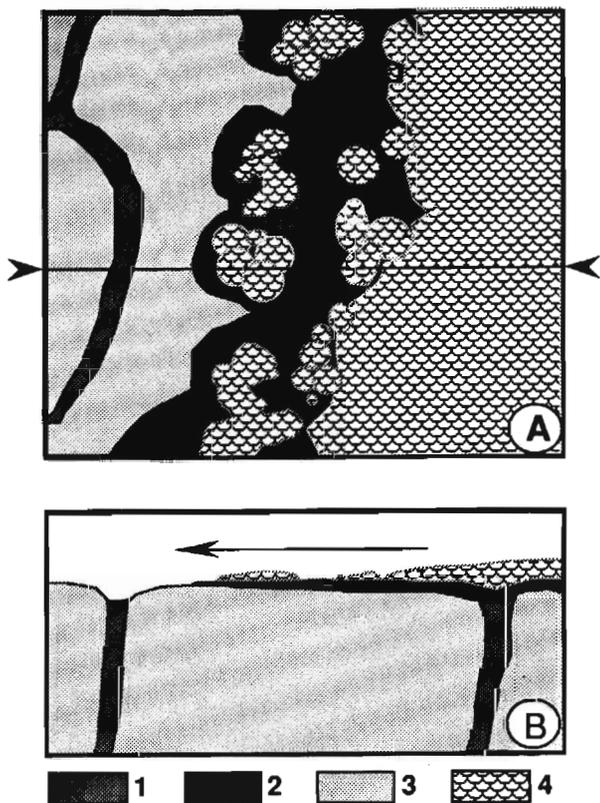
Production of mineral aragonite that occurs on the prism covering organic sheet is correlated with important changes in biochemical composition of mineralizing macromolecular compounds that are secreted by the mantle external epithelium. Soluble matrices demonstrate a high concentration of acidic amino acids (aspartic and glutamic acids) (table 1), in agreement with the models for calcareous biomineralization process (Mitterer, 1989; Weiner and Hood, 1975). Organic matrices of aragonitic nacreous layer differ from those of calcitic prisms by a less acidic isoelectric point.

### Crystal features and biochemical characteristics in nacreous layers of diseased shells

In diseased *Pinctada* shells, the disturbed parts are characterized by a deep brownish colour (plate 2: 1, 2 and 3). SEM examination of nacreous internal surfaces in disturbed shells reveals a number of nacreous units which exhibit a series of morphological changes (Dauphin and Denis, 1987). Crystals firstly lose the mineralization of their inner parts (plate 1: 9, 10

**Table 1.** – Amino acid compositions of soluble matrices in prismatic and nacreous layers of healthy shell (mol %). Acid-basic ratio: AC/BAS, calculated isoelectric points: pI.

	Asp	Glu	Thr	Ser	Pro	Gly	Ala	Val	Leu + Ile	Tyr	Phe	Lys	His	Arg	AC/BAS	pI
Prism	45.3	2.6	8.9	2	16.3	9	6.5	1.1	9	2.2	0.4	0.2	3.5	1	10.2	3.35
Nacre	17.6	4.2	0	3.8	2.2	38.2	17.5	2.6	3.5	2.6	1.6	1.6	2	7	2.1	4.95



**Figure 1.** – Diagrammatic sketch of plate 1: 4, showing separation of prismatic calcitic layer and internal aragonitic nacreous layer by a continuous organic sheet. A: view of the internal surface of the shell around the “aragonitic line”; line between arrows: plan of figure 1 B. B: transverse section (radial); arrow: progress of nacreous layer, covering the upper surface of calcitic prisms; (1) external organic envelope of prisms; (2) organic sheet covering prisms; (3) calcitic prisms; (4) aragonite.

and 11), the increasing disturbance leading to complete loss of morphological control (*plate 1: 12*). At the ultimate degree of alteration in biomineralization process, nacreous layers appear as an undifferentiated accumulation of organic compounds in which any indication of microstructural organisation is lost (*plate 1: 13*).

To study progress of disturbance during shell growth, corings (12 mm in diameter) were made in the more or less diseased parts of the shell (*plate 1: 14*), and using a wire-saw, the resulting cores were subdivided into thin sections parallel to the mantle growth surface (Dauphin and Cuif, 1990; Marin and Dauphin, 1992). This technique allowed us to compare healthy and disturbed nacreous layers that were produced in the same place, enabling us to assess the metabolic changes in mantle secretion.

In all cases, development of the perturbation in the biomineralization process is clearly related to important changes in amino acid proportions (*fig. 2*):

- production of acidic amino acids (Asp, Glu) and Phe, Lys, His is decreasing;

- glycine and alanine are highly variable, and generally in higher proportion than in healthy shells (Marin and Dauphin, 1991).

**Mineralization disturbances in nacreous layers of pearls: similarity with diseased nacreous layers of shells**

Recent observations carried out on pearl nacreous layers show that biomineralization disturbances can also occur during growth of nacreous layers of pearls. Two different degrees of disturbance, but very similar in symptoms, can be observed in this occurrence of non-mineralized layers in pearls.

*Occurrence of locally non-mineralized surfaces*

This type of disturbance is presented in *plate 2: 3* and *4*. Two characteristics are visible in these pictures: In the brown non-mineralized areas, rhythmicity in the secretion process is conserved. However, the thickness of successive layers is increased (possibly due to the absence of crystallization process), resulting in a protruding surface of the disturbed zones.

In fact, the brown non-mineralized zone appears as the centre of a larger surface in which the epithelial secretion is perturbed. We can observe in *plate 2: 3* that production of the coloured pigment (deep green) in the nacreous layers is interrupted before the major symptoms of disease become visible (brownish colour), and still persists after formation of the last brown layer.

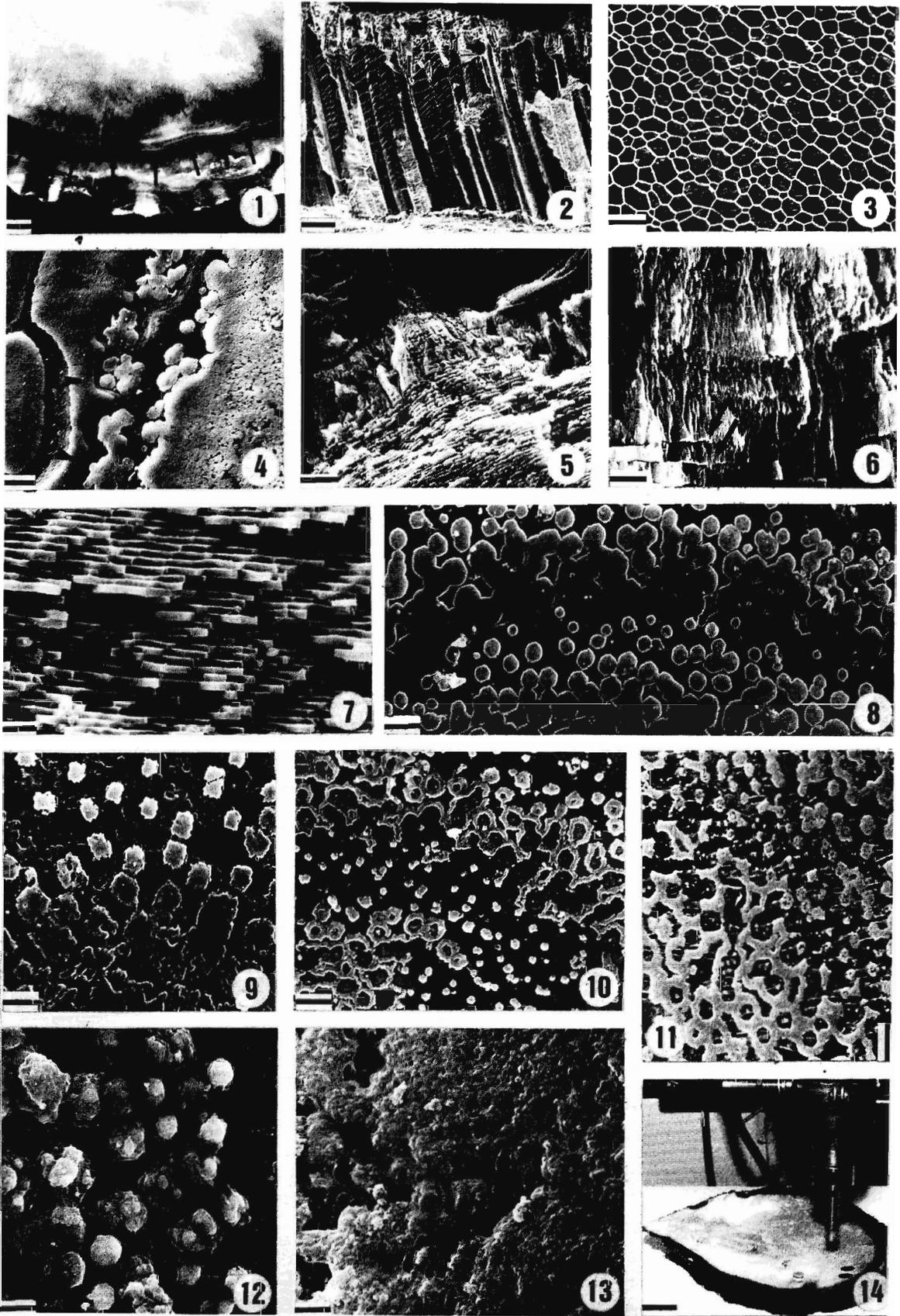
Thus, cessation of pigment production appears as the first symptom of a metabolic disturbance in the pearl-bag. However, even when mantle secretions are unable to develop the normal crystallization process to create nacreous units, the rhythmicity of the mantle secretion is not altered.

*Production of purely organic layers by large surfaces of the grafted epithelium, and sometimes on the whole pearl surface*

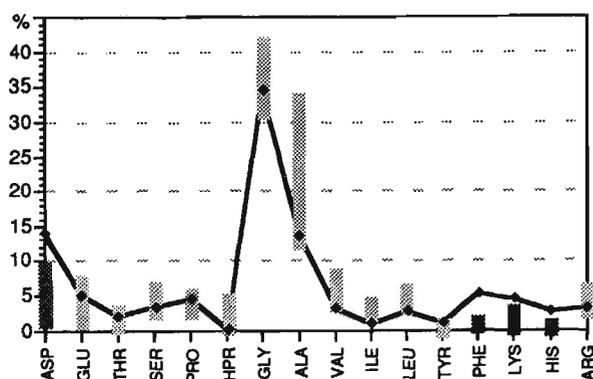
Very similar observations can be made on a number of pearls, most belonging to the category “baroque pearls”. These pearls are typically characterized by two strongly correlated features, resulting in low commercial values:

- they are not “round-shaped pearls” (a prominent quality in jewellery), but show an axis of symmetry;
- they show a circular growth zonation, with frequent changes in colour between successive zones.

It is of major interest to note that “baroque pearls” always exhibit extensive perturbations in mineralization processes. Most of these disturbances occur during the initial phase of the pearl-bag development, when production of nacreous crystals has to re-start after the extreme biologic stress caused by the grafting operation. Frequently, recovery of the ability to produce nacreous crystals is very slow and,



◀ **Plate 1.** – (1) Internal view of a “black-lip” shell valve. Arrows: “aragonitic line”, Bar: 1 cm. (2) SEM picture of the external prismatic layer, observed on a simple shell fracture surface, radial in direction, Bar: 400  $\mu\text{m}$ . (3) Growing surface of the prismatic layer. Prisms are surrounded by strong organic sheaths, Bar: 200  $\mu\text{m}$ . (4) Close view of the “aragonitic line”. On the left of the picture, the last growing surfaces of the prismatic layer. On the right, start of the aragonitic (but not yet nacreous) secretion. In the central part, note the continuous organic layer that covers perfectly the calcitic surfaces of prisms before deposition of first aragonitic spots, Bar: 10  $\mu\text{m}$ . (5) On a radial shell fracture surface this picture shows the contact between external prismatic layer (above) and the internal aragonitic layer. The replacement of radial fibrous aragonite (upper part) by true nacreous structure is clearly visible, Bar: 8  $\mu\text{m}$ . (6) Among fibrous aragonite, arrows indicate the first occurrence of parallel organic layers by which the nacreous secretion mechanism will be achieved, Bar: 1.5  $\mu\text{m}$ . (7) The classical view of nacreous layer: aragonitic biocrystals are separated by parallel organic sheets, Bar: 2  $\mu\text{m}$ . (8) Two successive nacreous laminae. The nucleating steps of crystals are produced on a linear basis (turning to strongly convoluted, or spiral in the most internal parts of the shells). By a rapid lateral growth, crystals initially separated become perfectly contiguous, Bar: 5  $\mu\text{m}$ . (9 to 13) Various examples of mineralization disturbance in nacreous layers: from loss of morphologic control (9) to complete absence of mineralization (13) - compare to (8), Bar: 5  $\mu\text{m}$ . (14) Diamond coring tool. The cylindrical resulting shell pieces are then subdivided into parallel thin sections, allowing study of biochemical changes in mantle secretion through time, Bar: 2 cm.



**Figure 2.** – Amino acid composition of soluble matrices of the nacreous layer in healthy (black line) and diseased shells. Dotted rectangle: range of amino-acid contents in analyzed samples (9 specimens).

during this time, the grafted nucleus is surrounded by purely organic layers (plate 2: 6, 8 and 9).

When recovery of biomineralization occurs, it can only be in restricted zones (not on the whole nucleus surface), and from these sectors biomineralization spreads in concentric zones (plate 2: 7 and 8), possibly resulting in the circular growth zones that are visible on the pearl’s external surfaces. A detailed view of a completely non-mineralized layer in pearl (plate 2: 7) shows that the successive organic sheets that normally separate the successive nacreous layers are produced continuously.

**CONCLUSION**

Perturbations of mineralization process in shells and pearls lead to very similar symptoms: the secretory epithelium (outer layer of the mantle or pearl bag) continues to produce the laminated organic structures that are a basic component of nacreous tissue. However, the aspect of nacreous crystals in shells (plate 1: 9 to 13) or nacreous layers in pearls (plate 2:

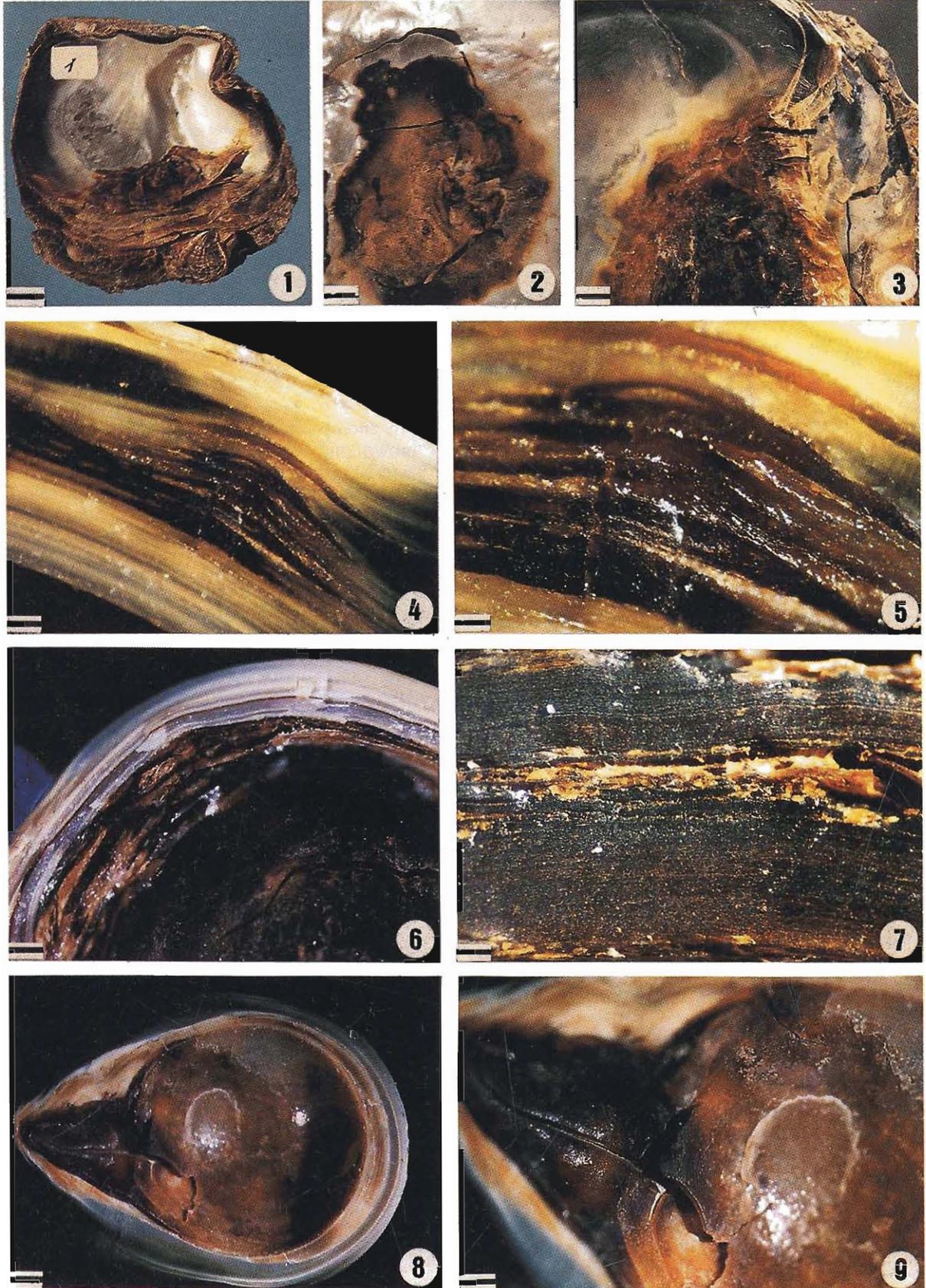
4 to 7) indicates that associated soluble matrices are no longer able fully to conduct the biomineralization process.

In another case of shell disease, the well studied “brown ring disease” of *Ruditapes philippinarum*, a bacterial origin of mineralization disturbance has been demonstrated by various authors (Paillard, 1992). Loss or perturbation of the mantle edge functions are related to the presence of this pathogenic agent.

Paillard (pers. comm.) pointed out that melanin pigments have a well known bactericide action. In altered layers or pearls we noted that loss of pigmentation appears as the first step in development of the biomineralization disturbance. Conversely, new production of the typical pigmentation in pearl nacreous layers after a localized biomineralization disturbance is associated with the recovery of biomineralization capacity (e.g. plate 2: 4). According to this hypothesis, influence on pigment production could be the initial cause, allowing the pathogenic agent to act as a perturber of the mineralization process.

It should be of interest to confirm the biological origin of this sequence of events. Cabral (June 17th 1994, oral communication during the Papeete “Journées de la Perle”) presented convincing data concerning the spread of biomineralization disease through Tuamotu archipelago, emphasizing its similarity with typical epizootic contamination. However, no precise microbial agent has so far been evidenced, despite similarity of symptoms.

Definite statement of the involvement of a pathogenic micro-organism in the biomineralization disturbances that occur in shells and pearls of French Polynesia should be of major importance for the pearl industry in Polynesia. This could lead to elaboration of an improved sequence of steps in grafting operations, obviously a critical phase in pearl production, in order to minimize the risk of contamination of the grafted epithelium.



◀ **Plate 2.** – (1 to 3) The usual aspect of mineralization disturbances in shells. This phenomenon leads to formation of purely organic sectors, deep-brown and finely laminated. Biochemical changes that occur in mantle secreted matrices result in a loss of calcification between the foliated structures that normally separate the mineralized compartments, Bar: 1.8 cm, 0.4 cm and 0.4 cm for (1), (2) and (3) respectively. (4 and 5) Examples of punctual loss in mineralization in pearl nacreous layers. Rhythmicity in pearl-bag secretion is preserved but locally, the mineralization process cannot achieve the crystal formation, Bar: 150  $\mu\text{m}$  and 60  $\mu\text{m}$  for (4) and (5) respectively. (6 and 7) Absence of mineralization on the whole pearl-bag surface. (7) shows the finely foliated aspect of the deep-brown non-mineralized layers. Symptoms are exactly similar with disturbances observed in shells, Bar: 420  $\mu\text{m}$ , and 15  $\mu\text{m}$  for (6) and (7) respectively. (8 and 9) A typical “baroque pearl” showing that eccentricity is due to asymmetrical development in mineralization in the nucleus surrounding mantle and occurrence of successive zones in recovery of the mineralizing capacities, Bar: 1.2 mm and 0.5 mm for (8) and (9) respectively.

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