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TITOLO TESI

Microbial mediation in the formation and alteration of minerals in shallow marine environments (Southern Calabria, Italy)

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Ciclo XXVII

Microbial mediation in the formation and alteration of minerals in shallow marine environments (Southern Calabria, Italy)

CoMAS

Abstract

This work wants to investigate the role that microorganisms have in the alteration and precipitation of minerals, in particular Ca-carbonates, on natural and artefact rocks of archaeological interest in shallow marine environments. This finds numerous applications in the recognizing of the abiotic vs. biotic nature of neo-formed minerals, such as in the reconstruction of depositional palaeoenvironment, in astrobiology and in medicine. In addition, nanostructured microbially induced minerals have several technical application varying from bioconservation, bioremediation, biogrouting to biomedic.

Furthermore, the knowledge of such complex interaction between bacterial communities and hard substrates is essential for the preservation and the valorization in situ of underwater cultural heritage, which represents a particularly important element in the history of peoples.

For this purpose, several key-samples of natural and artifact rocks, have been collected in Santa Maria di Ricadi bay and in the archaeological submerged park of Monasterace, respectively sited on the Tyrrhenian and Ionian coast of Southern Calabria.

Optical and scanning electron microscopy (SEM) have been used for micro- and nano-scale investigation of the role of microorganisms in the precipitation and alteration of minerals. Observations showed that biofilms are characterized by: (1) Skeletal elements, such as red algae, bryozoan, polychaete, bivalves and diatoms; (2) bacteria such as cocci, bacilli, spirochaete, filamentous bacteria, often associated with organic matter remains which result sometimes mineralized; (3) Neo-formed Ca-carbonate and pyrite minerals; (4) bioerosion elements, such as grooves and boreholes.

Neo-formed Ca-carbonate minerals occur in Monasterace biofilms as sheet deposits, triads or dumbbell- to spherical-shaped crystals; whereas in Santa Maria di Ricadi as aphanitic and peloidal deposits. All these deposits result made up by an assemblage of nanospheres, varying in diameter from 50 to 200 nm, tightly clotted together. The co-existence of degraded EPS and bacteria, strictly associated with Ca-carbonate nanospheres, implies that the organic matter and the microbial metabolism played a fundamental role in the precipitation of these minerals.

Moreover, in Monasterace biofilms, framboidal pyrite occur both in rock fracture up to 2 mm below rock surface and within cavities of encrusting red algae. The presence of framboidal pyrite and dumbbell- to spherical- shaped crystals imply they formed in anoxic environment by sulphate reducers bacteria.

As regards bioerosion products, these results formed by epilithic, endolithic and euendolithic organisms. In particular, in Ca-carbonate deposits forming part of Santa Maria di Ricadi rock samples, an intricate net of grooves is produced by cyanobacterium *M. testarum*.

All these elements suggest that biofilms are complex systems formed by different biotic processes occurred both in oxic and anoxic conditions. Epilithic and endolithic microorganisms resulted capable to modify chemical and physical micro-conditions leading to the precipitation and alteration of diverse rocky materials, in particular Calcium carbonate, in shallow marine environments.

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CHAPTER 1

STATE OF ART

1.1 Introduction

Since the early Precambrian (3.5 billion of years ago), microbial growth and metabolism have had a fundamental role in the geological and geochemical evolution of the Earth surface and atmosphere. Moreover, Bacteria of various kinds were the unique living forms on Earth during at least the first crucial 1.5 billion years. Obviously, how early microbes affected their habitat can be only supposed from knowledge about their activities in modern equivalent environments.

Microorganisms are ubiquitous settlers on every Earth solid surface and relatively shallow subsurface, and frequently their action significantly accelerates the effects of chemical and physical factors (Gaylarde et al., 2003).

The relationship between bacteria and the lithosphere can ranges from formation of minerals to microbial utilization of rocks and mineral as a nutrient source or as a habitat. Microbes may play a role in the authigenic and diagenetic formation of minerals.

The minerals may appear external to the microbial cell responsible for its genesis, on or in the cell envelope, or even within the cell. Some microbial mineral formation is active and may involve direct enzymatic intervention or metabolic production of specific chemical reactants that cause precipitates to form. Other microbial mineral formation is passive and may even be mediated by dead cells (Ehrlich, 1998).

Otherwise, alterations occur under the influence of physical, chemical and biological processes, though subaerial and subaqueous breakdown generally involves interaction between all three types (Brehm et al., 2004).

As a measure of the contribution of organic activity to rock weathering, it is estimated that 20–30% of stone weathering is the result of biological activity (Wakefield and Jones, 1998). Microbial marine communities represent key factors largely influencing minerals and global biogeochemical cycles of elements. So far, only a fraction of marine microbial organisms have been identified and it seems rather likely that numerous of unknown species survive performing chemical reactions that may either contribute precipitation and/or alteration of rocks and minerals (Ehrlich, 1998).

1.2 Microorganisms

A microorganism is, by definition, an organism that is unicellular or lives in a colony of cellular organisms.

This group encompasses Prokaryotes (Bacteria and Archea) and Eukaryotes (Fig. 1.1). Often, as well as in this study, bacteria is synonymous with the term prokaryote, not the domain.

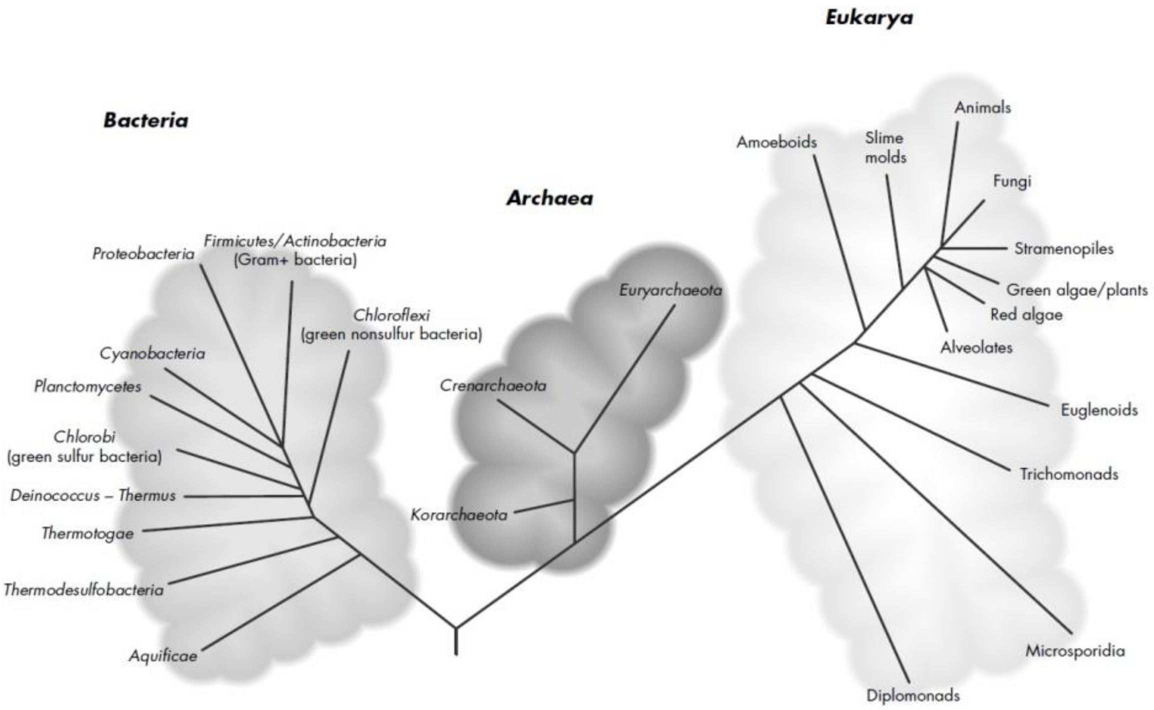

Fig. 1.1 - The “universal” phylogenetic tree based on 16S/18S rRNA sequence comparisons, showing the three domains of life: Bacteria, Archaea, and Eukarya. The Bacteria and Archaea are subdivided by representative phyla, and the Eukarya are outlined by familiar grouping for lack of general consensus among taxonomists. Note: all the branch lengths should be considered approximate (from Konhauser, 2007).

Most Bacteria and Archaea are between 500 nanometers (nm) and 2 micrometers (µm) in diameter, with a volume between 1 and 3 µm 3 and a wet weight approximating 10^{-12} grams. The only way to observe such small objects is by microscope, hence the term microorganisms or microbes. Individual cells occur basically occur as rods (known as bacilli), spheres (known as cocci), or helical shapes (known as spirilla, spirochete or vibrio) (Fig. 1.2). Individual cells are common, but they can form easily groups as clusters or filaments, when they grow end on end (Beveridge, 1988).

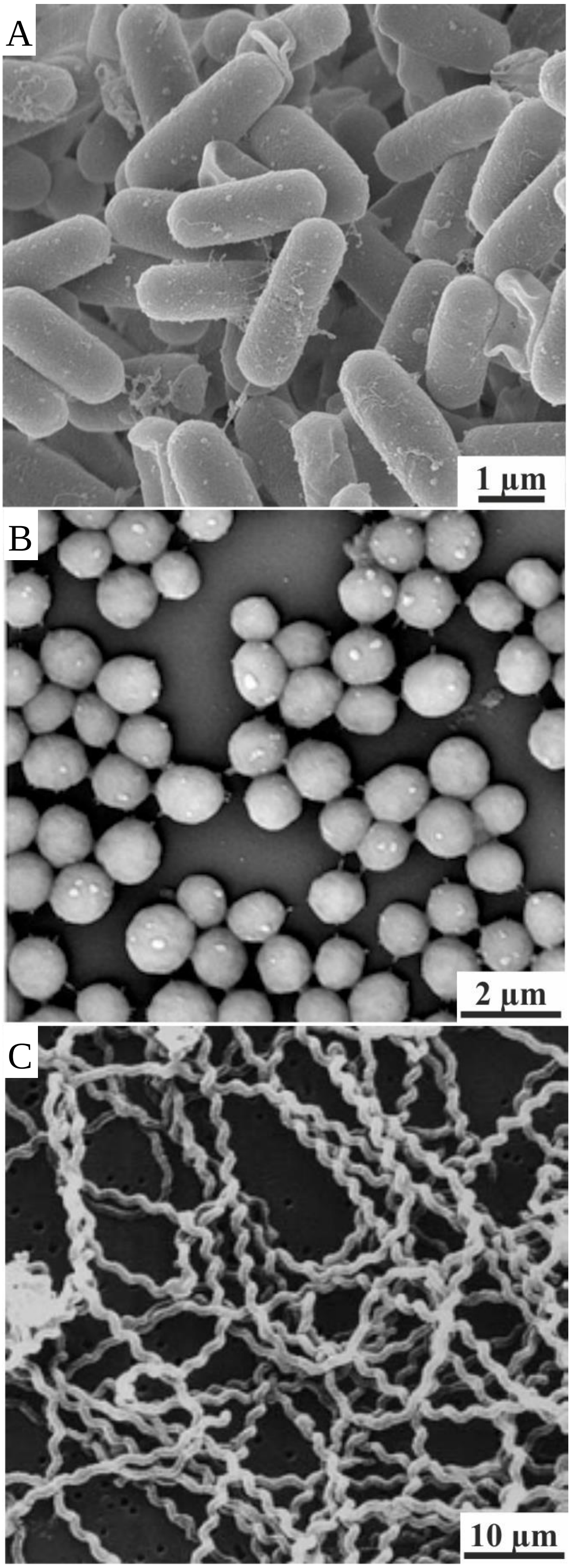

Fig. 1.2 – Basic bacterial shapes as seen under SEM. (a) *Bacillus methylotrophicus* sp., with rod shape (Madhaiyan et al., 2009). (b) *Staphylococcus aureus*, with sherical shape (Drake et al.,2013). (c) Leptospira sp. , with helical shape (Konhauser, 2007).

All the Prokaryotes are characterized by the lacking of organelles, unlike the Eukaryotes.

Eukaryotes include microorganisms, such as protists and fungi, as well as multicellular life, such as the plants, algae and animals. Algae are a diverse group of photosynthetic organisms that produce O_2 as a byproduct of their metabolism. They are typically found in fresh- and sea-water, but they can also grow on rocks and trees, or in soils when water is available to them.

Algae are broadly classified into green, brown, golden, and red that are mainly filamentous forms. Small algae are usually associated with microbial mats, where they trap and stabilize sediment (Riding, 2000). Collectively they display a wide variety of morphologies, ranging from unicellular forms to macroscopic seaweeds that can grow tens of meters in length (van den Hoek et al., 1996; Konhauser, 2007). Others can grow symbiotically with animals, such as the dinoflagellates associated with coral reefs.

In contrast, diatoms are small unicellular algal forms, but they attach by mucilaginous stalks, secrete mucilage and, when abundant, are important sediment stabilizers in marine (Awramik and Riding, 1988) and non-marine (Winsborough, 2000) environments.

Fungi also have a range of morphologies, from unicellular yeasts, to filamentous molds that clump together to form what is known as mycelia, to macroscopic forms such as mushrooms.

They get many important roles, as decomposers of organic material, leading to nutrient cycling and spoilage of natural and synthetic materials (such as wood or food); as symbionts of algae and cyanobacteria (e.g. as lichens); and as producers of economically important substances, such as ethanol, citric acid, antibiotics, and vitamins (Moore-Landecker, 1996).

With regard to the study areas, cyanobacteria play an important role, contributing notably to biofilms formation and alteration.

Cyanobacteria, also known as *Cyanophyta*, are a phylum of bacteria. In the past, they were formerly known as "blue-green algae". The basic morphology comprises unicellular, colonial and multicellular filamentous forms. The majority of them are aerobic photoautotrophs, but certain cyanobacteria show a distinct ability for heterotrophic nutrition (Fay, 1965).

Two morphological types are distinguished: filamentous species that form elongated cell chains, called trichomes, often bundled together (multitrichomous species) and coccoid species that form spheroidal cells often arranged into cell clusters (Whitton and Potts, 2000).

Their life processes require only water, carbon dioxide, inorganic substances and light. Photosynthesis is their principal mode of energy metabolism. In the natural environment, however, it is known that some species are able to survive long periods in complete darkness. Furthermore, the prominent habitats of cyanobacteria are freshwater and marine environments. They flourish in water that is salty, brackish or fresh, in cold and hot springs, and in environments where no other “microalgae” can exist. Most marine forms grow along the shore as benthic vegetation in the zone between the high and low tide marks (Humm and Wicks, 1980).

The cyanobacteria comprise a large component of marine plankton with global distribution (e.g. Gallon et al., 1996). From the geological point of view, cyanobacteria are important because they colonize the interface between sediments and water and affect fluid flow dynamics and structure formation, enveloping the mineral grains forming biofilms (Noffke et al., 2003).

In the case of lithobiontic cyanobacteria we differentiate between epilithic, chasmo-/crypto-endolithic and euendolithic cyanobacteria (Golubic et al., 1981; Fig. 1.3).

Epilithic forms live on the surface of hard substrates, while endoliths reside inside hard substrates. Endoliths include organisms that colonize existing cracks and fissures (chasmo-endoliths) or cavities in porous and cavities of substrates (cryptoendoliths), as well as those which actively penetrate carbonate substrates, such as euendoliths. These latter, actively excavate, bore tunnels or burrows within the mineral solid or a variety of calcareous substrates, such as shells, dead coral, etc. (Golubic et al., 1981). The first record of euendolithic cyanobacteria was found in 1.5 billion year old stromatolite rocks in China (Zhang and Golubic, 1987). As a result, they are believed to have played a major role in the production and destruction of carbonate, including reef frame builders and sediments, over long periods of geological time. The well fossilized microborings of euendoliths are used as proxies in paleoecological and paleobathymetrical studies (Glaub and Vogel 2004; Radtke and Golubic 2005) as their diversity, distribution and abundance depend on substrates and environmental conditions (Gektidis 1999; Vogel et al. 2000; Tribollet, 2008b).

They are also used for ichnological and ichnotaxonomic determinations. Those studies are based on comparisons between modern and fossilized microborings.

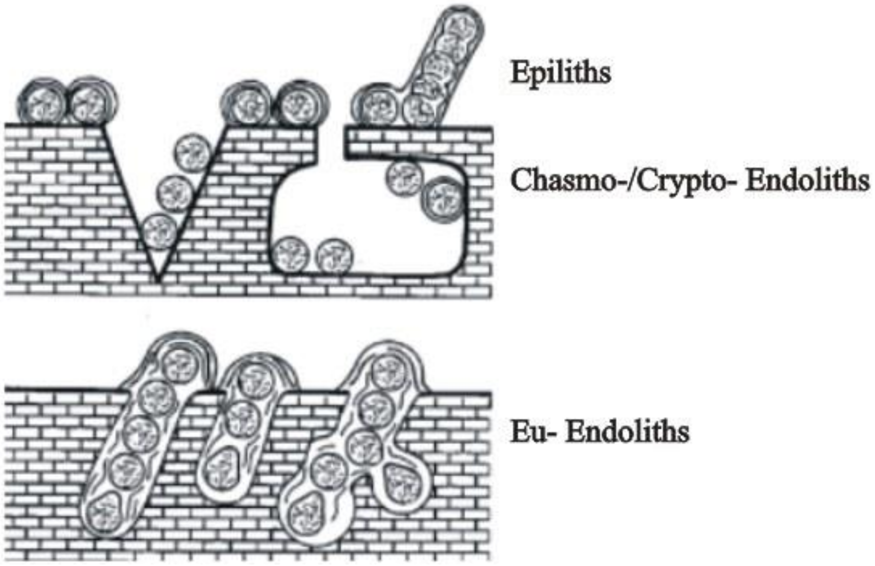

Fig. 1.3 – Mode life and ecological niches of cyanobacteria (Golubic et al., 1981).

1.3 Microbial biofilms and mats

Several studies testify that solids added to aquatic systems are rapidly coated with an organic microbial accretion, forming a more or less thick layer. Such microbial communities are complex structures, ranging from micrometers to few centimeters in thickness, respectively called *biofilms* and *mats*.

In addition to thickness, another fundamental difference between them is that microbial mats are characterized by high population densities of photoautotrophic microorganisms that act as primary producers in the top millimeters and build up the mat matrix, while many biofilms are heterotrophic and depend on substrate supply from the surface or the surrounding water (De Beer & Külh, 2001).

Functionally, a modern living mat is a vertically compressed ecosystem that supports most of the major biogeochemical cycles within few millimeters. Due to differing growth requirements, it is common for mat microorganisms to orient themselves into vertically stratified subsystems that are manifested as distinct, and often, visible layers, where the lamination is determined by the light quantity and quality. The decreasing light regime that occurs with depth results in a cyanobacteria-dominated blue–green layer near the surface, often underlain by a reddish-pink layer of purple sulfur bacteria, a deeper layer of green sulfur bacteria. The deepest layers of the mat are typically black, with occasional gray bands (Fig. 1.4). Mats are largely self-sufficient, in that sunlight or chemical reductants provide the primary source of energy used to convert CO_2 into biomass, while heterotrophy recycles the carbon and reducing equivalents back to other mat microorganisms.

The most considerable components are the filamentous microorganisms, either photosynthetic or chemolithoautotrophic, which, associated with EPS, contribute notably to the cohesion of the mat (Konhauser, 2007).

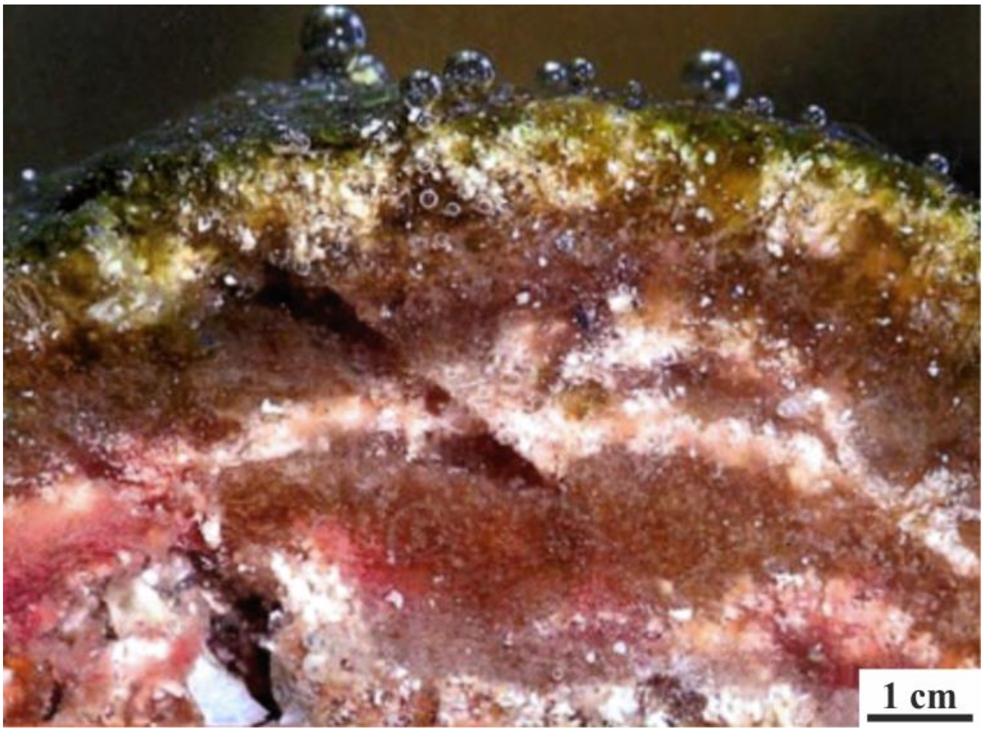

Fig. 1.4 - Cross-section of mineralized microbial mat. Different colors of layers reflect the differentiated microbial composition. Green layer on top is given by photosynthetic bacteria, followed by intervals of brownish layers, with heterotrophic bacteria; whereas red layers with purple sulphur bacteria and an underlying grey layer with sulphate reducers. White layers represent carbonate precipitates. (from Spadafora et al., 2010).

In a typical sedimentary system, the types of microbial metabolism found with depth exhibit a corresponding decrease in thermodynamic yields: aerobic respiration has the highest yield and is encountered at surface, while methanogenesis has the lowest energetic yield and occurs the deepest following the redox gradient (Fig. 1.5).

The activities of these microbes determine mat calcification and preservation (Canfield and Raiswell, 1991). In addition, algae add a further complexity to the bacterial surface community.

Biofilms are described as surface associated to bacterial communities, forming micro-colonies surrounded by a matrix of exopolymers and found in an extremely varied environment, from pure water systems to stream beds (Izano et al., 2007).

Biofilm formation occurs in response to a variety of environmental triggers including high cell density, nutrient deprivation and physical environmental stress

(Li et al., 2003). All these reasons lead to develop different biofilms structures expressed in various morphologies (Hermanowicz, 2001).

The primary stage for microbial communities formation is the attachment of bacteria (initially only one strain) to a surface followed by proliferation of attached cells which leads to the accumulation of multilayer clusters of cells and formation of EPS (Shakeri et al., 2007). Among the primary colonizers bacteria are usually dominant components owing to their high abundance in seawater (Dang and Lovell, 2000).

Microorganisms in biofilms display some particular features that are not shared with the same microorganisms in suspended form. Biofilms may contain mixed populations of bacteria, fungi, protozoa and if conditions allow, they can host even higher organisms in the food chain such as nematodes and larvae. Cyanobacteria, diatoms, and other micro-algae are dominant in the early stages of biofilm formation (Decho, 2000).

All bacteria within a biofilm live together and depend on other microorganisms for energy, carbon and other nutrients (Prakash et al., 2003).

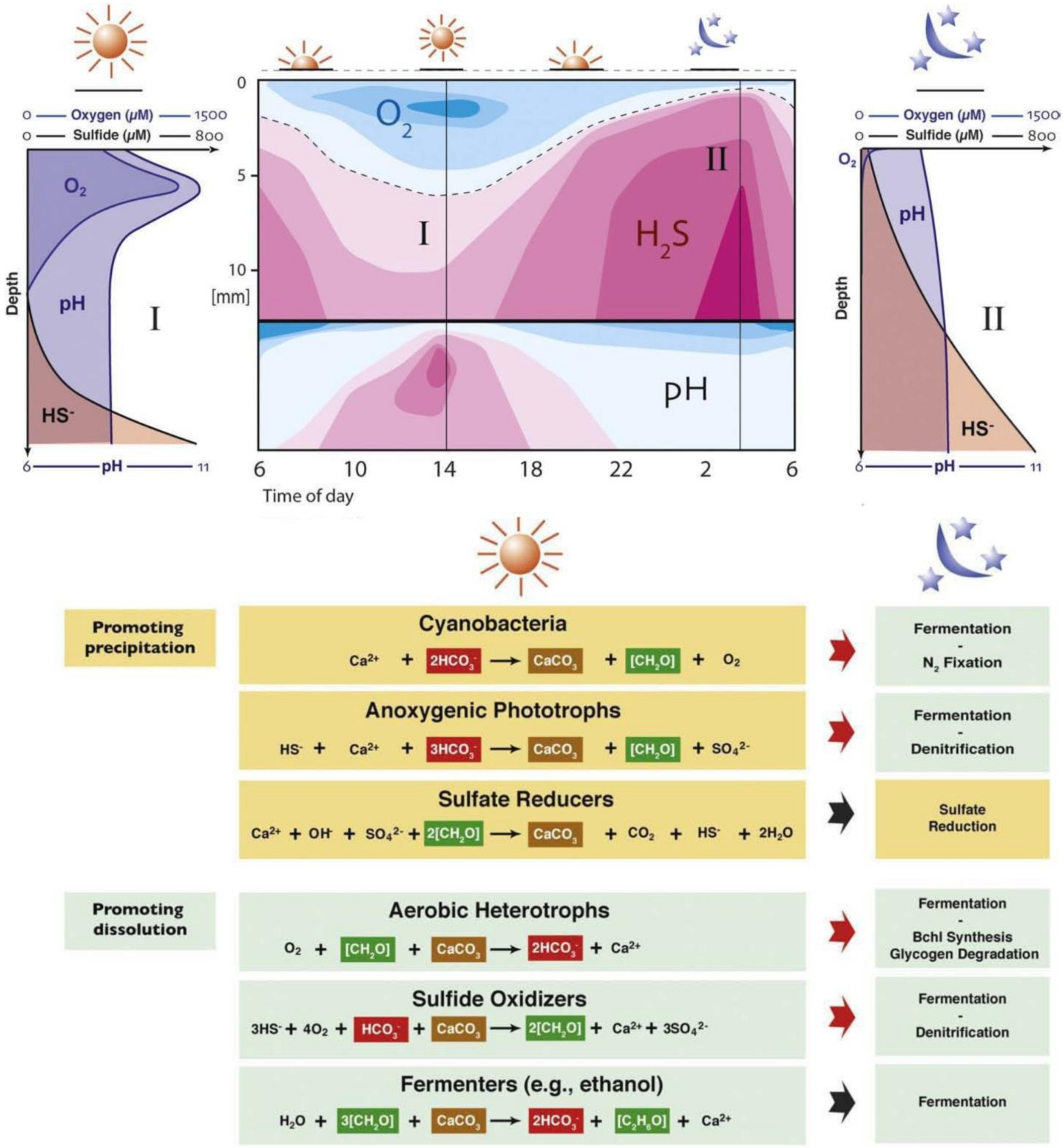

Fig. 1.5 – Diel fluctuations of vertical geochemical gradients in a microbial mat and combined metabolic–geochemical reactions leading to carbonate precipitation and dissolution. (A) Shows the variation in oxygen, sulfide and pH within a microbial mat over a 24 h period. Profiles I and II represent two geochemical "snapshots" taken at 2pm and 3am that show key differences in depth profiles between day and night. As soon as the dark period starts, the photosynthesis ceases and the mat turns completely anoxic because of rapid O^2 consumption by aerobic heterotrophs. (B) The six major guilds of microorganisms that compose a typical microbial mat are arranged by their respective effects on the precipitation process (Dupraz et al., 2009).

1.4 Extracellular Polymeric Substance

Extracellular polymeric substance (EPS) is a biosynthetic polymeric compound formed by prokaryotic (bacteria, archaea) and eukaryotic (algae, fungi) microorganisms, which either form (loose or tight) slimes around the microbial cells or excreted as discrete gels to the surrounding environment. Typically, EPS are heterogeneous mixtures of polysaccharides, proteins, nucleic acids, lipids and other polymeric compounds. The highly diverse chemical composition of EPS is a result of the different processes related to their production and their immediate environment: active microbial secretion, shedding of cell surface materials, cell lysis and adsorption from the environment (Wingender et al., 1999).

EPSs are often associated with the formation of biofilms and microbial aggregates, and some of them play a twofold role, either inhibiting or promoting carbonate formation, depending on the physicochemical characteristics (Decho, 2000) (Fig. 1.6). This work, in particular, refers to the above-mentioned kind of EPS.

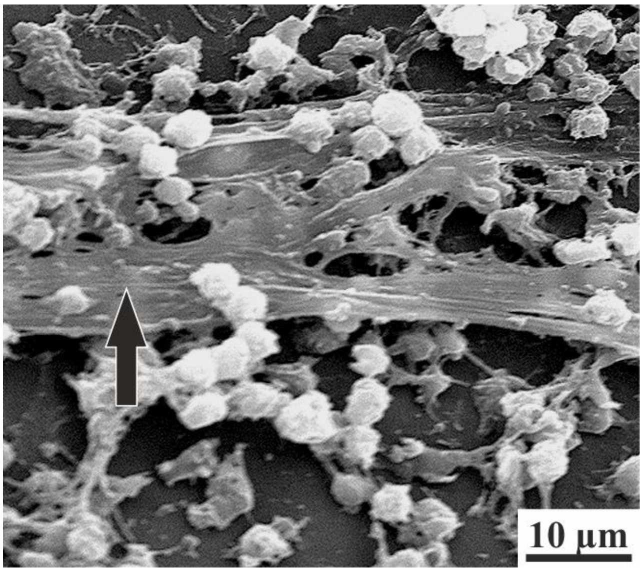

Fig. 1.6 – Scanning electron micrograph of a staphylococcal biofilm. Black arrow indicates EPS filaments (mod. from Donlan, 2002).

As reported by Dupraz et al. (2009), the EPS matrix represents an important component of marine biogeochemical processes (Decho, 1990; Bhaskar and Bhosle, 2005). The chemically-reactive EPS matrix is of considerable ecological importance because it is a physical barrier between the cell and organic and inorganic metabolic substrates, predators, antimicrobial agents, and other bacteria (Costerton et al., 1995). EPS may account for 50-90% of the total organic carbon of biofilms and can be considered as the primary matrix material of the biofilms (Flemming, 1998). The high molecular-weight (8 to N1000 kDa) matrix is composed of polysaccharides, and may include protein and peptides, nucleic acid, uronic acid, DNA, lipids and humid substances (Donlan, 2002; Cheng, 2007).

EPS is also highly hydrated because it can incorporate large amount of water into its structure by hydrogen bonding.

EPS can be produced by a wide array of microorganisms, both photoautotrophic and heterotrophic bacteria. In microbial mats, cyanobacteria are generally recognized as the most important EPS producers (Richert et al., 2005). Recently, Bosak and Newman (2005) and subsequently Braissant et al. (2007) demonstrated the potential role of heterotrophic bacteria, such as sulfate-reducing bacteria, in the production of the extracellular matrix.

Although some free-living bacteria can produce large amounts of extracellular polymers (Kives et al., 2006), EPS production is a seminal feature of benthic communities, enabling the formation of microbial mats and biofilms.

The EPS matrix fulfills many functions within microbial mats (Decho, 1990; Bhaskar and Bhosle, 2005):

1) It allows communities to attach to surfaces and create micro-domains, where various types of metabolism can coexist in microspatial proximity;

2) It physically stabilizes microbial cells under variable hydrodynamic regimes;

3) It may also help the microbial mat community to resist multiple stress conditions, such as nutrient shortages, UV exposure or desiccation.

EPS within a microbial mat can exist in a continuum of physical states, ranging from particulate to dissolved, or from a ‘cohesive gel’ to a ‘loose slime’ to a ‘dissolved solute’ state. The physical state is largely a function of the EPS concentration (or, the water activity), and the abundance and types of bonds or interactions among individual EPS molecules.

The EPS is a key player in organomineralization, having a distinct impact on the morphology and mineralogy of mineral products (Braissant et al., 2003). The physicochemical properties of the polymer matrix, such as the acidity or functional group composition, are important factors in the metal binding potential (initially inhibiting calcium carbonate mineral formation) and biotic and abiotic degradation or alteration of the EPS (favoring calcium carbonate precipitation).

Negatively-charged acidic groups within the EPS matrix can bind a large amount of mono- and divalent cations, which can help to maintain the structural integrity of EPS by promoting gel formation through bidentate bridge formation (Sutherland, 2001). The cation-binding capacity of EPS removes free Ca^{2+} ions from solution, inhibiting the precipitation of carbonate minerals by depleting them from the proximal surrounding environment. The role of acidic amino acids (e.g., aspartic or glutamic acid) and carboxylated polysaccharides (e.g., uronic acids) as strong inhibitors of calcium carbonate precipitation has been well documented (e.g. Gautret and Trichet, 2005). The functional group characteristics are therefore key in the mineral formation process. Although several functional groups contribute to the overall negative charge of the EPS and consequently to metal binding, carboxylic acids and sulfate groups are generally considered to be the most important ligands within EPS.

Differently, in order to precipitate calcium carbonate minerals within a microbial mat, the Ca-binding capacity of the EPS matrix has to be greatly reduced. This can be accomplished through via one of the two following mechanisms 1) diagenetic alteration of EPS or 2A, B) supersaturation of the cation-binding sites, (Fig. 1.7).

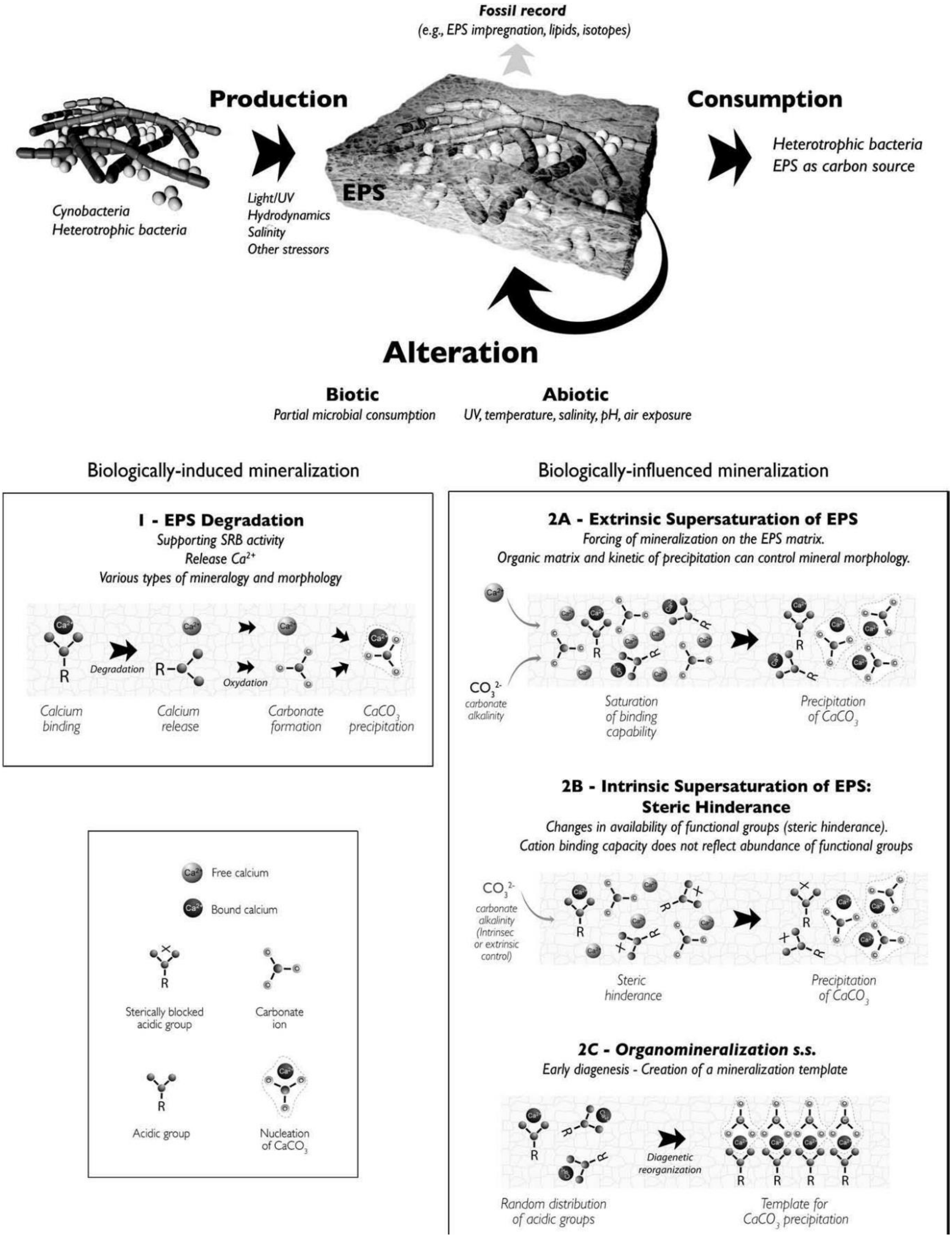

Fig. 1.7 – Synthetic scheme representing the role of EPS in biological mineral precipitation (from Dupraz et al., 2009).

1.5 Biomediated mineral formation

Biomineralization refers to processes in which organisms are involved in the formation of minerals. Most biominerals are calcium carbonates, silicates, iron oxides, and sulfides (Bazylinski, 2004).

In order to define the genesis of minerals produced dependently on biological activity, clear definitions of the relevant processes are required.

According to the definition proposed by Perry et al. (2007), *organomineral* includes any minerals precipitated by interaction with organopolymers, bioorganic, and/or non-biological organic compounds, without evidence of direct skeletal, intracellular or extracellular biological control. The term *biomineral* generally refers to a mineral that was produced by living organisms, forming both minerals and organic components (Weiner and Dove, 2003; Dupraz et al., 2009).

In particular, organomineralization process can be intrinsically (microbial metabolisms) or extrinsically driven (e.g., degassing, evaporation), therefore be respectively either active (biologically- induced) or passive (biologically-influenced) process.

Two different modes of biomineral precipitation can be distinguished (for further details see Dupraz et. al., 2009): (1) biologically controlled mineralization and (2) biologically induced mineralization (Fig. 1.8).

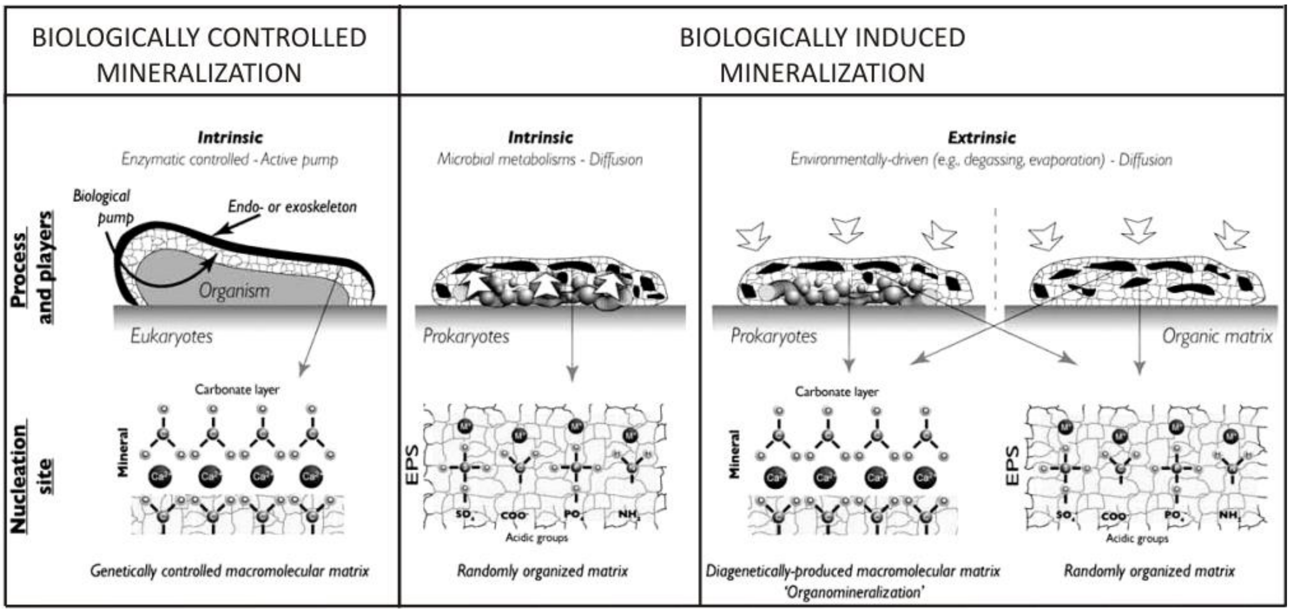

Fig. 1.8 - Classification of mineralization processes showing the different types of biomineralization (mod. after Dupraz, 2009).

1.5.1 Biologically controlled mineralization

In this kind of mineral precipitation, organisms exert a considerable control over all the aspects of the nucleation and mineral growth stages (Mann, 1988).

This kind of mineralization serves the function of specifically producing structural components, such as skeletons, bones, teeth, shells, etc. The molecular structure of nucleation sites are usually genetically controlled to ensure defined mineral precipitation in terms of shape and mineral composition. Minerals produced under biological control are usually well defined in terms of structure and chemical composition. Additionally, a high level of spatial organization is encountered, including complex morphologies and controlled aggregations and textures. Minerals produced in biologically controlled environments also exhibit a preferential crystallographic orientation.

Biologically controlled biomineralization is generally rare in the bacterial community. However, an example is given by magnetotactic bacteria (Komeili, 2004), which provide to the formation of intracellular magnetic crystals (magnetosoma), allowing movement in marine sediments in response to the environmental magnetic field (Blakemore, 1975).

Others, or the same type of bacteria are capable to form greigite (Fe_3S_4), mackinawite (tetragonal FeS) and/or a third phase cubic FeS with the sphalerite structure (Pósfai, 1998) by the same controlled intracellular mineralization process.

1.5.2 Biologically induced mineralization

Formally, two processes known as biologically induced and biologically influenced mineralization were distinguished from each other as microbially or environmentally driven (fig. 1.6).

However, as microbial activity and environmental conditions are close dependent associated, in this study the two processes will not be distinguished from each other and solely referred to as biologically induced mineralization, as suggested by Frankel and Bazylinski (2003) and Konhauser and Riding (2012).

Microorganisms interact in numerous ways with the surrounding environment, involving metabolic reactions and the mode of life. Re-mineralization is a primary ecological process where microbes degrade organic matter. This process is mandatory for element cycling. Microbial activity modifies spatial distribution of chemical components by liberation, production, transport, enrichment, and incorporation into biomass. The resulting alteration of geochemical properties as pH, Eh redox potential, and gas concentrations may induce dissolution and also precipitation of various minerals.In numerous instances, inorganic minerals are precipitated as the result of metabolically produced ions, released into the adjacent environment (Vasconcelos et al., 1995). Metabolic products are transported outside the cell passively via diffusion (e.g. inorganic carbonate ions) or actively via ion channels and excretion (Goodman, 2008). Continued supply of metabolic products to the environment surrounding the cell may lead to supersaturation for one or several minerals, resulting in the subsequent precipitation and deposition of mineral particles.

Thus, biologically induced mineralization is considered an unintended and uncontrolled consequence of metabolic activities (Frankel and Bazylinski, 2003). As there is no cellular control, precipitates size, shape, structure, chemical composition, and organization are generally heterogeneous and often poorly defined (Weiner and Dove, 2003).

The minerals that form through this passive process have crystal habits and chemical compositions similar to those produced by precipitation under inorganic conditions (Konhauser and Riding, 2012).

Several biogenic minerals are passively formed. Some of them have been widely studied such as iron hydroxides, magnetite, manganese oxides, clays, amorphous silica, carbonate (calcium carbonate, dolomite, strontianite, siderite, etc.), phosphates, sulfates (gypsum, celestine and barite), sulfide minerals, etc. (Konhauser, 2007).

1.6 Calcium carbonate precipitation by bacteria

Bacterial carbonate deposits can be traced back for at least 2.6 billion years (Altermann, 2006). They are locally abundant sediments that record not just bacterial growth, but even environmental factors that promote calcite/aragonite ($CaCO_3$) and dolomite $Ca:MgCO_3$ precipitation in, on and around bacteria and the organic matter that they produce. These sediments include carbonate mud, produced by *whitings*, and stromatolites (Konhauser and Riding, 2012).

Calcium carbonate precipitation by bacteria evidenced close dependence on environmental conditions, particularly the carbonate saturation state of ambient waters. It is, therefore, a good example of ‘induced’, as opposed to ‘controlled’ biomineralization.

Benthic cyanobacterial communities can form a wide variety of calcareous deposits. When cyanobacteria growing in biofilms calcify, they can form micritic coatings, crusts, and layers on submerged substrata.

Cyanobacteria can live as single cells (coccoid cyanobacteria) or several cells may be linked to form cell rows, called trichom. If the trichom is surrounded by EPS sheath, the structure is called a filament. The occurrence of cyanobacteria is frequently linked to the deposition of carbonate. By trapping sediment between the filaments, they are involved in the formation of stromatolites in both sea water and freshwater. Cyanobacteria can also calcify when carbonate is precipitated in association with the organism. The result of precipitation is often a micritic tube surrounding the filament or the trichom (Merz, 1992).

The role of cyanobacteria in carbonate precipitation is twofold: metabolic fixation of inorganic carbon tends to increase pH, leading to a state of supersaturation, while cation adsorption to the cell surface promotes heterogeneous nucleation (Fig. 1.9).

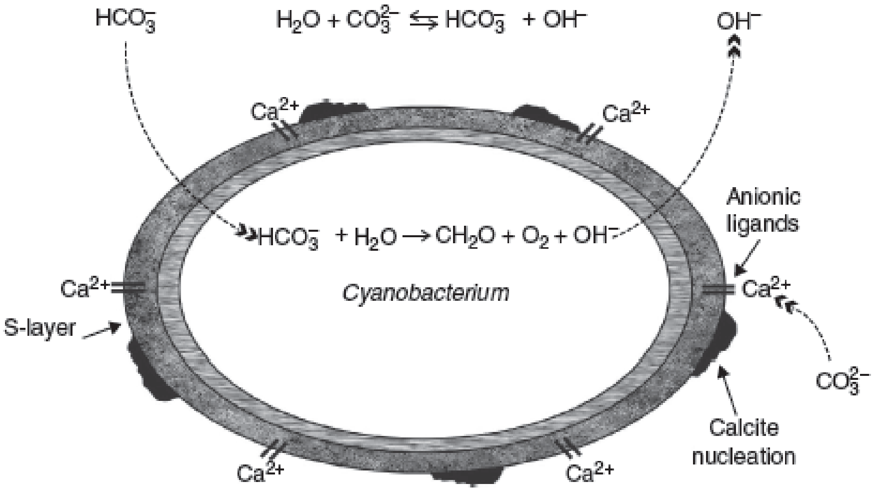

Fig. 1.9 – Schematic of metabolically-induced biomineralization in a cyanobacterium. The uptake of the bicarbonate anion leads to excretion of OH^-, which in turn changes the alkalinity and inorganic carbon speciation proximal to the cell surface. The generation of carbonate anions and the pre-adsorption of calcium cations to the cell's sheath can then induce calcification (mod. after Konhauser, 2007).

With respect to photosynthesis, in waters with neutral to slightly alkaline pH, cyanobacteria use HCO^{3-} instead of, or in addition to, CO_2 as a carbon source (reaction 1.1). Hydroxyl ions are byproduct of this reaction, are then excreted into the external environment where they create localized alkalinization around the cell. This, in turn, induces a change in the carbonate speciation towards the carbonate (CO_3^{2-}) anion (reaction 1.2):

$$HCO^{3-} \longleftrightarrow CO_2 + OH^- \qquad (1.1)$$

$$HCO^{3-} + OH^- \longleftrightarrow CO_3^{2-} + H_2O \qquad (1.2)$$

Cyanobacteria also provide reactive ligands towards metal cations and, once bound, they can react with the CO_3^{2-} anions to form a number of carbonate phases, such as aragonite or calcite (reaction 1.3):

$$CO_3^{2-} + Ca^{2+} \longleftrightarrow CaCO_3 \qquad (1.3)$$

Extracellular layers are particularly favorable sites for nucleation, and cyanobacterial species that produce sheaths or EPS generally precipitate more calcium carbonate than those species without such structures (Pentecost, 1978).

When calcium carbonate nucleates on the sheath surface it grows radially upwards and, in some cases, this may lead to the complete encrustation of the cell. On the contrary, when Ca-carbonate nucleates within the intermolecular spaces of the sheath, this latter may become filled by mineral (Verrecchia et al., 1995).

EPS promotes carbonate precipitation by providing diffusion-limited sites that create localized alkalinity gradients in response to metabolic processes, while simultaneously attracting Ca^{2+} to its organic ligands (e.g., Pentecost, 1985).

Furthermore, the type of functional groups in EPS affects carbonate morphology and mineralogy, e.g., spherule vs. euhedral calcite or calcite vs. aragonite (Braissant et al., 2003). One line of thought suggests that the phase and morphology of calcium carbonate are bacterium or strain specific (Hammes et al., 2003). Cyanobacteria grown in the presence of various combinations of Sr^{2+}, Mg^{2+}, or Ca^{2+} can precipitate instead strontianite, magnesite, or mixed calcite-strontianite carbonates (Schultze-Lam and Beveridge, 1994). In general, cyanobacteria are equally capable of incorporating Ca^{2+} or Sr^{2+} during carbonate mineral formation, while magnesite is easily inhibited from forming by the preferential binding of the former two cations over Mg^{2+}. Other studies have documented that cyanobacteria can partition of up to 1.0 wt% strontium in calcite (Ferris et al., 1995).

Filamentous cyanobacteria, such as *Schizothrix* species, have been heavily implicated in the growth process because they produce EPS that binds Ca^{2+} cations, and their metabolism changes the physicochemical properties at the ooid-water interface (Davaud and Girardclos, 2001).

Ca-carbonate precipitation, caused by microorganisms, may be exploitable in the preservation of monuments and statuary made from carbonate rock, and concrete as studied, for example, under laboratory conditions, respectively with

Bacillus subtilis (Barabesi et al., 2003), and with *Bacillus sphaericus* (De Muynck et al., 2008) as inducers of $CaCO_3$.

1.7 Mineral bio-alteration

Every rock and artifact presents in the uppermost lithosphere and hydrosphere is exposed to chemical and physical weathering. Physical erosion (e.g. storms) is temporary and localized, and chemical erosion is considered as negligible due to the actual seawater chemistry, so biological weathering operated by macro- and micro-organisms, including various kinds of bacteria, fungi, algae, and protozoa, have a primary role in both kind of weathering (Tribollet and Golubic, 2011).

Light availability in relation to bathymetry is one of the main factors influencing the distribution of macro- and micro-organisms and determining the composition of the borer communities.

Agents of bioerosion comprise internal and external bioeroders of different sizes and from a wide range of taxonomic affiliation. Internal bioeroders excavate carbonate substrates in search of shelter or food, while external bioeroders graze on both epilithic and endolithic organisms thereby abrading the substrates. Epiliths are attached to the surface of hard substrates, whereas endoliths reside inside hard substrates. Internal agents comprise micro-borers (<100 µm) and macro-borers (> 100 µm).

Microborers are phototrophic and organotrophic microorganisms (Tribollet, 2008). Microboring phototrophs are, for example, prokaryotic cyanobacteria (Fig. 1.10) and eukaryotic chlorophytes and rhodophytes. Organotrophs (heterotrophs) are fungi foraminifera, and other prokaryotic and eukaryotic light-independent microorganisms.

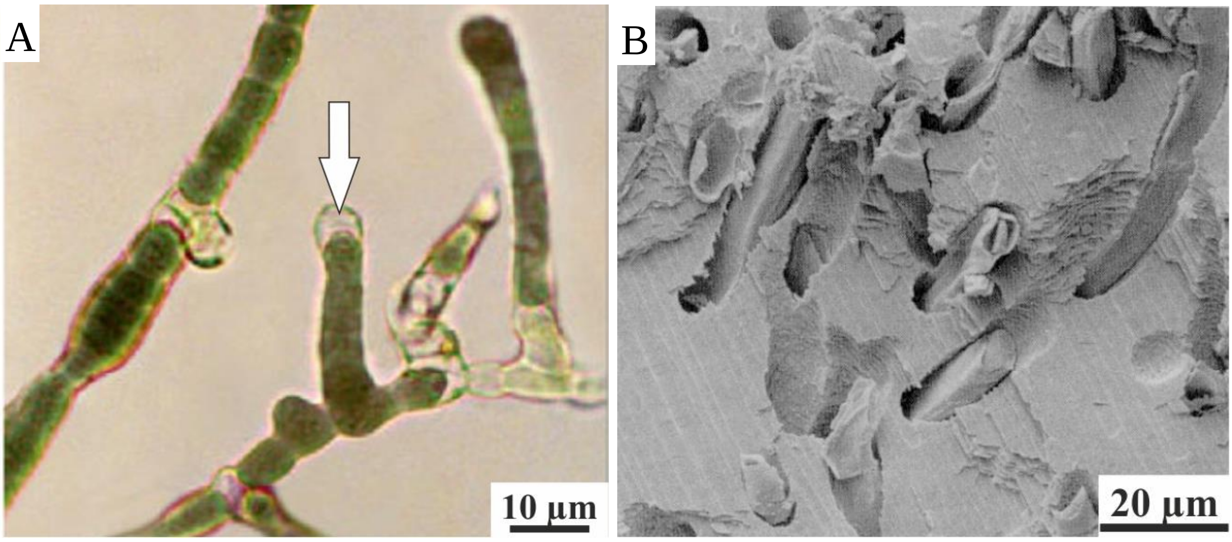

Fig. 1.10 – (A) *Mastigocoleus testarum* cultivated in marine water. White arrow indicates an heterocyst cell (Montoya Terreros et al.,2006). (B) Details of *Mastigocoleus* sp. tunnels in a mussel shell (Webb and Korrûbel, 1994).

Macro-borers are comprised of various organisms (Hutchings, 1986 for review), including protists (foraminifera), sponges, bryozoans, polychaetes, sipunculids, bivalves, and crustaceans Organisms such as some foraminifera, serpulids (polychaetes) and bryozoans only etch the surface of the substrates to anchor themselves (Fig. 1.11).

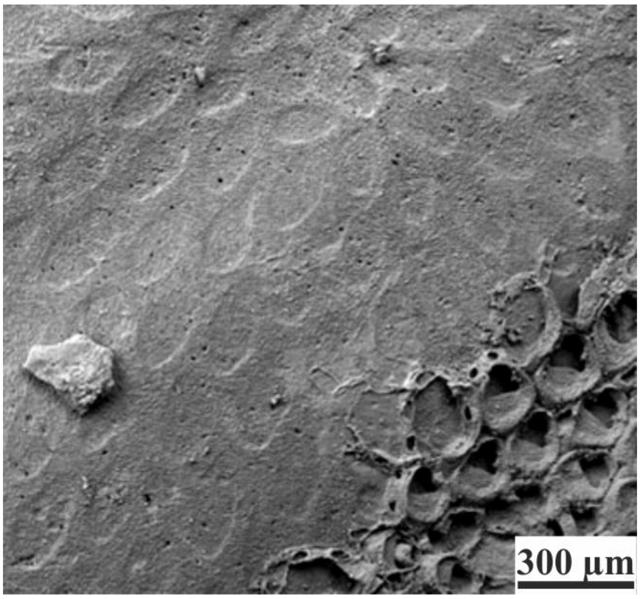

Fig. 1.11 –Micrograph taken under SEM of the shell etched by a bryozoan colony (Taylor et al.,1999).

In the shallow sub-littoral zone, biological corrosion and biological abrasion form bored limestones underneath the biogenic micro-reefs (Golubic, 1962; Schneider et al., 1983). Through the process of boring, euendoliths will leave clear traces of their activity in the substrates that they excavate, and these fossilize well. The fossil record presents evidence of euendoliths populating the Earth since the Precambrian. The oldest cyanobacterial euendolithic microfossil, *Eohyella campbellii,* was recorded in carbonate rocks as old as 1.5 billion years (Zhang, 1987).

Other evidence of euendolithic fossils is found in microbial assemblages from the Neoproterozoic, some 700-800 million year ago, where the cells penetrated aragonitic ooid grains (Knoll, et al., 1989, Ramírez-Reinat and Garcia-Pichel, 2012).

Microorganisms attach exposed mineral surfaces, coat them with extracellular polymers substance (EPS) causing, subsequently, a physical disruption of the grains to gain access to nutrients and energy in the underlying substrata.

Endolithic cyanobacteria, algae, and fungi have been found to cause local dissolution of limestone, thereby forming tubular passages in which they can grow (Golubic et al., 1975). The kinds of limestone they attack in nature include coral reefs, beach rock, and other types. Active algae include some green, brown, and red algae (Golubic, 1969). The mechanism by which any of these organisms bore into limestone is not understood. Some filamentous boring cyanobacteria possess a terminal cell that is directly responsible for the boring action, presumably dissolution of the carbonate (Golubic, 1969). Different boring microorganisms form tunnels of characteristic morphology (Golubic et al., 1975). The depth to which cyanobacteria and algae bore into limestone is limited by light penetration in the rock, because they need light for photosynthesis. Boring cyanobacteria may have unusually high concentrations of phycocyanin, an accessory pigment of the photosynthetic apparatus, to compensate for the low light intensity in the limestone. In contrast, boring fungi are not limited by light penetration. Being incapable of photosynthesis, they have no need for light (Ehrlich and Newman, 2009).

Moreover, they create a complex microenvironment at the mineral–water interface, where metabolic reactions and generation of acids lead to pH and concentration gradients markedly different from the bulk solution. This often promotes a state of thermodynamic disequilibrium that increases rates of chemical weathering (Konhauser, 2004).

Intended mineral weathering as a strategy for nutrient and trace element acquisition, as well as an energy source, has been documented on numerous different microbes (e.g. Shock, 2010; Edwards et al., 2005).

Acidification is of prime relevance for silicate and carbonate minerals, as their dissolution is positively correlated with decreasing pH. Once microorganisms are fixed to the substratum, they begin to produce organic acids, such as lactic, pyruvic, citric and oxalic acids, accelerating dissolution. The majority of organic acids are byproducts of fermentation and/or various intermediate steps of the aerobic respiration of glucose, but some microorganisms further excrete organic acids when growth is limited by the absence of an essential nutrient.

In aquatic systems, mineral dissolution can be attributed to the presence of carbonic acid, resulting from microbial respiration (Soetaert et al., 2007), and nitric and nitrous acid, produced by nitrifying bacteria. Also, the release of organic acids largely contributes to mineral dissolution (Mailloux et al., 2009). Even upon death, microorganisms are important agents in weathering because their decay, via the action of respiring heterotrophs, leads to elevated soil CO^2 partial pressures, which, in turn, creates carbonic acid (Konhauser, 2004).

Silicate weathering normally undergo incongruent dissolution, in which most of the cations (as Ca^{2+}, Mg^{2+}, K^+, $Al^{2+}$and Na^+) are leached out from the lattice, leaving a residual phase as clays or metal oxides.

In addition, cellular organic functional groups and chelating metabolites also induce mineral weathering via three simultaneously occurring mechanisms (Uroz et al., 2009). Organic acids and chelating molecules adhere to mineral surfaces and liberate nutrients from mineral phases by electron transfer. These compounds also break the oxygen links and chelate dissolved ions in carboxyl and hydroxyl groups.

Results from relatively recent laboratory experimentation on the effects that solutions of various organic acids reagent grade have on rock minerals, are still discussed as though, in nature, these reactions were purely abiotic (Ehrlich, 1998).

Furthermore, the dissolution of limestone by biological corrosion is promoted by the grazing activity of different organisms (nematodes, copepods, gastropods), which abrade the rock surface with the aim to remove endolithic microorganisms sited in (Fig. 1.12). This process of bioerosion develops gradually and accelerates between the calcified patch reefs where more light is available. In this way the biokarst relief of the furrowed limestones develops.

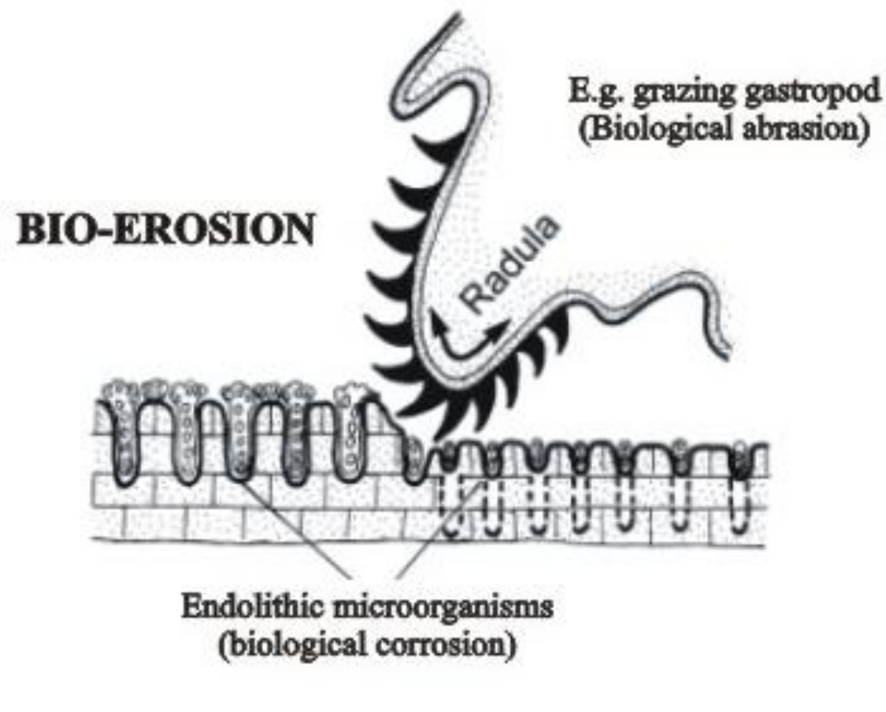

Fig. 1.12 – An example of bioerosion scheme by the radula of the marine gastropod Patella. The endolithic cyanobacteria receive new stimuli to bore to the phototactically controlled light compensation depth after the surface is rasped away (after Schneider & Torunski, 1983).

The corrosive activity of epi- and endolithic cyanobacteria affects the substratum directly. On marine limestone coasts the carbonate rock surfaces are very densely populated with euendoliths (Schneider, 1976). More than half a million individuals per square centimeter are not unusual, as shown by the casting-embedding.

Tudhope and Risk (1985) showed that euendolithic autotrophs, present in Davies reef, Australia, penetrate rapidly into new available substrate by dissolving chemically its crystals. Crystals of substrates show a specific arrangement around microborings suggesting a precisely controlled excavating process (Golubic et al. 1975; Le Campion-Alsumard 1975, 1979, Tribollet et al., 2006).

Garcia-Pichel (2006) revisited mechanisms to explain dissolution of carbonate substrates by euendolithic cyanobacteria. Those mechanisms are (1) a temporal separation of photosynthetic and boring activities during the daily cycle (dissolution due to the CO_2 produced during respiration at night), (2) a spatial separation of photosynthetic and boring activities, and (3) the use of calcium pumps. A combination of those mechanisms is possible but the use of calcium pumps seems the most probable mechanism. Garcia-Pichel (2006) showed that an active transport of Ca^{2+} from the apical cell of euendolithic filaments to their trailing end would make dissolution thermodynamically favorable around the apical cell, while interstitial pH is high due to photosynthesis. This is consistent with the known range of bored substrates including aragonite, calcite, granite and hydroxylapatite, and with precipitation of micrite and brucite observed around euendolithic filaments at the surface of dead substrates. The reasons why several taxa of euendoliths cyanobacteria bore holes into minerals are not fully understood. However, advantageous aspects for boring include availability of nutrients, avoidance of competition, protection from extreme environments and prevention of detachment (Ramirez et al., 2010).

1.8 Application of nanostructured biologically induced minerals

The use of microbially induced carbonate biominerals, formed by primititve nanostructures, is becoming increasingly popular day by day.

Nano-structured biologically induced minerals have unique structural features; many physical and physicochemical properties render them different from usual nano-particulate materials and single-crystal materials. This provides better performance in numerous applications.

Bioremediation, the range of processes that use microorganisms and their products to return contaminated environments to their original condition, uses microorganisms, their enzymes and, increasingly, microbial induced Ca-carbonates to deal with contaminated soils, for example by lead (Pb), Cadmium (Cd), Copper (Cu), Arsenic (As), oil spills or chemical waste (Kang et al. 2014; Achal et al., 2011, 2012a, 2012b).

Nanostructured materials are widely used even as biomedical materials, such as tissue engineering scaffolds and drug carriers, and lightweight structural materials.

In construction materials, calcinogenic bacteria added to concrete and cement mortar improve compressive strength and the remediate cracks (e.g. Achal et al. 2010; De Muynck et al., 2010).

Biomineralization processes have been used also for the bioconservation of the built heritage and statuary made of stone. Structurally and mechanically coherent, and therefore durable, bacterial calcite cement is applied to calcitic stones such as limestone and marble consolidating their external structure, filling pores, in order to protect them from deterioration and alteration (e.g. González-Muñoz, 2003).

Recently, microbial induced carbonate precipitates are used for increase strengthening of sand and soil (so called *biogrouting*), for instance, at the base of railroad embankments, dikes, etc. In addition, they are used as reducer of CO_2 emission into the atmosphere, filler for rubber, plastics and ink (Dhami et al., 2013).

Furthermore, nano-scale study of the fabric and the morphology of neo-formed Calcium carbonate crystals results essential for recognition of the abiotic versus biotic nature of carbonate minerals in several fields such as in the reconstruction of depositional palaeoenvironments, in astrobiology (numerous similar features have been found on Mars and in meteorites; Folk and Lynch, 1997); in medicine, where different biologically induced minerals are formed by pathogenic bacteria (e.g. kidney stones, dental tartar; Kirkland et al., 1999).

CHAPTER 2

GEOGRAPHICAL SETTING AND HISTORICAL INTRODUCTION

2.1 Geographical setting

Samples used for this work have been collected in two different places, selected for their historical relevance: Monasterace, sited along the Calabrian Ionian coast, and Santa Maria di Ricadi, sited along the Calabrian Tyrrhenian coast (Fig. 2.1).

Fig. 2.1 – Maps illustrate the position of the two different sites where samples have been collected, respectively Santa Maria di Ricadi, on the Tyrrhenian coast, and Monasterace, on the Ionian coast.

In Monasterace site, sampling occurred in the submerged archaeological park in front of the ruins of the ancient village of Kaulonia, between the actual Assi River and Doric temple (Fig. 2.2).

Differently, Santa Maria di Ricadi is a small village located along the Calabrian Tyrrhenian coast, on the southeast margin of the Capo Vaticano promontory (Fig. 2.3), facing the sea along more than 2 km of coastline.

Samples were collected from a stratified conglomerate which extends for about 1 km in the bay area, characterized by covering the seafloor for hundreds meters in length, parallel to the coastline, tilted about 10° seaward and cropping out from the medium sea level up to 50 cm (Fig. 2.4).

Fig. 2.2 - Part of the archaeological site of Kaulonia ancient village ruins. In close-up view, the remains of the Doric temple of Kaulonia represented by the rocky basement. On the right side the votive area. Red circle indicates approximately the submerged archaeological area, where abundant archaeological material, including samples used in this study, has been found (Photo mod. after www.panoramio.com "ruins of Doric temple").

Fig. 2.3 - Planar view of Santa Maria di Ricadi bay that extends for about a 1 km. Red line indicates the sampling area (mod. after www.viamichelin.it).

Fig. 2.4 - Part of Santa Maria di Ricadi bay (looking southward) is characterized by a stratified conglomerate extended for hundreds meters in length. In particular, red arrow shows remains of a millstone, carved on edges and in the center and broken in the upper part.

2.2 History of Monasterace

The site of Monasterace represents the remains of Kaulonia, a rather modest-size Magna Graecia colony, founded at about 700 years BC by the Achaeans, a people from the Peloponnesus, and perhaps also by settlers from Kroton (Crotone), another Greek settlement located to the northeast. Kaulonia was conquered in 389 B.C. by Dionysius the 1st, tyrant of Syracuse, and its settlers deported to Locri, a colony positioned about 40 km to the SSW. A settlement was then rebuilt at the original Kaulonia site by the Brettii, a nomadic Indo-European tribe in the 3rd century BC, and was conquered once again, this time by the Romans in 205 BC (Stanley et al., 2007)

Archaeological excavations at Kaulonia are positioned immediately north of the small town of Monasterace Marina. The gentle arcuate headland immediately to the north of the site is known as Punta Stilo, or Capo Colonna (Fig. 2.6). It has had this name at least as far back as about 2000 years ago when the locality was cited by Pliny the Elder in his The Natural History.

This suggests that columns may still have been visible to the Romans in this sector to about 2000 years ago, i.e. perhaps as long as 3 to 4 centuries after exile of the Greek settlers in 389 BC (Stanley, 2007).

Monasterace shoreline varied in mid and late-Holocene. At the pre-Greek time, about 2500 years BC, was behind the present dunes, at Greek time (ca. 500 BC) advancing seaward of the present one by little more than 100 m; after migrating back landward during the post-Roman age and finally to present shoreline (Fig. 2.6). Actually, the coastline undergoes several storms that frequently, during the winter season, erode the sandy cliff behind which are settled the basement of the temple and a votive area.

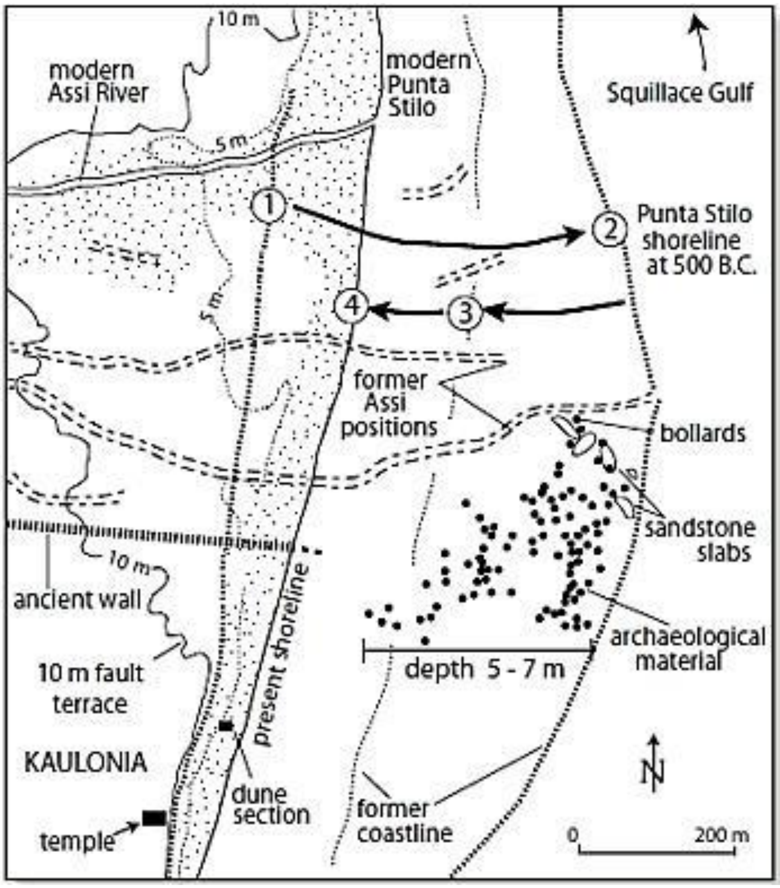

Fig. 2.6 - Paleogeographic scheme of the Kaulonia margin shows major Holocene coastal shifts: from former shoreline position (1), to seaward (2), and then return to the present coastline (3, 4). (After Stanley et al., 2007).

At 500 BC, sea level was only about 2 m or less below the present m.s.l., according to some presently used world curves (Fairbanks, 1989; Pirazzoli et al., 1997).

This eustatic rise, plus seafloor erosional downcutting phenomenon would thus account for total lowering of shoreface elevation to about 3 to 4 m below present m.s.l. at the time of Greek settlement (Stanley et al., 2007).

Abundant archaeological material as column sections, column bases, construction blocks, bollards, anchors associated with numerous potsherds, bronze, lead, and copper fragments (Lena and Medaglia, 2002) were discovered by divers in the mid- to late 1980’s at water depths ranging from 5 to 7 m up to 300 m offshore Monasterace (Fig. 2.7).

The columns are dated stylistically to about 480-470 BC. Archaeologists have determined that the large number of column sections (length to 113 cm, diameter to 80 cm) and other materials on the seafloor were not distributed randomly as a result of shipwrecks (Lena and Medaglia, 2002). Rather, these and other archaeological items are positioned at, or proximal to, once exposed coastal near shore surfaces upon which they were worked and/or from which they were discharged from vessels.

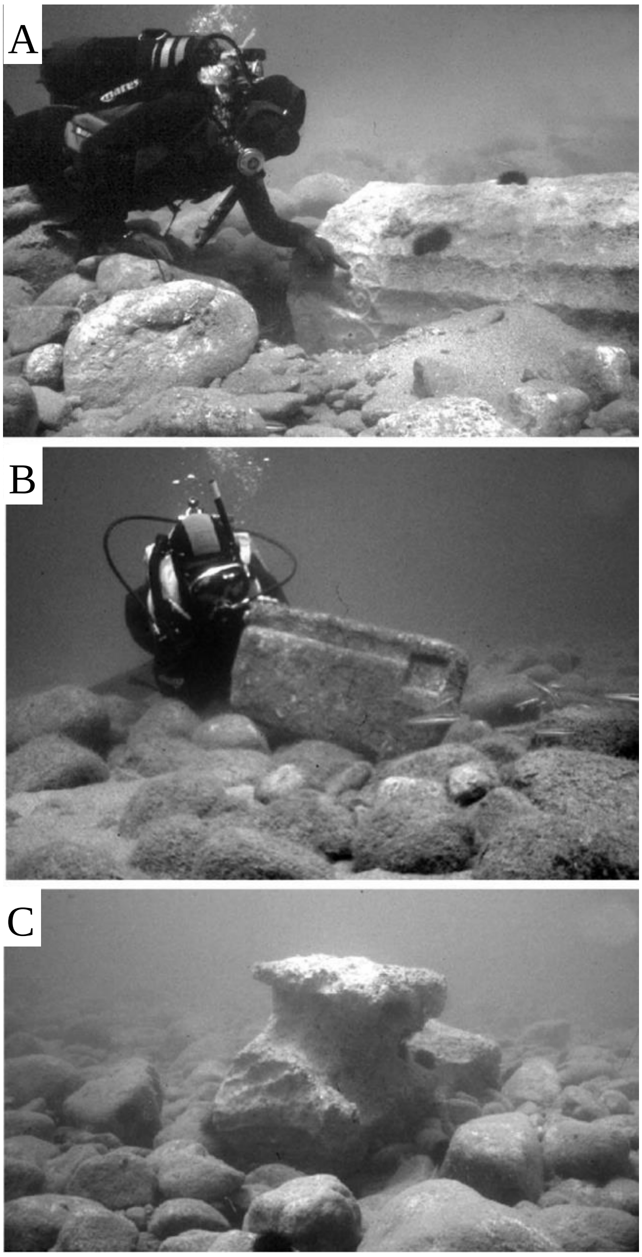

Fig. 2.7 - Underwater photographs taken in the 1980s show examples of archaeological material off Kaulonia, including (A) fluted Ionic column section, (B) construction block, and (C) bollard (after Iannelli, 1997; Iannelli et al. 1993). Large, rounded boulders (some up to 50 cm diameter) of igneous and metamorphic material of Assi River derivation are widely distributed in this offshore sector (photos by S. Mariottini).

More recent geo-archaeological analysis, using data from geophysical and coring surveys, indicates that the seafloor on which the columns were discovered was actually only a small part of a broad arcuate (not small hook-shaped) headland that subsided after time of Greek occupation. Moreover, other analyses show that a sector of the now-submerged surface, currently lying at a depth of 5 to 7 meters below m.s.l., had been partially covered by stratified sandstone originally formed along the beach-line to foreshore zone during the latter half of the Holocene (Stanley et al., 2007).

This surface seaward of Kaulonia, comprising sandstone slabs, cobbles and boulders and archaeological material, appears to have been submerged completely beneath sea level during approximately four to five hundred years from roughly 400 BC near the end of Greek occupation to the time of Roman rule in about the 1st century AD.

2.3 History of Santa Maria di Ricadi

The origin of the village of Santa Maria di Ricadi is not clear, and just few information are available but, according to Pantano (1972), the Ausones, an italic tribe, settled there about XII centuries B.P. After them, several populations colonized that area, even Greeks and Romans. In 1987, in Santa Maria di Ricadi, the excavations revealed a large settlement of the III-IV century BC with remains of Roman villas and a kiln with a wide deposit of cereal amphora. Jars have been recognized belonging to the same type, suggesting that there were used for shipping local alimentary products (mainly cereals).

Santa Maria di Ricadi bay was used for hundreds years as natural harbor. Last discoveries indicate that Romans built the first real port, known in the past as "portus Erculis" (port Hercules), cited in several ancient sources (Pliny the Elder and Strabo), but not yet identified (Pantano, 1972).

Rock formation, cropping out the medium sea level, is characterized by having many remains of millstones (Fig. 2.5). Millstones have been probably left in place because broken during carving operations. Several unconfirmed sources indicate Romans as first people carving this tough rock were that used them for grinding cereals.

Various examples of well, or sometimes bad, preserved millstone quarries are found in the southern coasts of Italy, such as Palinuro in the region Campania; Crotone, Roccella Ionica, Capo dell'Armi in the region Calabria, Capo d'Orlando and Giardini-Naxos in the region Sicily.

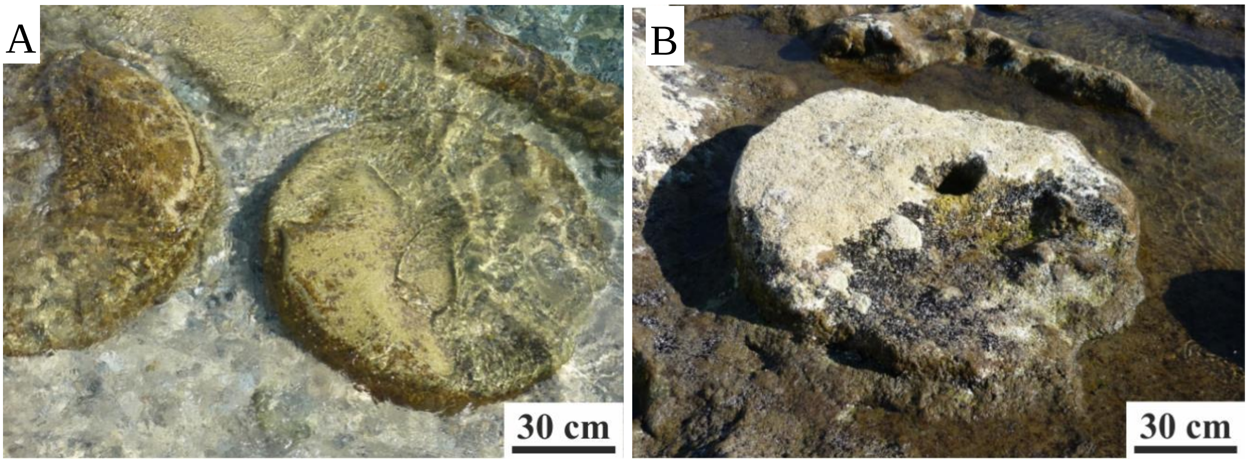

Fig. 2.5 - Semi-manufactured millstone carved in place. They show a typical cylindrical shape and a diameter up to 1,3 m.

This kind of cylindrical-shaped wheels with a diameter up to 1,30 m, are well documented since the beginning of the Hellenistic period in Israel and Cyprus, and widespread within the whole Mediterranean basin from the Roman to the modern age, until the XIX century (Amouretti and Brun, 1993).

According to Antonioli et al. (2006, 2009), the average uplift rate for the Capo Vaticano area is 1.5-1.6 mm/year. This means that the millstones whose actually settle at a maximum 60 cm below medium sea level, 2000 years ago where at least 2.7 m above m.s.l.

CHAPTER 3

METHODS

3.1 Sampling

Samples have been collected in collaboration with a diving team belonging to "Associazione culturale Kodros" and headed by Stefano Mariottini.

In Monasterace, sampling took place during the summer period at water temperature of 23°C and depths ranging from 5.8 to 6.7 m and about 250 m far away from the actual coastline. Among the various rests of artifacts present in the submerged archaeological area of Monasterace, ten key-samples that well represent the variety of natural rocks and artifacts used in that area by ancient people were collected.

Differently, in Santa Maria di Ricadi, two samples were extracted with chisel from the conglomerate, outcropping few centimeters up to the medium sea level. These samples result sufficient for well representing the conglomerate and its own biofilm.

3.2 Fixation and dehydration

Fixation is the first crucial step in the preparation of specimens. After a half hour from sampling, every specimen was quickly immersed in a solution composed of 1/4 of 4% paraformaldehyde and 3/4 marine water, with the purpose to preserve all the biological organisms and tissues.

Subsequently, samples were gradually dehydrated and dried in HMDS (hexamethyldisilazane; Arp et al., 1998; Fratesi et al., 2004). Dehydration process consists in the chemical removal of water from the specimen.

3.3 Optical microscope and SEM-EDS analyses

All the samples collected were prepared for optical and scanning electron microscope (SEM) analyses.

All 12 samples, respectively 10 of Monasterace outcrop and 2 of S. Maria di Ricadi, were worked in order to obtain for each sample a thin section and a fresh cutting. Cross-section cuttings were embedded in resin and cut to obtain thin sections of 30 μm in thickness and dimensioned 28 × 48 mm. As the same time, for every sample was extracted a piece, cut sub-perpendicularly to biofilm surface, carbon coated and fixed on stubs for SEM analyses.

Optical microscope analyses allowed evaluating physical, chemical and biological variations in the rock from the top, where biofilm is sited, to the inner 2 centimeters. Thin sections were analyzed in plane polarized light (PPL) and cross polarized light (CPL).

Scanning electron microscopy (SEM) has been finalized to micro and nano-scale observation of the biogenic elements forming biofilm and the recognition of the abiogenic or biogenic nature of the neo-formed precipitated minerals. Furthermore, mechanical/chemical, biological and weathering products were observed.SEM analyses occurred using FEI Quanta 200 F instrument, in high-vacuum condition with backscattered-electron-detention (BSE) and secondary-electron-detention (SE), even indicated as Everhart-Thornley Detector (ETD), imaging system at 20 kV, at an average working distance of 12 mm.

In addition, SEM instrument was equipped with an energy-dispersive X-ray spectrometer (EDS) for semi-quantitative analyses.

CHAPTER 4

RESULTS

4.1 Introduction

Among the several samples collected in the areas of Monasterace and Santa Maria di Ricadi, twelve key-samples were analyzed.

Foremost, every sample has been described at naked eye, in order to characterize the biofilms present on them.

Successively, further investigations were carried out with optical microscope and Scanning Electron Microscope with the purpose to observe micro-and nano-elements characterizing biofilms, such as skeletal organisms, bacteria, organic matter and neo-formed minerals.

Moreover, biological weathering features have been observed.

4.2 Monasterace

Samples collected in the submerged archaeological area of Monasterace vary notably in composition.

Samples made of calcilutites, calcarenites and marble represent probably scraps produced during rock carving (Fig. 4.1 A, B, C).

Two samples of brick, probable fragments of tiles, have been found on the seabed isolated from the rests (Fig. 4.1 D).

Calcschist specimens resulted very tough, hardly breakable, but along cleavage planes (Fig. 4.1 E). These samples come from semi-manufactured slabs, widespread only in the submerged area.

Biofilms, observed on the surfaces of the samples collected in Monasterace, have variable thickness, from few microns to ≈ 1 cm, extension and are made up by numerous components varying from sample to sample.

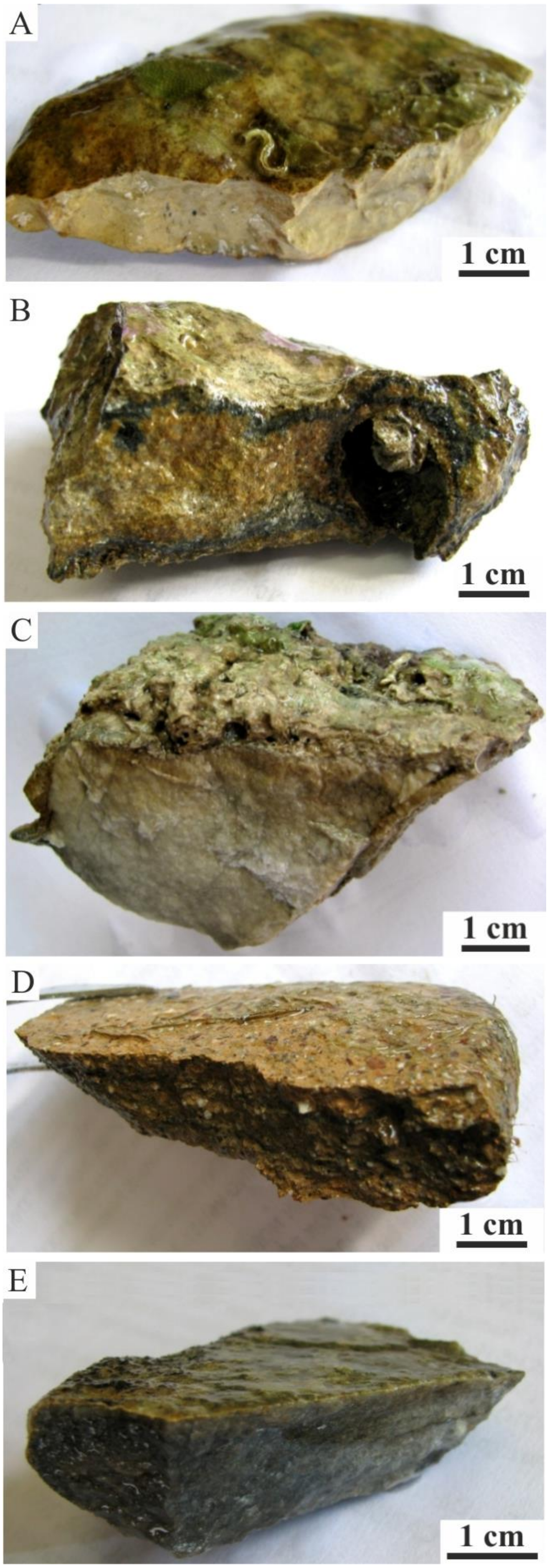

Fig. 4.1 - Hand-samples in photos represent the different categories of specimens collected in Monasterace. (A) Calcilutite; (B) calcarenite; (C) marble; (D) tile; (E) calcschist.

4.2.1 Components of the biofilm

4.2.1.1 Skeletal organisms

Skeletal organisms occupy great part of the exposed samples surfaces. At the sampling moment, many organisms resulted alive, but for many others, only the skeleton remained adhering on the rock surface.

Red algae

Highly calcified encrusting red algae (*Rhodophyta*), thick in average 1 mm (up to 5 mm), occur in many collected specimens. Some samples are characterized by the presence of a strong incrustation of red algae (Fig. 4.2 A, B), whereas other samples show just few patches of red algae appearing with reddish color (Fig. 4.2 C). Cellular structures vary from bad preserved (Fig. 4.2 D) to quite well preserved, especially in the upper part of the reticulate cellular structure, called *hypotallus*, (Fig. 4.5 E, F). Moreover, EDS analyses indicates that, in average, red algae is composed of Ca-carbonate with 14-16 moles % of Mg.

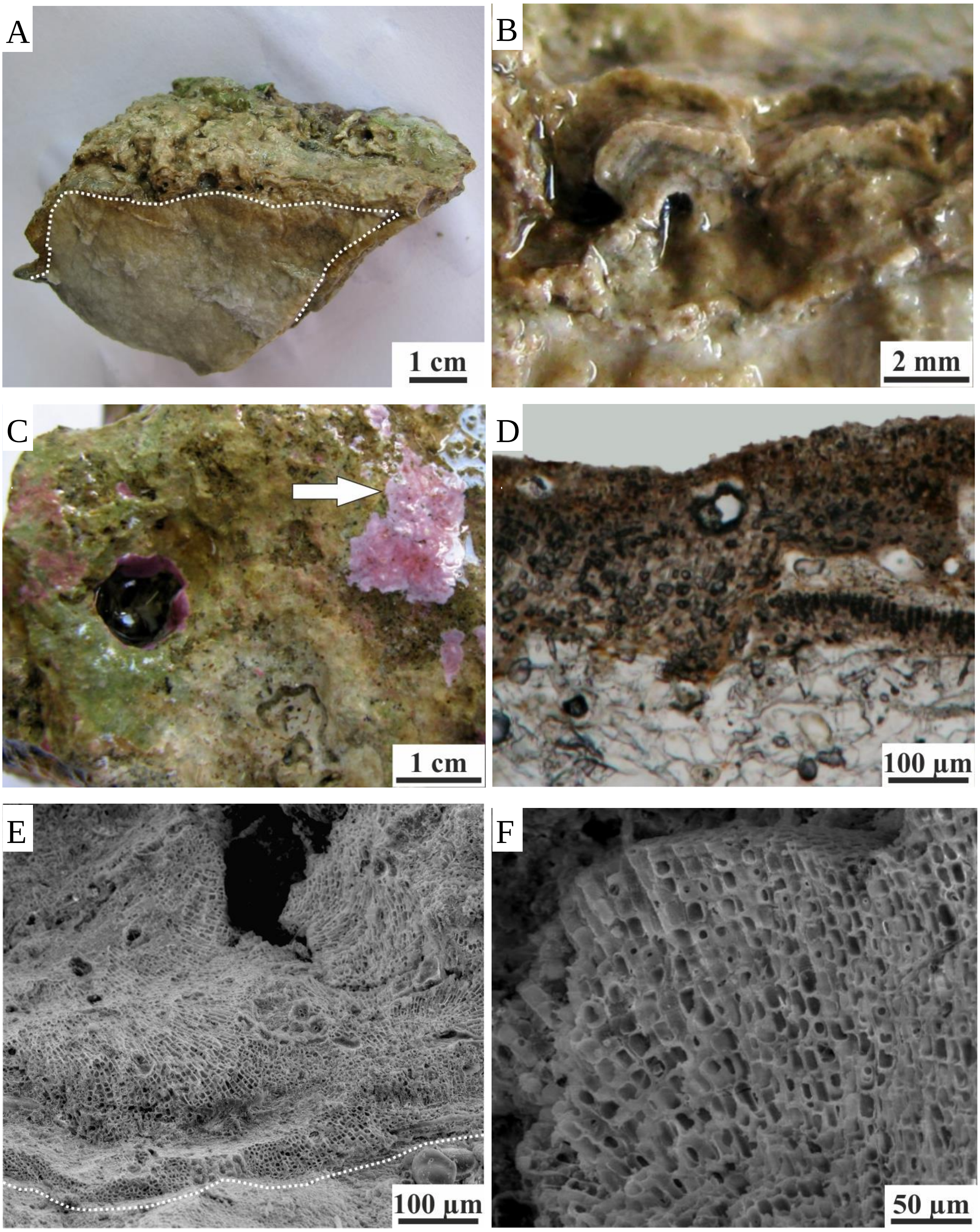

Fig. 4.2 – (A) Marble sample strongly encrusted by thick overlapped layers of red algae. (B) Close up view of the previous photo show a crust thick ≈ 6 mm. (C) Hand-sample surface shows several reddish patches (biggest indicated by white arrow) representing red algae. (D) PPL micrograph shows a bad preservation of the red algae skeleton. (E) SEM photomicrograph shows the upper part, called *perithallus*, of encrusting red algae. White dashed line indicates the limit algae/rock. (F) Close up view of *perithallus* shows its reticulate cells structure.

Bryozoans

Encrusting bryozoan are widespread as colonies, called *zoarium*, on many surface samples, appearing as a white patina, but most of the time showing different colors, since they are covered by cyanobacteria or others microorganisms that mask the original appearance (Fig. 4.3 A).

Every single element forming the zoarium is called *zooid*. This latter has sub-rounded to arcuate shape and diameter up to 300 μm, and resulted generally well preserved (Fig. 4.3 B, C, D). Zooids have a double-wall (*zooecium*) (Fig. 4.3 B).

Sometimes bryozoan results associated with filamentous bacteria (Fig. 4.3 C, D). The majority of the observed bryozoan lack in living organism, but in few cases its terminal part, called *lophophore*, is still visible with a ring of tentacles around the buccal opening (Fig.4.3 E).

Zoaria (pl. of zoarium) form widespread and patchy crusts, reaching 300 μm of thickness, on the top of the samples.

Skeletal nanostructure is layered, made up by numerous thin isoriented lamellae, composed of Ca-carbonate with ca. 6 mole % of Mg (Fig. 4.6 F).

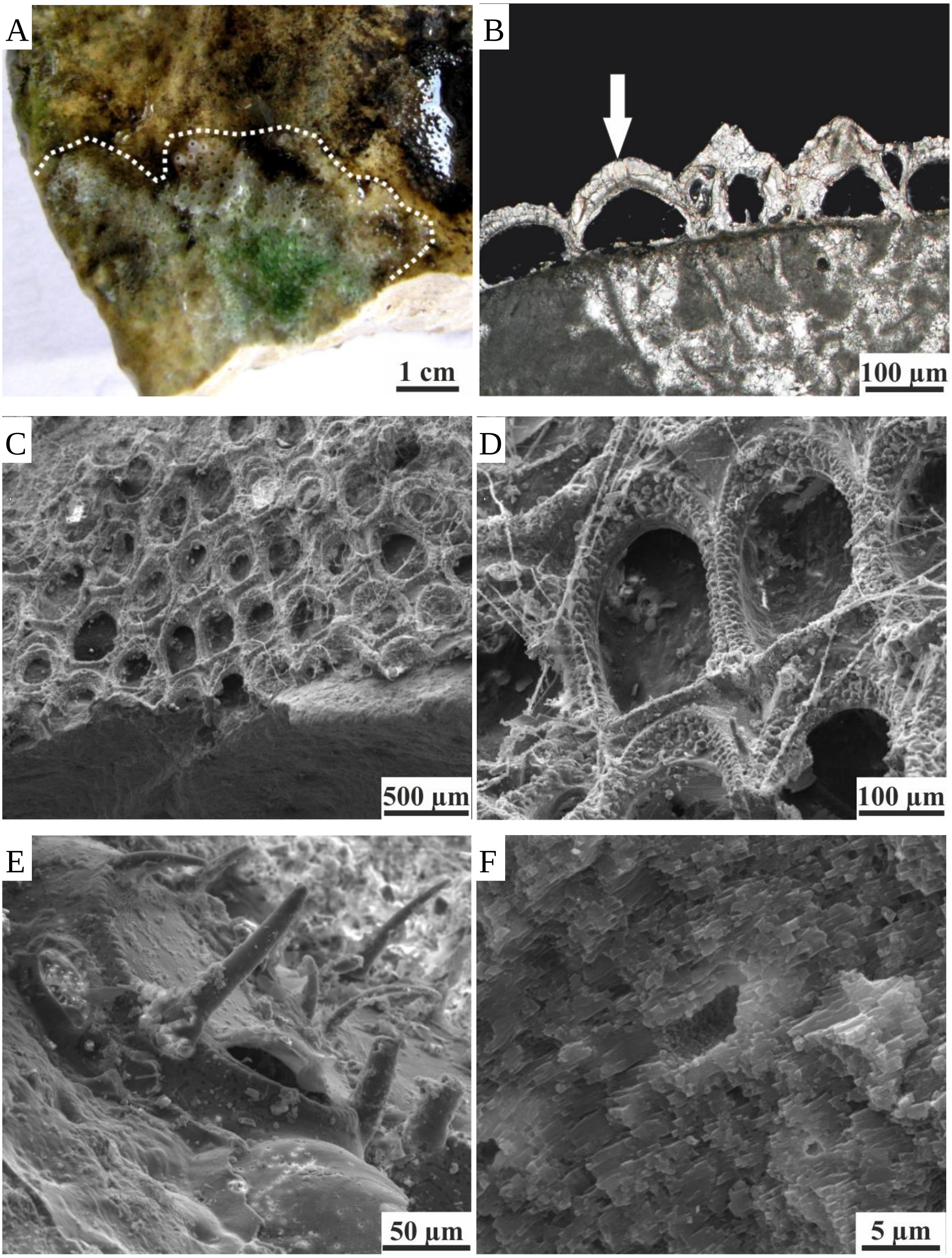

Fig. 4.3 – (A) Bryozoan are widespread on samples surfaces. They appear as a white patina, but often show different colors, as green or black. (B)Transverse section of a colony fixed on rock surface. White arrows indicate the two walls forming zoecium. XPL. (C,D) SEM micrographs show a tangential section of a zoarium partially covered by cyanobacterial filaments. (E) Micrograph shows numerous tentacles around the buccal opening. (F) Thin, isoriented lamellae form the bryozoan skeletal structure.

Diatoms

Diatoms, unicellular photosynthetic algae, represent a diffuse component in analyzed biofilms, where they usually occur as individual organisms.

Occurring diatoms are benthic, belonging to the order *Pennales* (elongated form with bilateral symmetry in valve view) up to 40 µm in length.

Diatoms occur usually encrusted within biofilm among cyanobacterial filaments and neo-formational calcite (Fig. 4.4 A); rarely observed as fixed directly to rock substratum in poor biofilm colonized areas (Fig. 4.4 B).

Diatoms hard parts remains, termed *frustules*, are composed of amorphous silica.

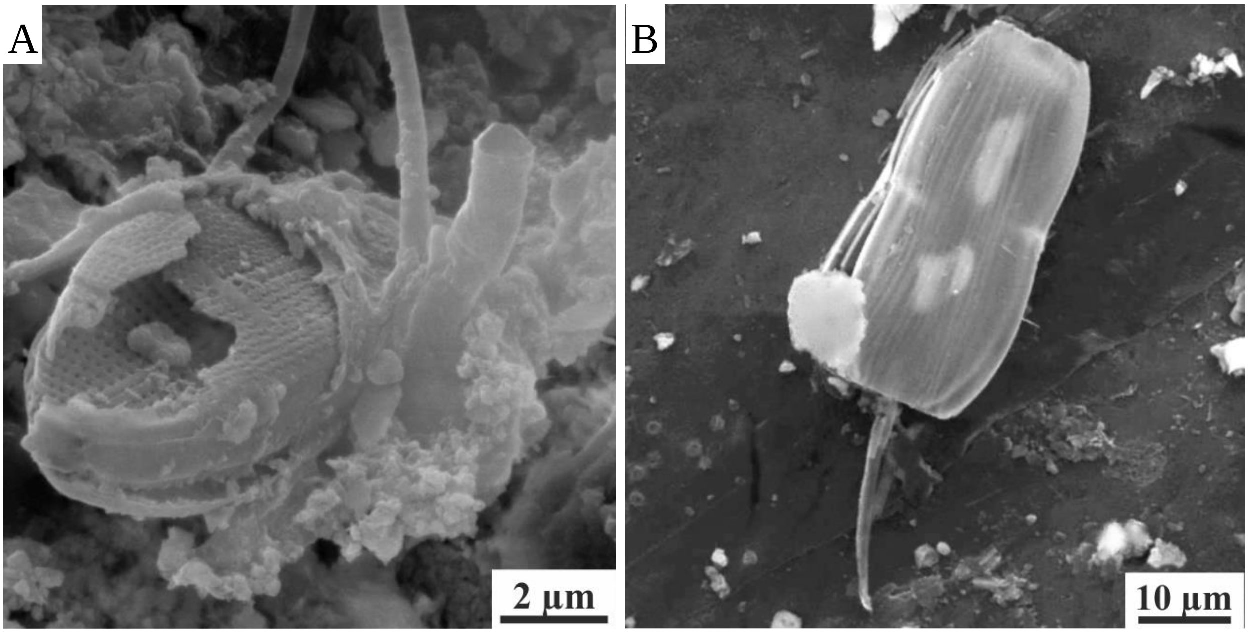

Fig. 4.4 – (A) Photomicrograph shows a diatom encrusted by bacterial filaments and Ca-carbonate crystals. (B) Diatom fixed on rock substratum, with its organic parts still in place.

<u>Other skeletal microorganisms</u>

Others less common skeletal microorganisms have been observed:

- **Annelid worms**, polychaete, with tubular shape and circular cross-section, encrusting hard surfaces are commonly associated with bryozoan (Fig. 4.5 A, B);

- **Coccolithophores**, are unicellular phytoplanktonic algae. They are formed by calcareous exoskeletal plates overlapped each other. Coccolithophores have a diameter of about 3 μm and occur trapped within the EPS of some bacterial colonies (fig. 4.5 C)

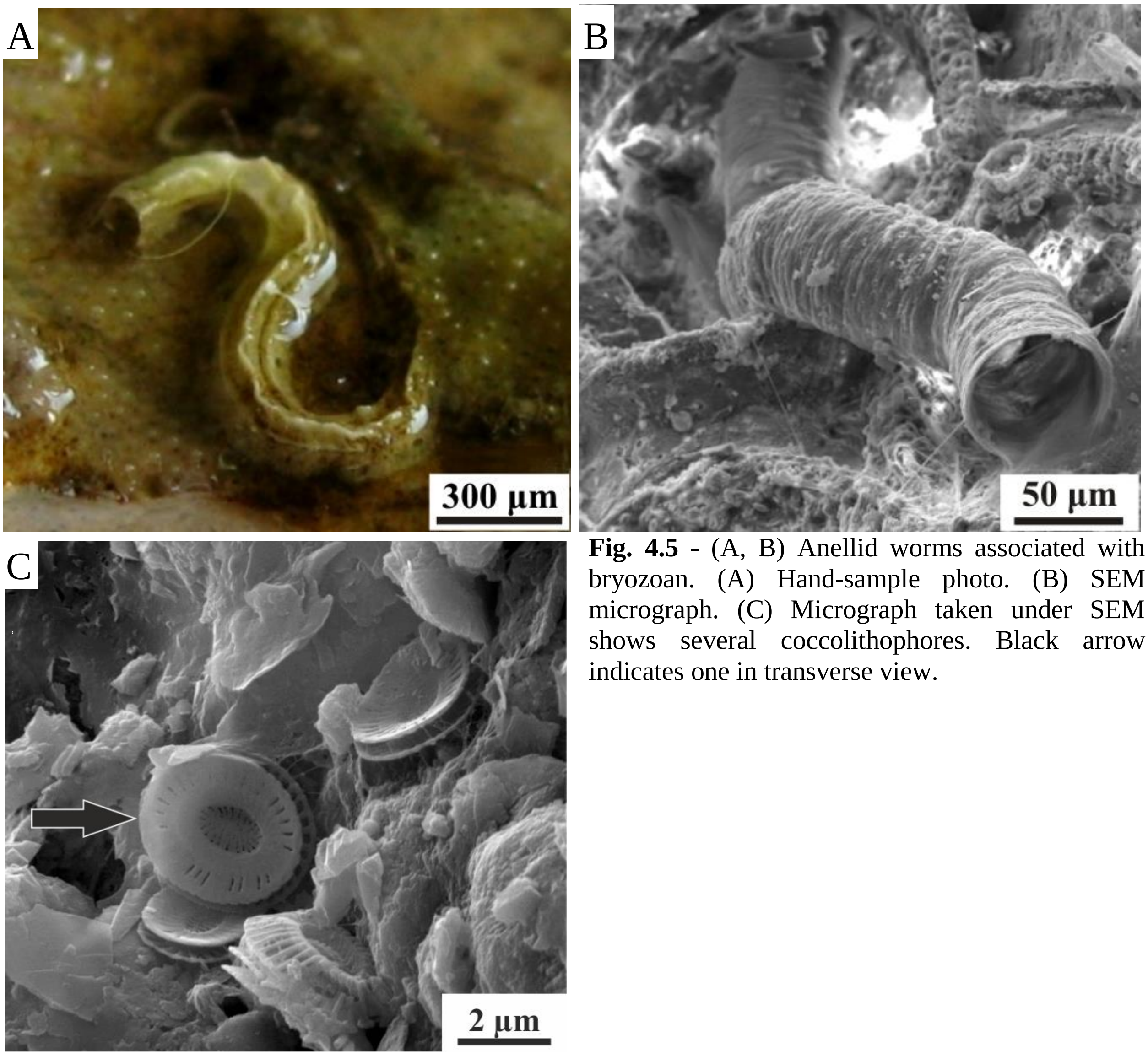

Fig. 4.5 - (A, B) Anellid worms associated with bryozoan. (A) Hand-sample photo. (B) SEM micrograph. (C) Micrograph taken under SEM shows several coccolithophores. Black arrow indicates one in transverse view.

4.2.1.2 Bacteria and organic matter remains

Observed surfaces resulted poor in bacteria, which occurred on few samples, typically in scattered colonies.

Filamentous cyanobacteria occur rarely in patches. These bacteria occur as unbranched single filaments with uniform diameter (≈ 5 µm) and length up to 1 mm (Fig. 4.6 A). Cyanobacteria result partially mineralized by Ca-carbonate neo-formed crystals that grow and develop on their surfaces. On the samples collected in the submerged archaeological area of Monasterace, others common recognized bacteria belong to cocci (Fig. 4.6 B) and bacilli (Fig. 4.6 C, D, E), which often result enclosed in EPS. Extracellular polymeric substance occurs often, but not abundantly, within colonies of coccoid and bacilli as thin filaments.

Cocci have spherical to ovoid shape and diameter up to 700 nm; whereas bacilli occur as rod-shaped up to 3 µm in length (Fig. 4.6 E). Both cocci and bacilli can occur as mineralized by Ca-carbonate nano-crystals (Fig. 4.6 D, E).

Analyzes revealed that this thin mineral covering is made up mainly by Ca-carbonate with ≈ 16 moles % of Mg.

Moreover, microorganism with a hyphal morphology with diameter of filaments < 1 µm, occur in few samples (Fig. 4.6 F). They could belong to actinobacteria and/or fungi.

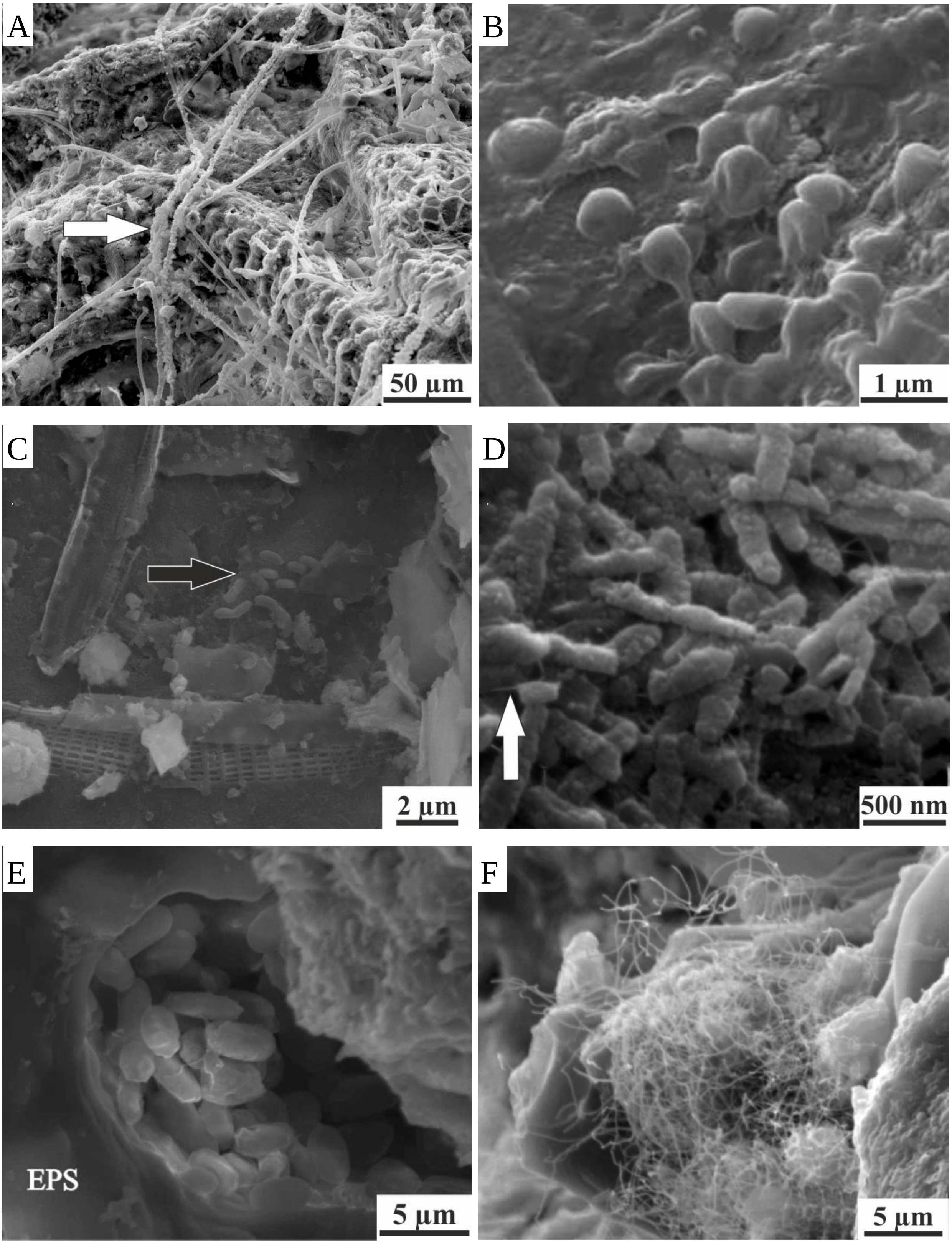

Fig. 4.6 – (A) Filamentous cyanobacteria (white arrow) partially mineralized occur on bryozoan. (B) Coccoids with diameter of about 700 nm, on a calcite sheet deposit. (C) A cluster of bacilli (black arrow) occurring among diatoms rests. (D) Rod-shaped bacteria mineralized by Ca-carbonate nano-crystals. White arrow indicates a filament of EPS. (E) A cluster of rod-shaped bacteria surrounded by EPS which covers them partially with apparently whitish filaments. (F) A rare *shrub* formed by a tangle of thin filaments, belonging probably to actinobacteria.

4.2.1.3 Neo-formed minerals

SEM analyses showed the occurrence of neo-formed minerals, i.e. calcium carbonate and pyrite.

These cited neo-precipitated minerals are widespread, especially Ca-carbonate, over the analyzed samples surfaces both in Monasterace that in S. Maria di Ricadi.

Calcium carbonate elements

Scanning electron microscopy observation on samples surfaces revealed that Ca-carbonate minerals are widespread, and exhibit numerous shapes.

Recognized Ca-carbonate elements can be resumed as follows:

- Sheet deposits;
- Triads deposits;
- Dumbbell- to spherical-shaped.

Ca-carbonate flat deposits up to 10 μm in thickness and hundreds of microns (< 800 μm) width, result common on Monasterace samples, mostly on surfaces where encrusting skeletal organisms, such as red algae and bryozoan are lacking (Fig. 4.7 A). Sometimes, sheets overlap each other, forming thicker crusts of Ca-carbonate (Fig. 4.7 B). These flat deposits are characterized by the presence of numerous kinds of bacteria, such as cocci, bacilli, filamentous and remains of diatoms. In addition, a thin patina of organic matter occurs on the top of deposits, covering bacteria and neo-formed minerals (Fig. 4.7 C).

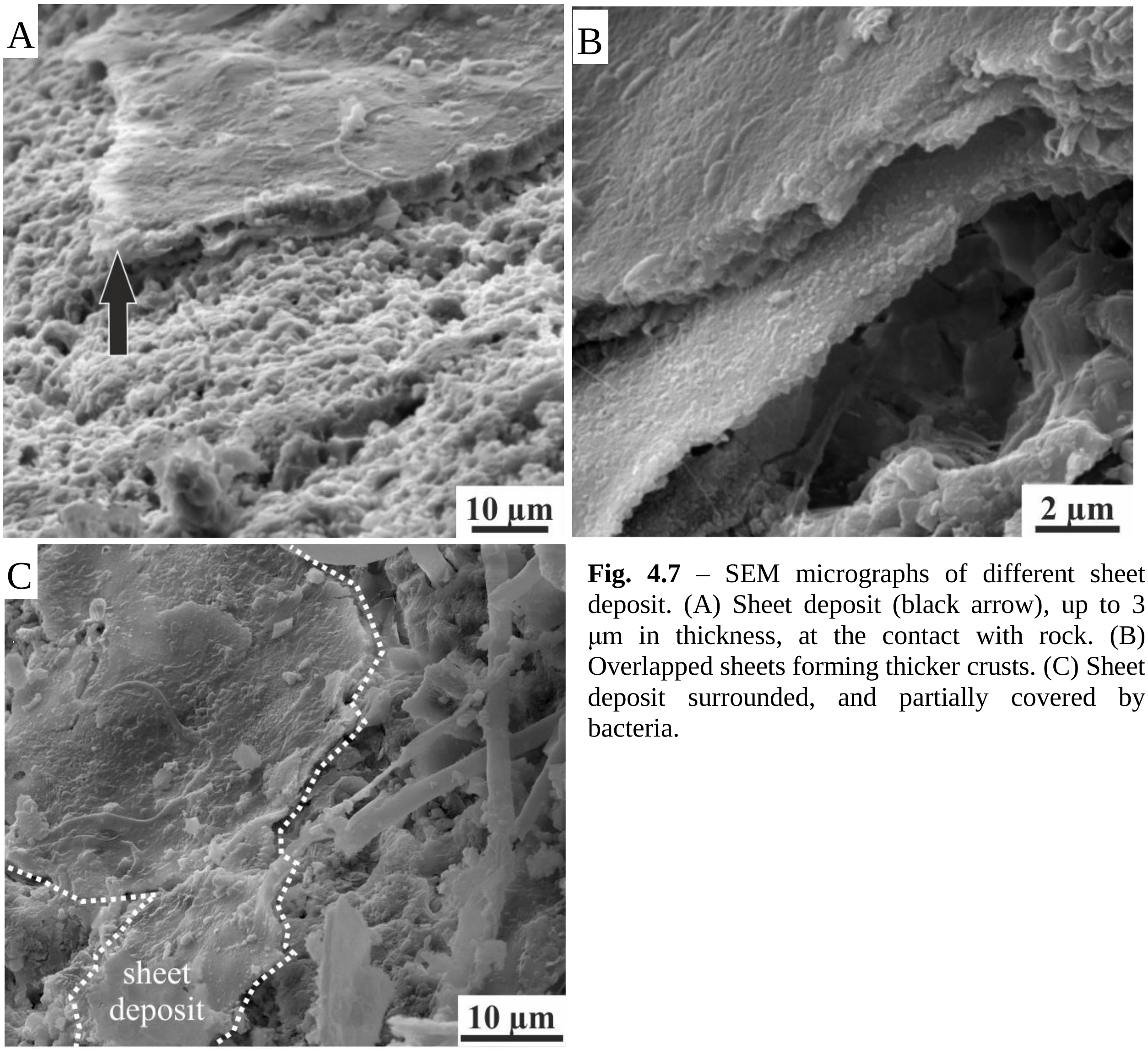

Fig. 4.7 – SEM micrographs of different sheet deposit. (A) Sheet deposit (black arrow), up to 3 µm in thickness, at the contact with rock. (B) Overlapped sheets forming thicker crusts. (C) Sheet deposit surrounded, and partially covered by bacteria.

Differently, deposit formed by triads occur irregular shaped, scattered and rarer. Triads are formed by, more or less defined, rod-shaped crystals growing in geometrical order, diverging from a common cusp and with variable angle of ca. 110-120° between them (Fig. 4.8 B).

Both sheet and triads deposits are formed by Ca-carbonate with high content of Mg (13-17 moles %).

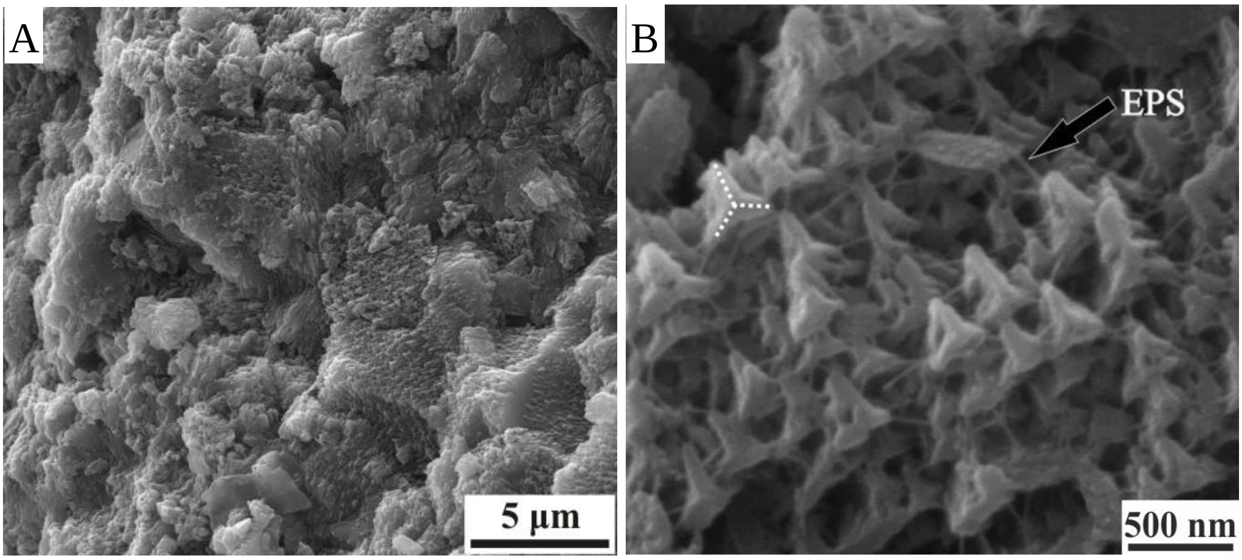

Fig. 4.8 – (A) An irregular deposit formed by triads. (B) Triads grow diverging from a common cusp and forming a variable angle of ca 100-120° between them (white dashed line). Numerous EPS filaments are visible (black arrow).

Dumbbell- to spherical-shaped crystals occur rarely in few samples. They have diameter of about 5 µm (Fig. 4.9 A, B, C), and are close each other forming clusters.

Dumbbell- to spherical-shaped crystals are made up by Ca-carbonate with high content of Mg (≈ 23 moles %) and S (about 4% moles).

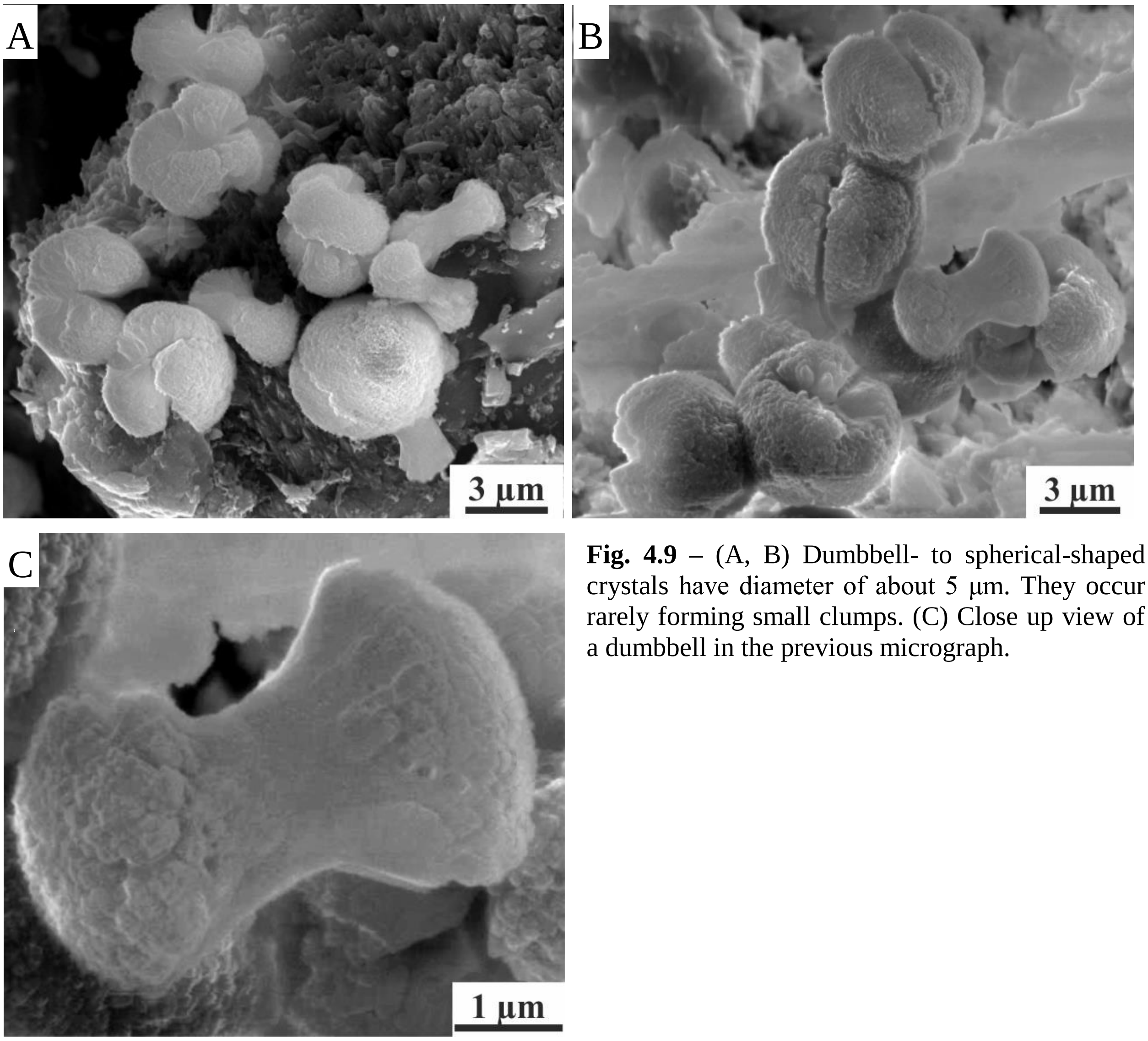

Fig. 4.9 – (A, B) Dumbbell- to spherical-shaped crystals have diameter of about 5 µm. They occur rarely forming small clumps. (C) Close up view of a dumbbell in the previous micrograph.

Observations at higher magnification showed that all the previously described Ca-carbonate elements are formed by sub-spherical to ovoid shaped nano-crystals that vary in diameter from about 50 nm to 200 nm, called formally *nanospheres*. These elemental bodies are widespread patchily above all the samples surfaces.

These primitive shapes of Ca-carbonate may either clot together forming more developed elements or, differently, may even not clot together forming a granular texture (Fig. 4.10 A, B).

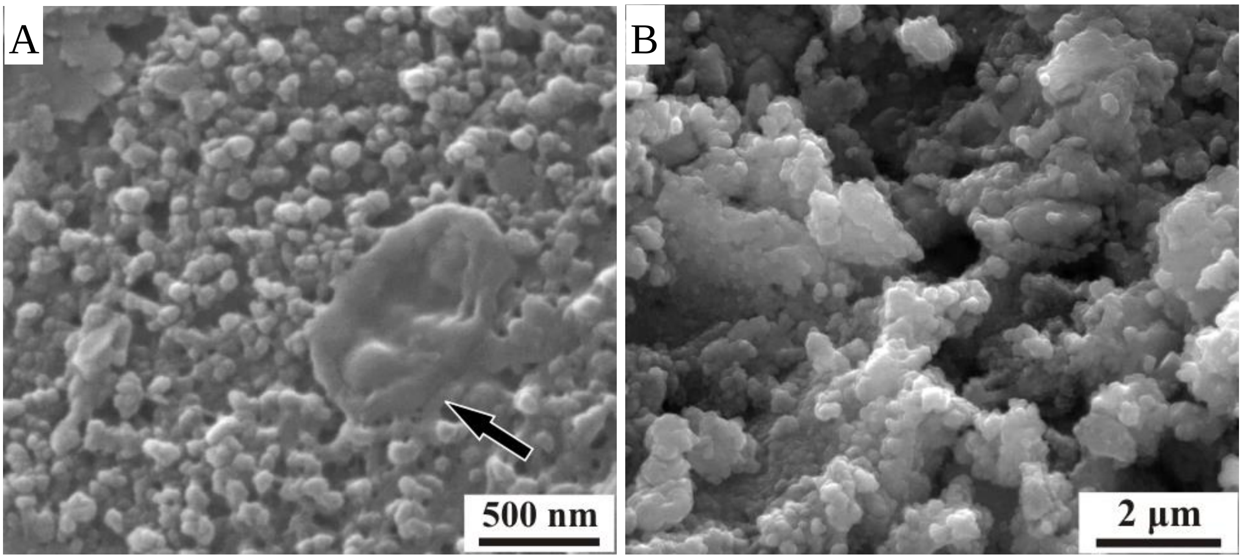

Fig. 4.10 – (A, B) Photomicrographs taken under scanning electron microscope, show calcium carbonate nanospheres, varying in diameter from 50 to 200 nm, form a granular texture. Nanospheres are partially covered by EPS (black arrow).

Pyrite

As observed with optical microscope, numerous pyrite crystals occur in many samples, often in rock fractures up to ≈ 2 mm inward (Fig. 4.11 A). In a calcarenite and in a calcilutite sample, framboids occurred both in fractures, within rock ca. 1 mm below surface, and in hollows (likely conceptacles) of algal skeletons (Fig. 4.11 B).

These spherical bodies, of about 25 µm in diameter, called framboids occur as cluster, forming veins (Fig. 4.11 B, C). Framboids are composed of an assemblage of interlocking minute crystallites of about 500 nm (Fig. 4.11 D, E).

Crystals result often associated with extracellular organic substance that occurs in different quantities (Fig. 4.11 B, D).

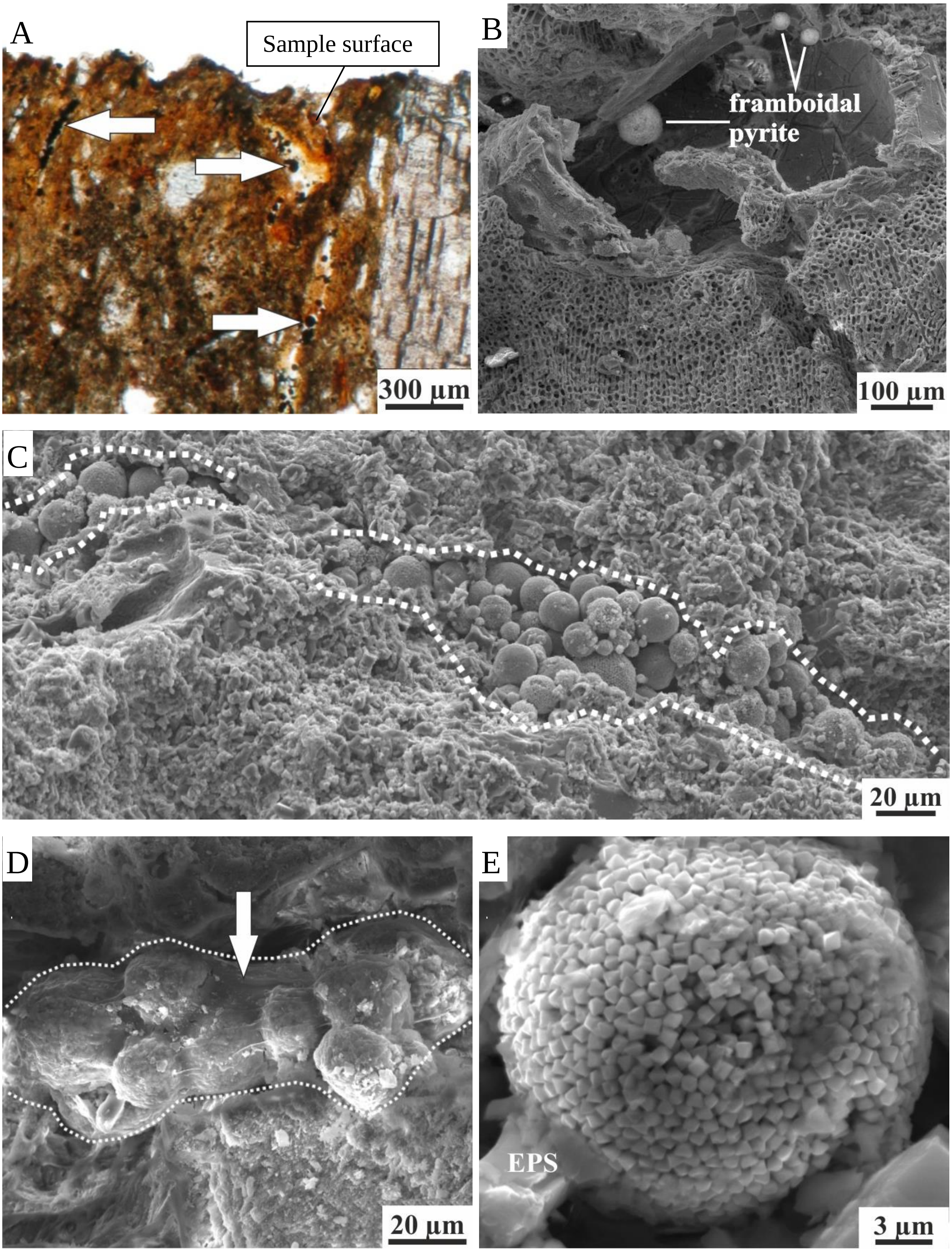

Fig. 4.11 – (A) Photomicrograph taken under optical microscope in plane polarized light. White arrows indicate pyrite, sited within fractures below sample surface. (B) Framboidal pyrite crystals occurring within hollows in algal skeleton. (C) A vein of framboidal pyrite, tightly clotted together, fill a fracture. (D) A framboidal pyrite cluster abundantly surrounded by a film of EPS (indicated by white arrow). (E) A framboidal body, of about 20 µm in diameter, partially covered by EPS. Minute crystals of Fe_2S , less than a 1 µm in diameter, compose framboids.

4.2.2 Biological weathering features

Analyses carried out on Monasterace samples, showed numerous macro- and micro-scale bio-weathering products highly variable in occurrence, dimension and shape from sample to sample. Weathering features occur both on rocks surfaces and on skeletal organisms, such as bryozoan.

Macro-scale bio-products are represented by grooves, often associated to serpulids (polychaetes) that anchor themselves to the substrate (Fig. 4.12).

Micro-scale bio-products, as grooves and boreholes, interest the surficial part of skeletons of red algae, bryozoan and rocky surfaces.

Grooves have variable length, at maximum 1 mm, and width up to 5 µm, whereas boreholes, appearing as hemispheric shaped, exhibit circular sections and diameter ranging from 5 to 10 µm. These products resulted strictly associated with filamentous and coccoid bacteria, which sometimes still lie in the grooves (Fig. 4.13 B). Moreover, boreholes of about 5 µm in diameter occur up to 2 mm below samples surface (Fig. 4.13). The amount of grooves decrease at the increasing of depth.

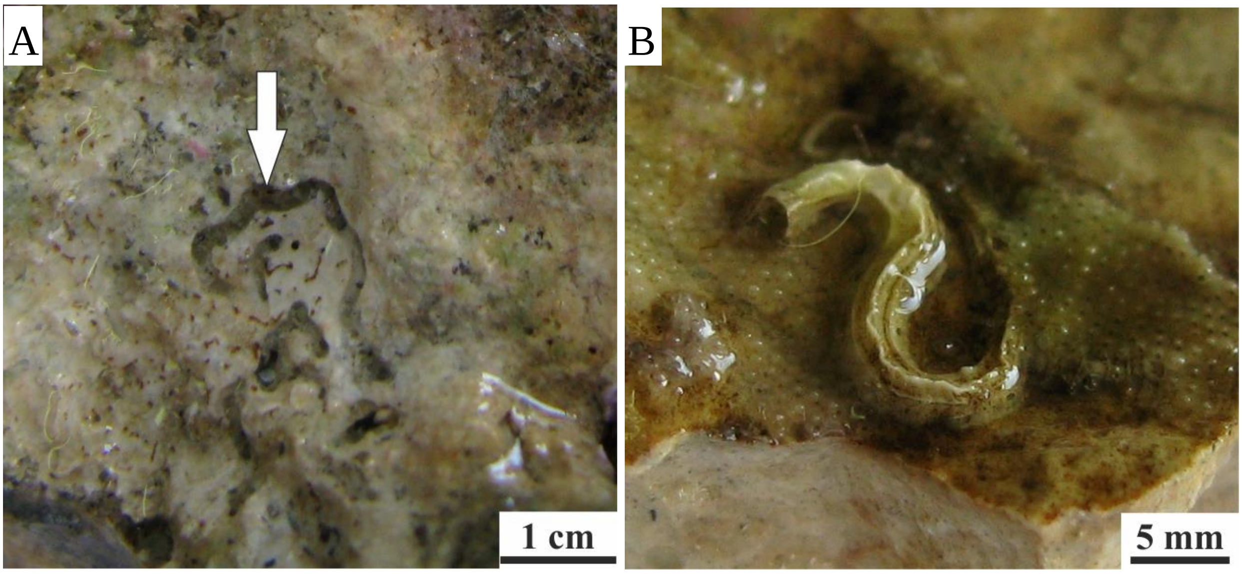

Fig. 4. 12 – (A) Hand-sample view of an etched groove (white arrow) on a sample surface. (B) A serpulid and a bryozoan colony encrust the surface of a sample.

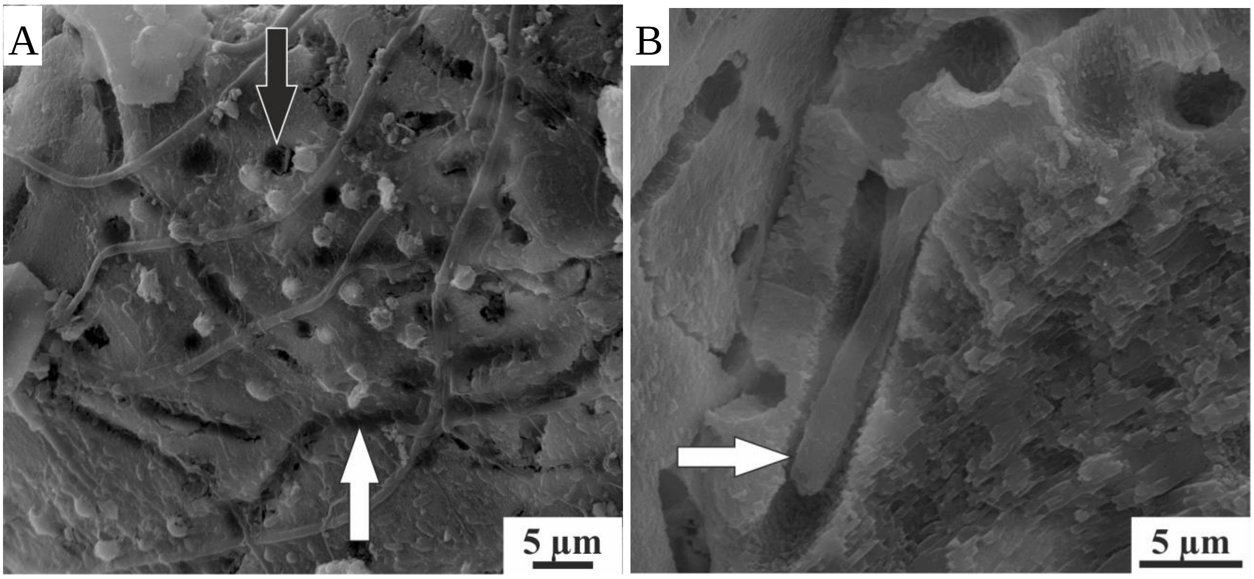

Fig. 4.13 – (A) A bryozoan wall on which occur different products of bio-erosion: A borehole (white arrow) and a groove (black arrow). (B) Cross section of bryozoan wall viewed in the SEM. White arrow indicates a rest of a cyanobacterium still lying within a bio-eroded groove.

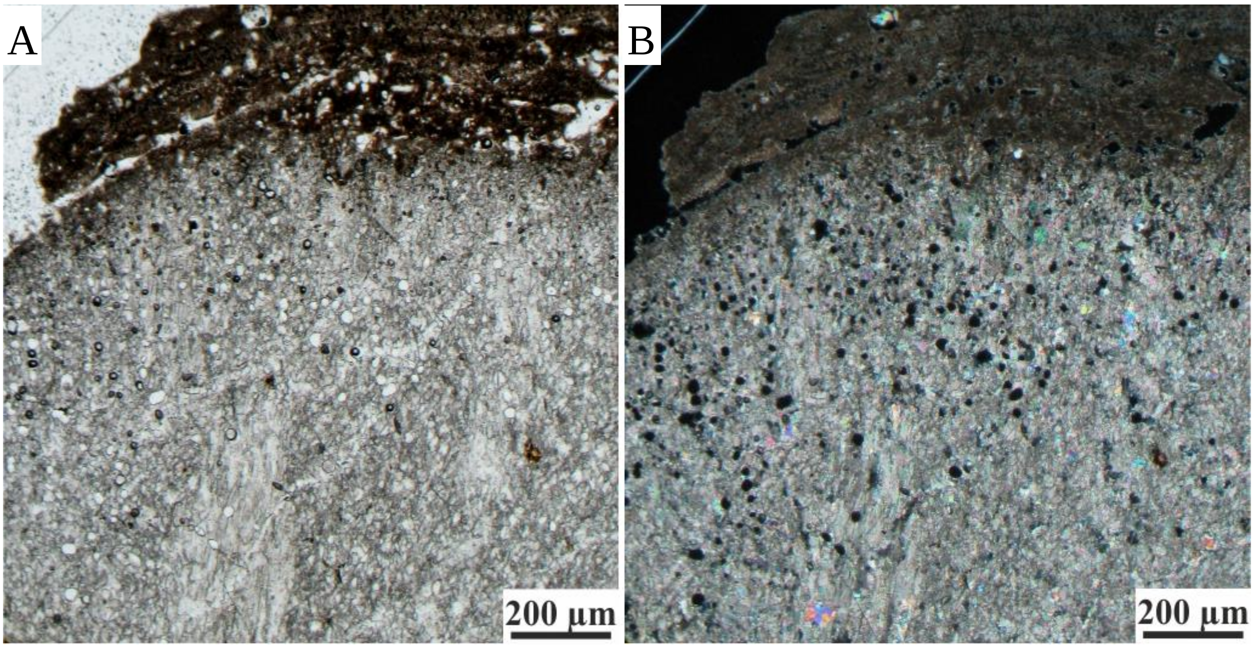

Fig. 4.14 – (A, B) Photomicrographs taken from a thin section, in plane polarized light (left) and cross polarized light (right). Bore holes, appearing as black spots, occur numerous below the algal encrustation up to 1 mm below the surface (brown color).

4.3 Santa Maria di Ricadi

Samples collected from the conglomerate outcropping in Santa Maria di Ricadi bay are composed mainly by quartz, K-feldspar and biotite, cemented together by a thin brownish to yellowish carbonate micritic cements (Fig. 4.15).

Biofilms grown up on the top of S. Maria di Ricadi samples are much more extended and thicker than those occurring on Monasterace samples.

At naked-eye biofilm is 1.5 cm thick and olive-green to bright-green color, interspersed with reddish patches, extended from the top to the bottom of it. Filamentous, highly branched, likely brown algae (*Phaeophyceae*) and filamentous bacterial community result enclosed and bound together by abundant extracellular polymers conferring typical gelatinous aspect (Fig. 4.16).

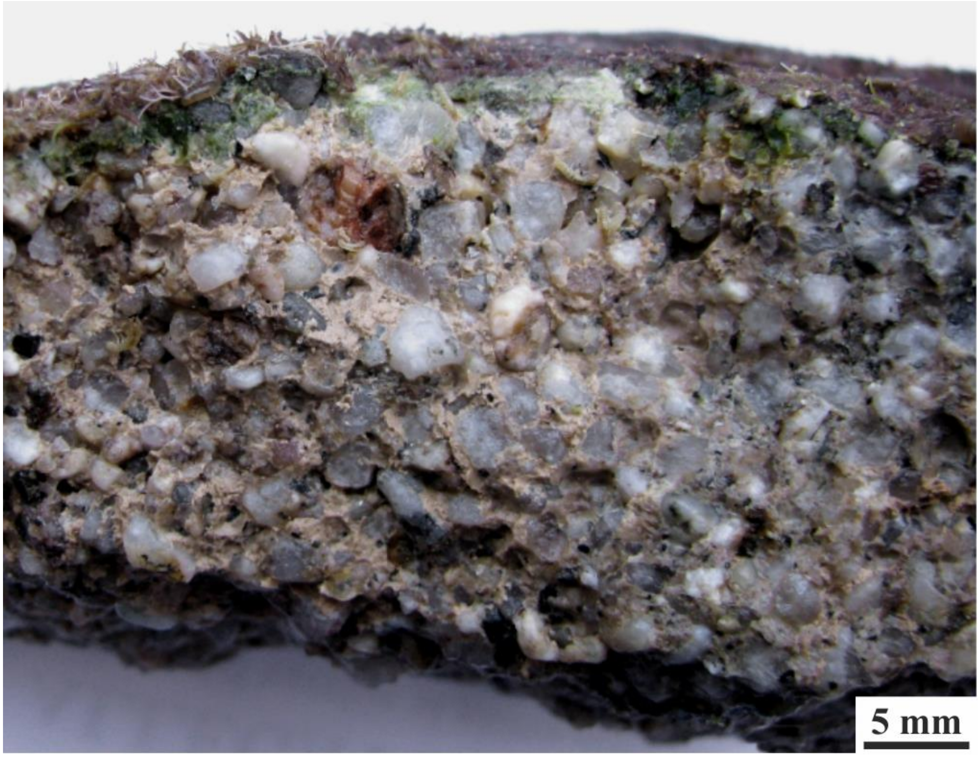

Fig. 4.15 - (A) Hand-sample taken from the conglomerate outcropping in Santa Maria di Ricadi foreshore, air-dried for a couple of days..

Fig. 4.16 - Biofilm occurring on S. Maria di Ricadi samples.

Furthermore, this biofilm is characterized by the presence of allochtonous grains trapped and bound together by microorganisms and EPS, and sometimes covered by neo-precipitated minerals that give them a granular aspect under SEM analyses (Fig. 4.17). Grains consist mostly of terrigenous clasts and bioclasts with diameter < 100 µm.

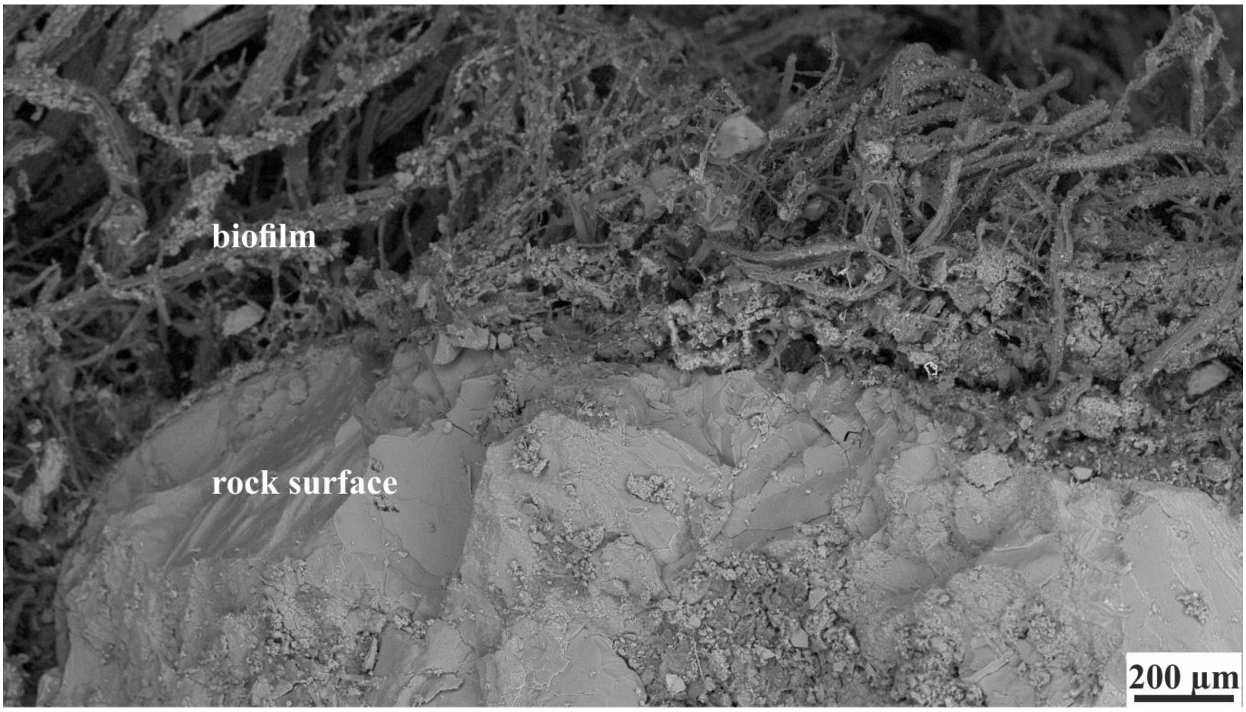

Fig. 4.17 – Micrograph taken under SEM, showing part of biofilm at the contact with sample surface.

4.3.1 Components of the biofilm

4.3.1.1 Skeletal organisms

Diatoms

Diatoms, unicellular photosynthetic algae, are less common in the Santa Maria di Ricadi outcrop than Monasterace.

They occur individually or in clusters, fixed to bivalve exoskeleton (Fig. 4.18). Occurring diatoms are benthic, belonging to the order of *Pennales*, varying from 20 to 25 μm in length.

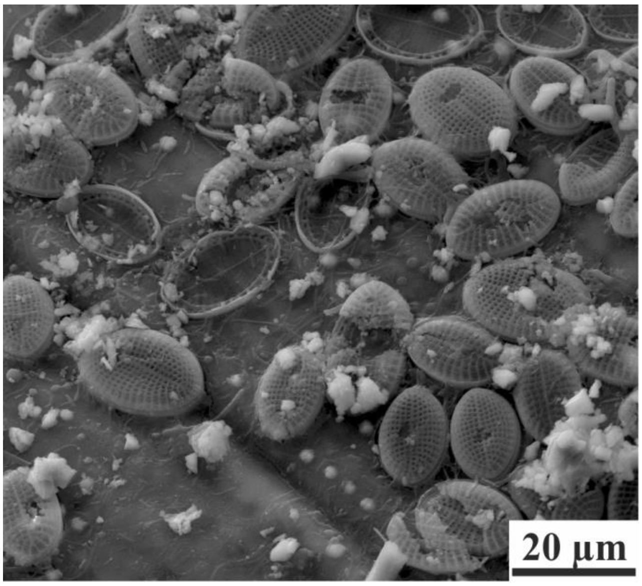

Fig. 4.18 - Diatoms encrusting a valve are partially covered by probable abiogenic calcite neo-precipitate crystals.

Bivalves

Bivalves occurring in S. Maria di Ricadi samples, belonging to the family *Mytilidae*, are conspicuous and easily recognizable, even in hand sample (Fig. 4.19 A). Bivalves have uniform dimension, with an average length of ≈ 1.5 mm (Fig. 4.19 B). They occur attached especially to air-exposed rocks in the upper part of the inter-tidal zone. In addition, bivalves are often used as hard substrate for other sessile organisms, like diatoms and cyanobacteria (Fig. 4.18).

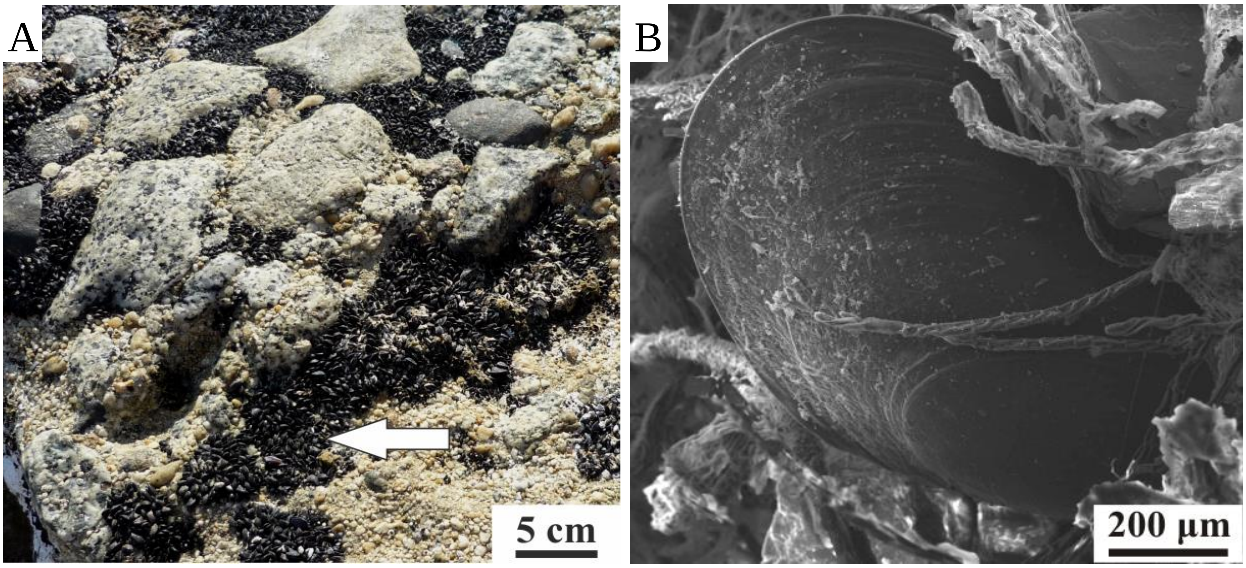

Fig. 4.19 – (A) Rock surface viewed in the field. White arrow indicates the colony of bivalves encrusting rock surface. (B)SEM photomicrograph of left valve of bivalve, partially enclosed by plants.

4.3.1.2 Bacteria and organic matter remains

S. Maria di Ricadi surfaces samples are characterized by plentiful occurrence of cyanobacteria.

Filamentous cyanobacteria are the primary authors of a complex and dense biofilm, in which they trap and bind few detrital clasts.

A vast majority of these filaments result non-mineralized, lacking in EPS, occurring from single isolated filaments to densely arranged, tube-like shaped, long up to 700 µm in length and ≈ 6 µm in diameter (Fig. 4.20).

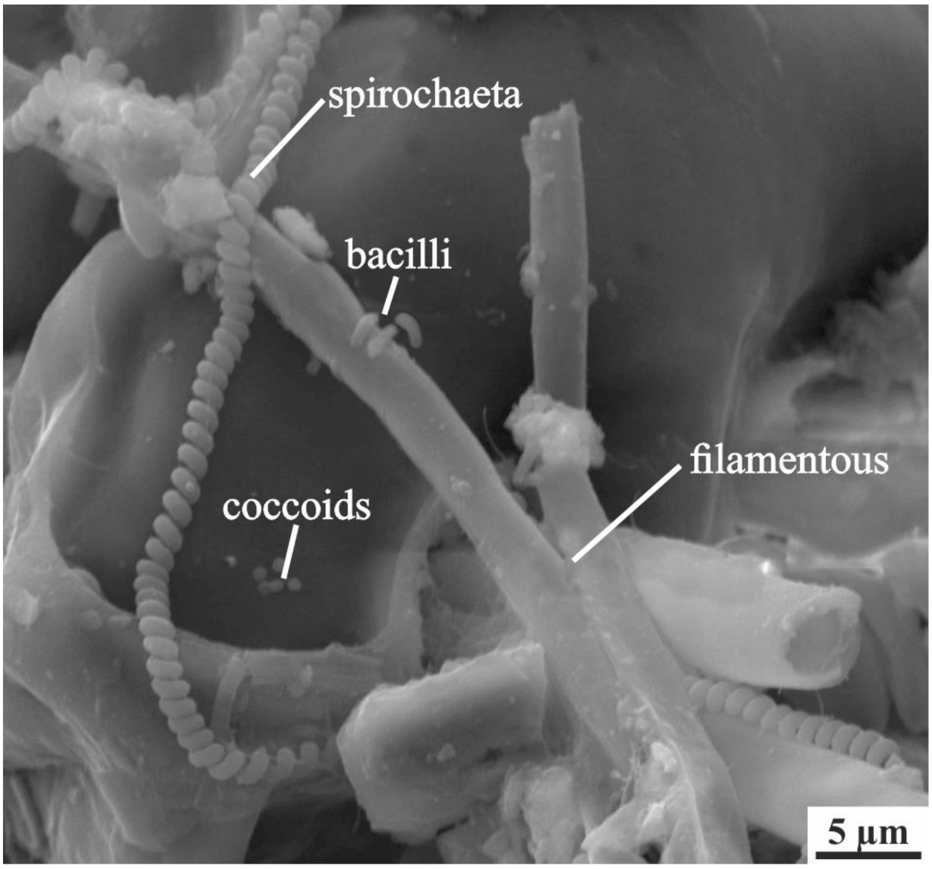

Fig. 4.20 - Micrograph shows different bacterial shapes occurring in the biofilm.

Moreover, many other kinds of bacteria have been observed under scanning electron microscopy, such as:

- Cocci, sub-spherical to ovoid shaped with ≈ 800 nm diameter (Fig. 4.20);
- bacilli, rod-shaped and slightly curved ≈ 2 µm long (Fig. 4.20);
- spirochaete, corkscrew-like shaped with length up to 200 µm and diameter of 2 µm (Fig. 4.20).

Differently, few others filamentous bacteria are strongly mineralized by small Ca-carbonate crystals (<1 µm in diameter) tightly clot together forming a crust (Fig. 4.21).

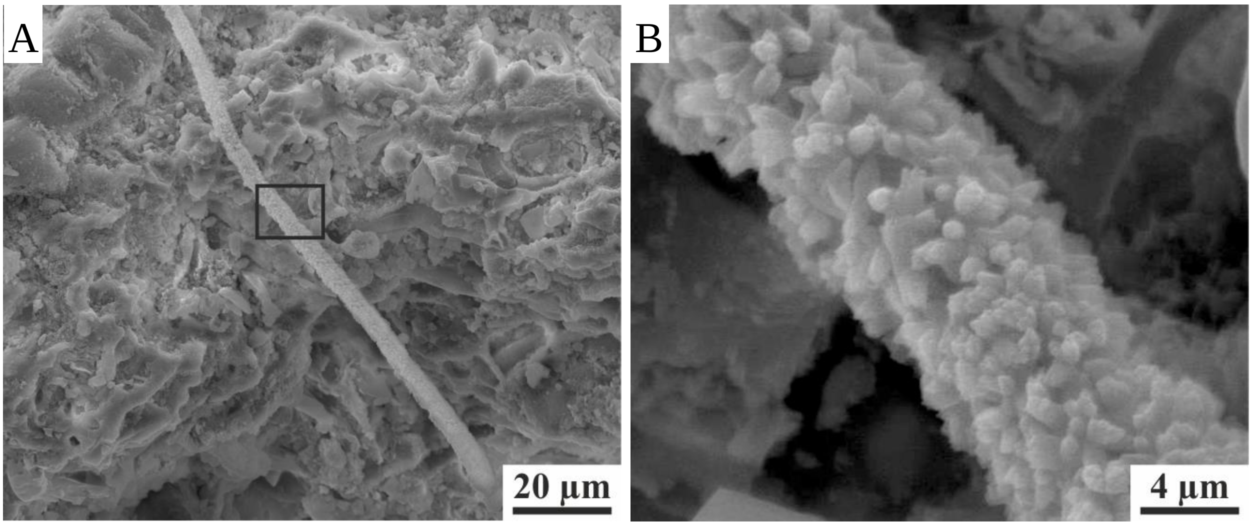

Fig. 4.21 – (A) Micrograph, taken under the SEM, shows a mineralized cyanobacterial filament. (B) Close up view of black rectangle in (a) shows the strong encrustation of calcite interesting filament.

Organic matter, in the form of polymeric substance secreted by bacteria (EPS), is widespread on the top of the samples where envelopes the living biofilm.

On the contrary, it occurs scarcely as filaments or small masses among neo-formed Ca-carbonate crystals.

EPS occurs within grooves, made up by microorganisms on rock surface, strongly mineralized sheathes, forming inner molds of grooves (Fig. 4.22). Sheathes are ≈ 250 nm in thickness, mineralized by Ca-carbonate nanospheres tightly clotted to each other. Nanospheres vary from 20 to 200 nm in diameter (Fig. 4.22 B).

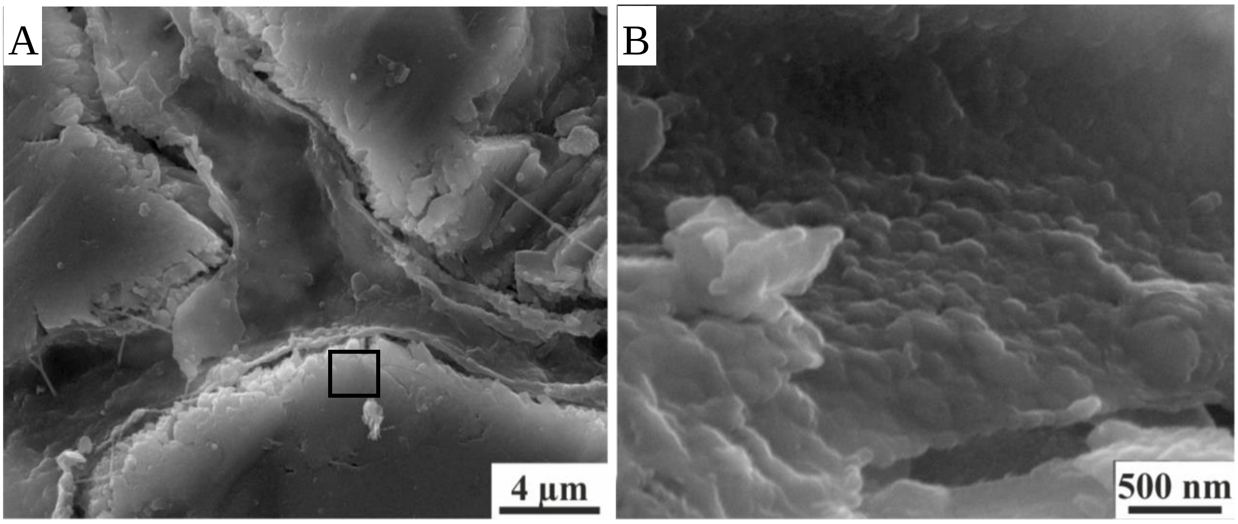

Fig. 4.22 – (A) Mineralized EPS sheath (white arrow) remain occurring within grooves. (B) Close up view of the black square of A, shows the strong mineralization operated by nanospheres.

4.3.1.3 Neo-formed minerals

Calcium carbonate elements

Samples collected in S. Maria di Ricadi outcrop are compositionally very similar to each other. Rocks forming the outcrop are clast-supported by terrigenous clasts, such as quartz, K-feldspar, biotite and carbonate grains as pelletoidal limestone.

Different types of cements were distinguished within spaces among clasts.

Isopachous micritic cements, with aphanitic texture, envelop all the clasts, forming a coating up to 20 µm in thickness, for the most part laterally continuous (Fig. 4.23).

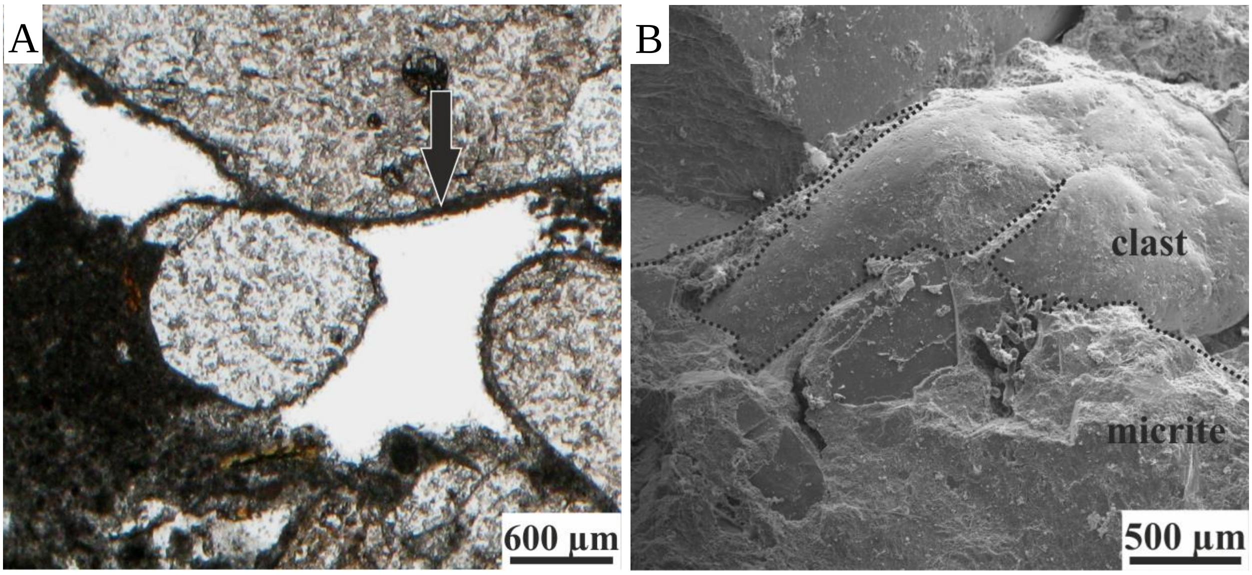

Fig. 4.23 – (A) Micritic crusts, indicated by black arrow, surround clasts (PPL). (B) Micrograph taken under SEM. Micrite covering partially a K-feldspar clast.

Furthermore, peloidal micrite fills either partially or totally voids among clasts. Peloids vary from 30 to 40 µm in diameter, from sub-rounded to ovoid shape and occasionally clot together forming shrub-like or denser structures (Fig. 4.24).

Aphanitic and peloidal deposits are composed of Ca-carbonate with ≈ 4 moles % of Mg and ≈ 1 moles % of S.

These different types of neo-formed mineral deposits are made up by an assemblage of minute nano-spheres up to 150 nm in diameter.

Thin fringes (up to 30 μm in thickness) formed by subhedral micro-sparite crystals and, rim both aphanitic and peloidal deposits (Fig. 4.26).

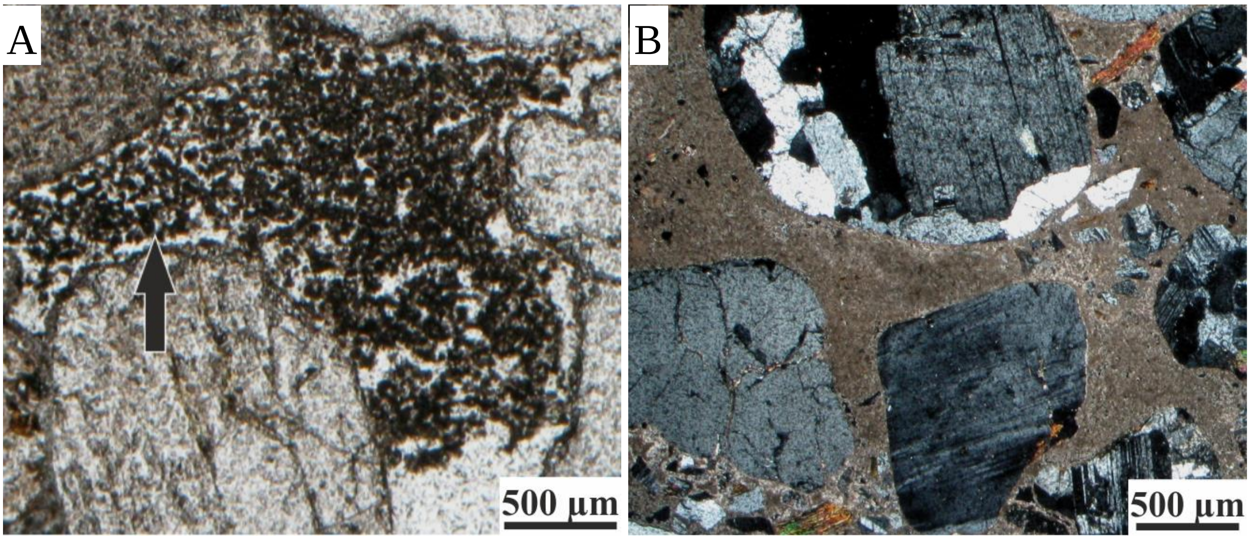

Fig. 4.24 – (A) Micrograph taken under optical microscope in plane polarized light. Black arrow indicates peloidal micrite, with shrub-like texture, filling partially void among clasts. (B) Micrograph in cross polarized light showing a dense deposit of micrite (brown in color) filling almost entirely voids among clasts.

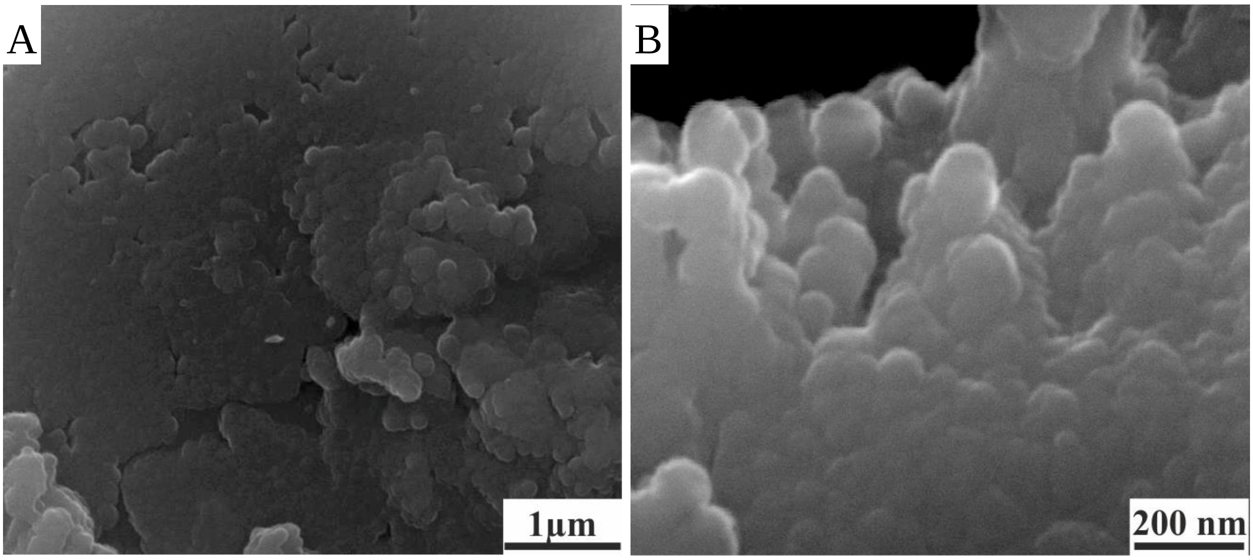

Fig. 4.25 – (A, B) Ca-carbonate deposits made up by an assemblage of minute nano-spheres up to 150 nm in diameter.

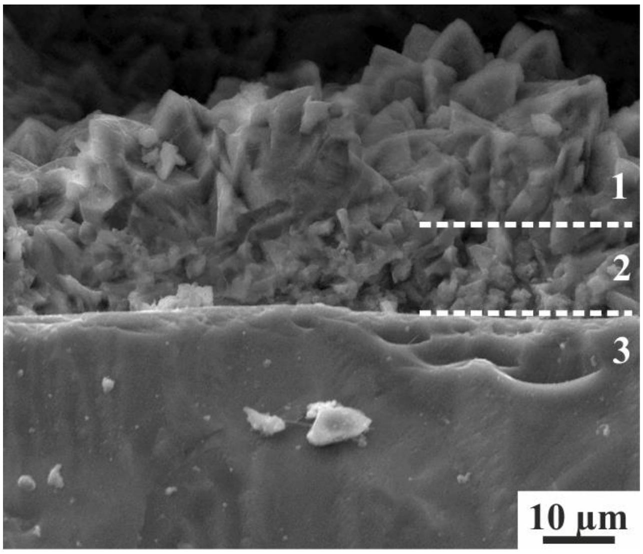

Fig. 4.26 – Ca-carbonate deposits on a clast. (1) Microsparitic fringe; (2) micritic cements with aphanitic texture; (3) clast.

4.3.2 Biological weathering features

Biological weathering processes interest Santa Maria di Ricadi samples from rock surface, at the contact with the biofilm, to 1 cm inward. Physical (mechanical) alteration interests essentially Ca-carbonate deposits among grains, where reeds of aquatic plants plug in fractures and rock weakness.

Bivalves, that occur numerous, fix themselves on rock surfaces causing bores on the substrate. Scanning electron microscopy observations permitted to appreciate the strong biological alteration that occurs at the micro-scale up to 1 mm in depth from the rock surface.

Micro-erosion is very evident on Ca-carbonate deposits surfaces, which are furrowed by numerous grooves forming intricate textures (Fig. 4.27 A, B).

These grooves are characterized by having abundant and strongly mineralized organic mucus, previously described and showed in Fig. 4.22, and by few desiccated filamentous cyanobacteria remains, occurring horizontally, oriented parallel to the substrate surface. The network of furrows (5–8 μm in diameter) radiate and repeatedly bifurcate in a uni- or bilateral mode in angles between 45 and 90◦. Grooves may exhibit slight terminal swellings (up to 10 μm in diameter). Along them, many characteristic lateral swellings or distinct nodular appendices (up to 10 μm in width) are developed. (Fig. 4.27 C).

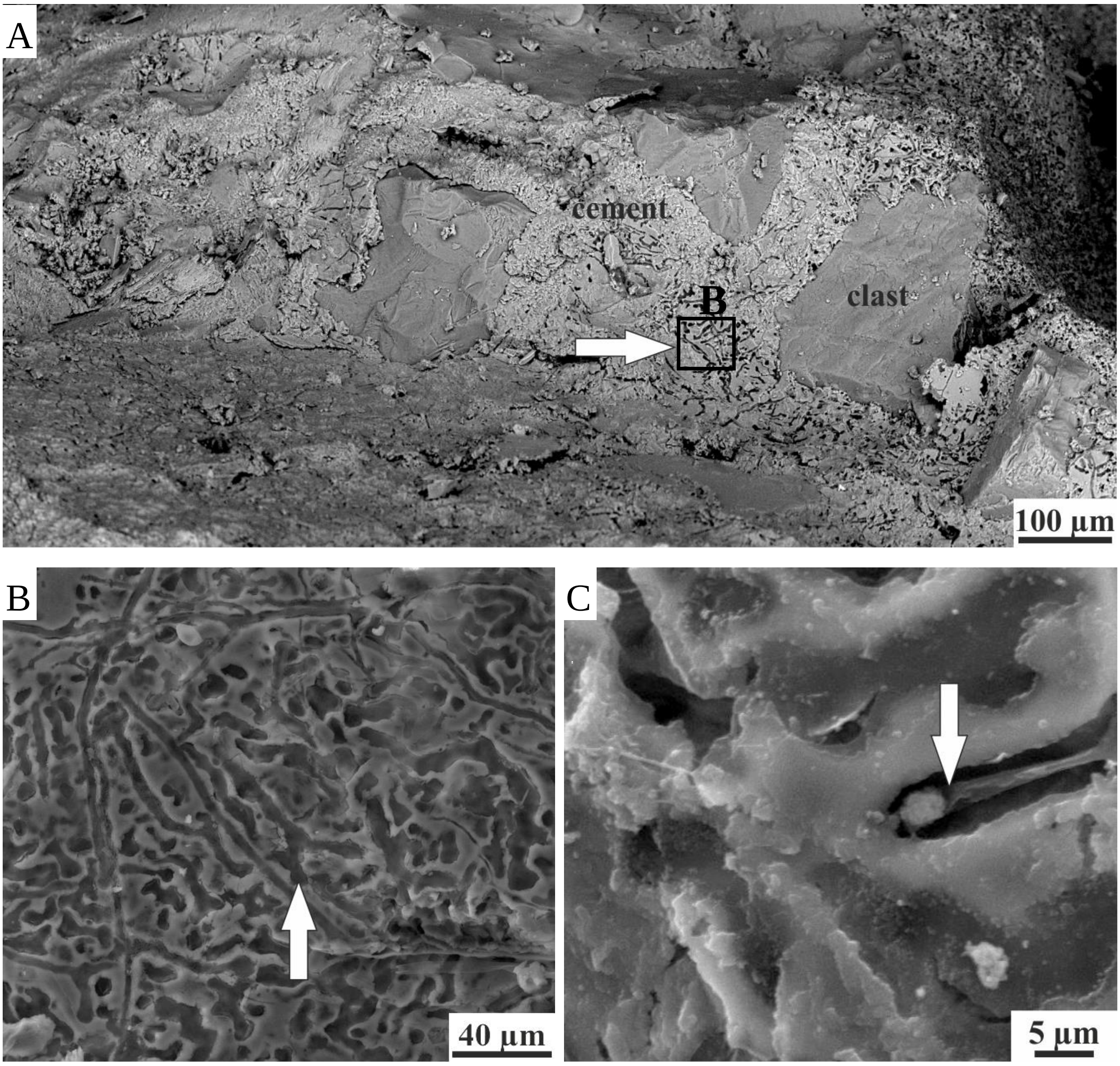

Fig. 4.27 – (A) Exposed surface shows some clasts partially surrounded by Ca-carbonate. Micrite is characterized by numerous grooves (white arrow). (B) Close up of the previous view shows the intricate textures, formed by grooves, appearing as darker than sample surface under SEM image. (C) A desiccated filamentous cyanobacterium remain (white arrow) still in living place within a groove.

Differently, quartz clasts show several alteration areas, irregular in extension and depth (Fig. 4.28 A), on which are present coccoid cyanobacteria colonies and EPS filaments remains (Fig. 4.28 B).

These areas differ from typical mechanical alteration, which show normally sharper and linear edges (Fig. 4.28 C).

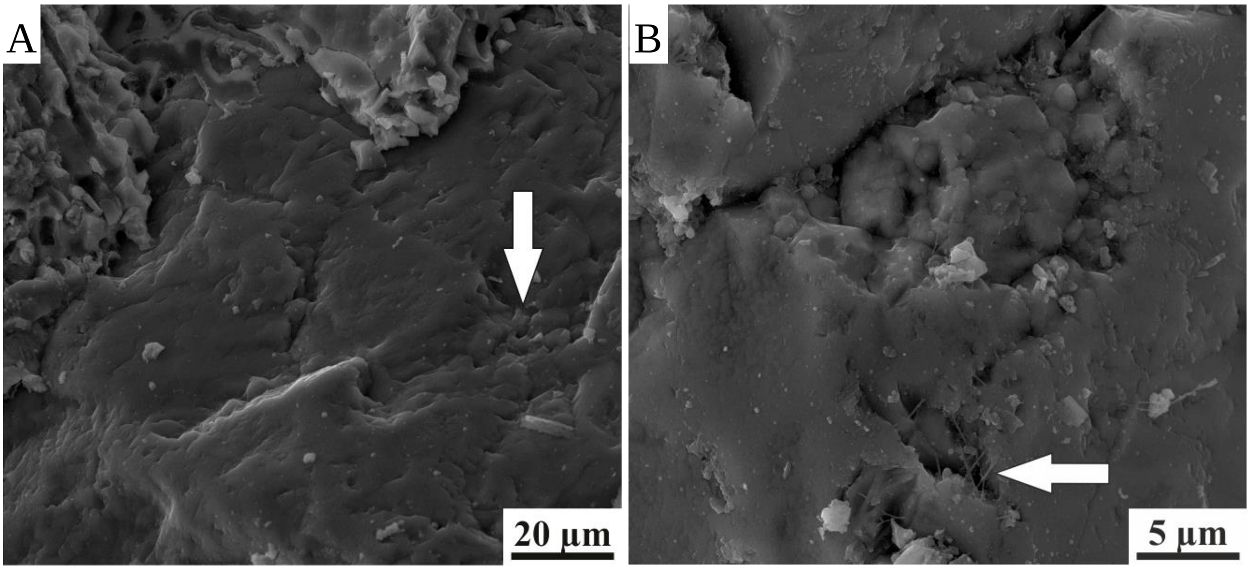

Fig. 4.28 - (A) A quartz grain showing several alteration areas irregular in extension and depth (white arrow). (B) EPS filaments remains e Ca-carbonate nanospheres occur numerous in some alteration irregular areas (white arrows).

CHAPTER 5

DISCUSSION

5.1 Biofilms formation

In the present study, observations carried out on rock samples collected in Monasterace and Santa Maria di Ricadi sites, show that biofilms are characterized by numerous skeletal organisms, bacteria, different products of precipitation and many others of bioerosion. Furthermore, as suggested by several authors (e.g. Awata, 2012), sampling has been performed during summer, when environmental parameters, such as temperature, salinity, nutrient availability and light intensity are higher than the rest of the year, influencing positively the growth of microbial biofilm.

Samples collected in the submerged archaeological park of Monasterace, resulted very heterogeneous in composition (calcilutites, calcarenites, marble, tiles, calc-schists).

As well, every biofilm, characterizing analyzed samples, represents a unique microstructure varying in thickness, color and superficial extension, suggesting variation in type and quantity of composing elements. Nevertheless, biofilms observed in Monasterace can be synthetized in an “ideal biofilm” in which is possible to indicate a formational order of every category of elements characterizing biofilms (Fig. 5.1).

Furthermore, this ideal biofilm is subdivisible in different layers such: Rocky substrate; bacteria, EPS and neo-formed minerals; skeletal organisms (Fig. 5.2).

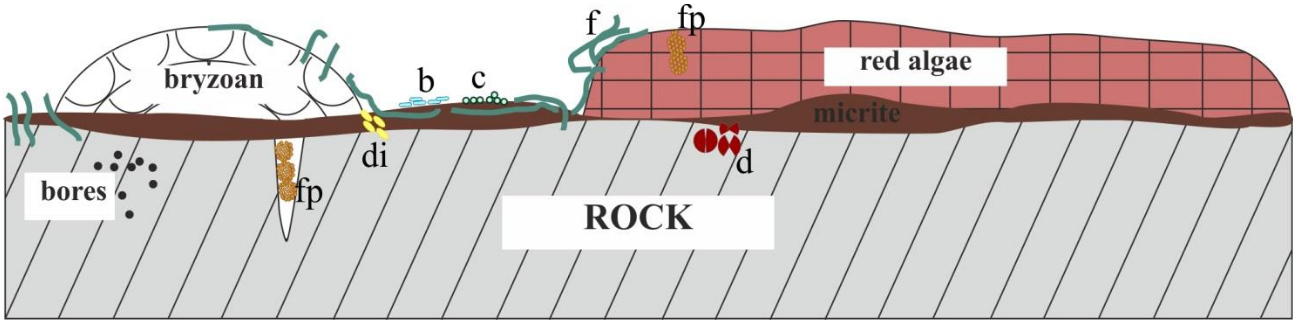

Fig. 5.1 – Ideal cross-section of a Monasterace rock sample showing organisms and products that form biofilm. (b) bacilli; (c) coccoids; (f) filamentous; (d)dumbbell to spherical crystals; (fp) framboidal pyrite; (di)diatoms.

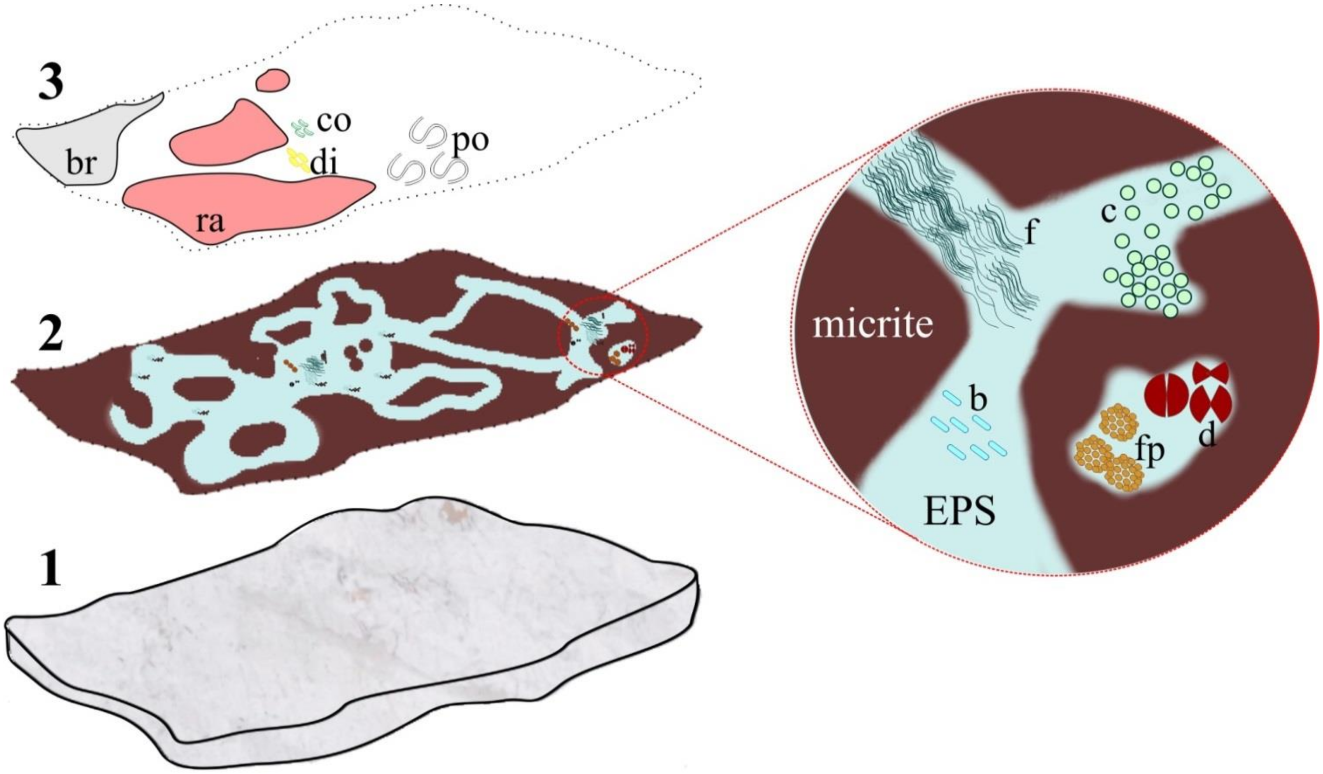

Fig. 5.2 – Formational model of Monasterace biofilms. (1) Rocky substrate. (2) EPS and neo-formed minerals layer. Filamentous (f), coccoid (c) and bacilli (b) bacteria occur associated with EPS (light blue), and where it is degraded form neo-precipitated minerals, such as micritic sheet deposits (brown), dumbbell to spherical shaped minerals (d), and framboidal pyrite (fp). (3) Skeletal organisms layer. red algae (ra), bryozoan (br), diatoms (di), coccolithophores (co) and polychaete (po) often overlie bacterial and micritic sheets. Elements are not in scale.

Rock surfaces result characterized by the presence of boreholes up to 2 mm in depth and by framboidal pyrite within fractures.

In the second layer epilithic and euendolithic forms, such as filamentous cyanobacteria, scattered colonies of cocci, bacilli and spyrochaete bacteria occur on rocks surface. Bacteria, though dimensionally irrelevant, as occupying few tens of micrometers in scattered areas, contribute notably to the formation and stability of biofilm. In fact, they are often the first organisms colonizing rocks in marine environments and the main producers of the extracellular organic substance which allows communities to attach to surfaces, physically stabilizes microbial cells under variable hydrodynamic regimes and may also help the microbial mat community to resist multiple environmental stress conditions (Mur, 1999; Decho et al., 2005).

Neo-formed Ca-carbonates occur as sheet and triads deposits, frequently associated with EPS and bacteria.

In third layer, skeletal organisms such as red algae, bryozoan, diatoms, coccolithophores and polychaete are chronologically the last organisms to settle, often superimposing on micritic deposits or directly on rock surfaces previously colonized and bored by filamentous cyanobacteria and diatoms (Fig. 5.3).

When they adhere to the preexisting biofilm, they do not oust bacteria, which, contrariwise, stabilize on skeletal organisms causing also the alteration of these latter ones.

Furthermore, framboidal pyrite crystals are present both in rock fractures and in algal hollows, likely conceptacles (Fig. 5.4). As regards samples collected in Santa Maria di Ricadi, these are composed essentially by micritic cements and clasts such as quartz, K-feldspar and biotite.

Also for Santa Maria di Ricadi biofilms has been possible to elaborate a ideal model (Fig. 5.5). This ideal model is subdivisible in different layers, showing fundamental elements forming biofilm (Fig. 5.6).

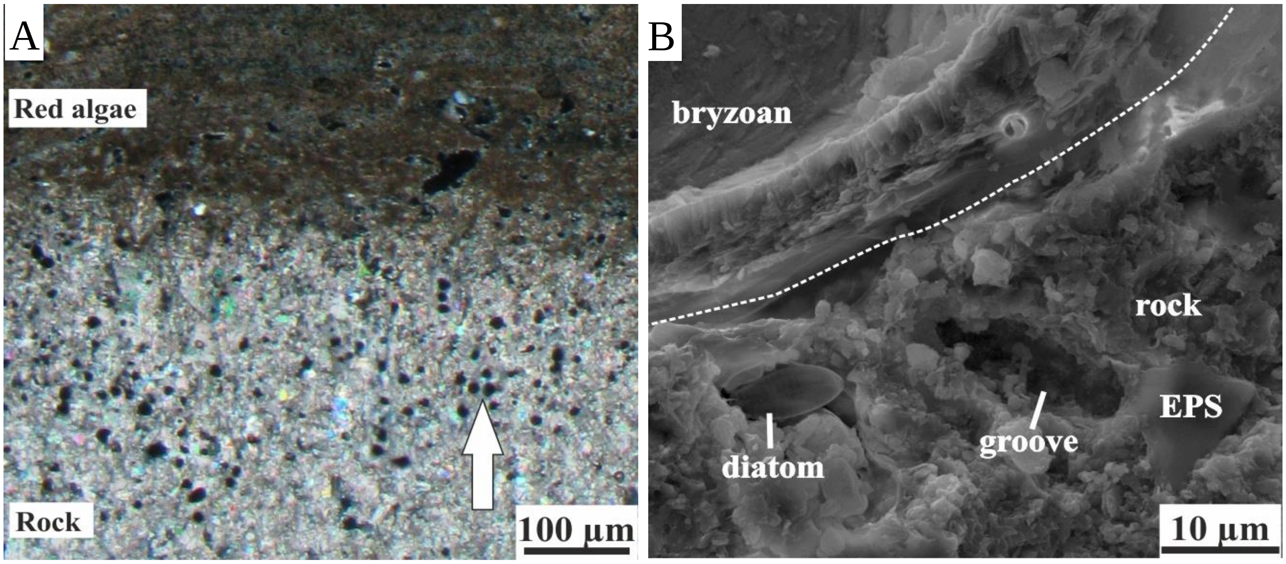

Fig. 5.3 – (A) Encrusting red algae adhering directly on rock surface. This later appears bored by numerous holes (white arrow) interpreted as formed by filamentous cyanobacteria. (B) Bryozoan lying on rock surface. This latter is colonized by diatoms, and elements such as EPS and alteration grooves suggest the previous occurrence of filamentous cyanobacteria.

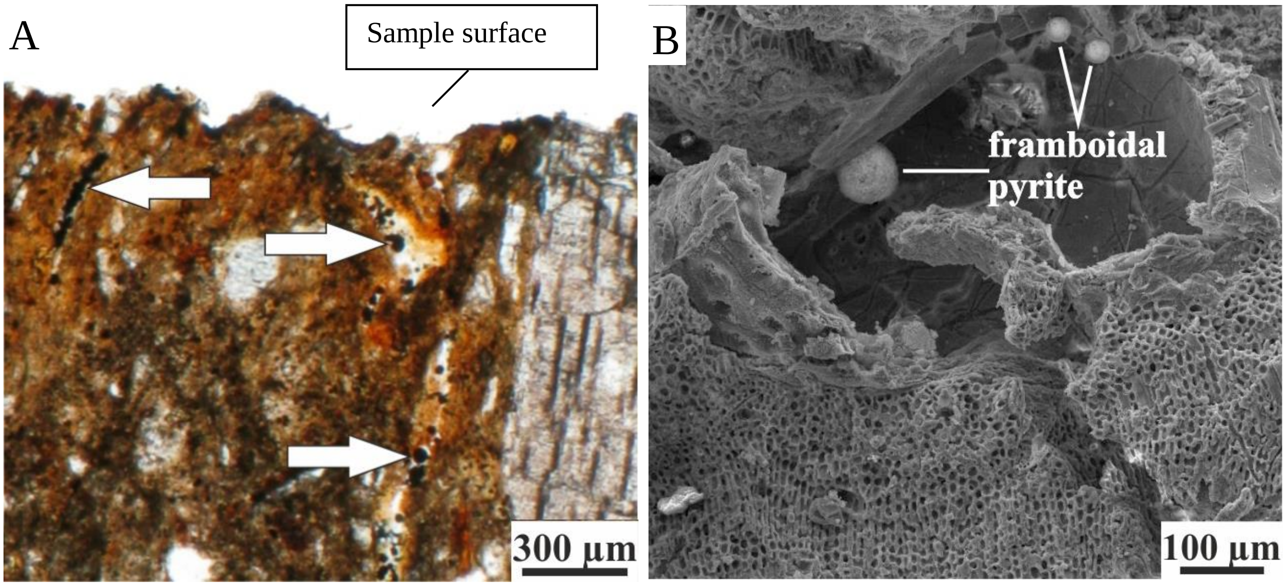

Fig. 5.4 – (A) Framboids occurring within fractures below rock surface. (B) Framboidal pyrite crystals formed within an algal conceptacle.

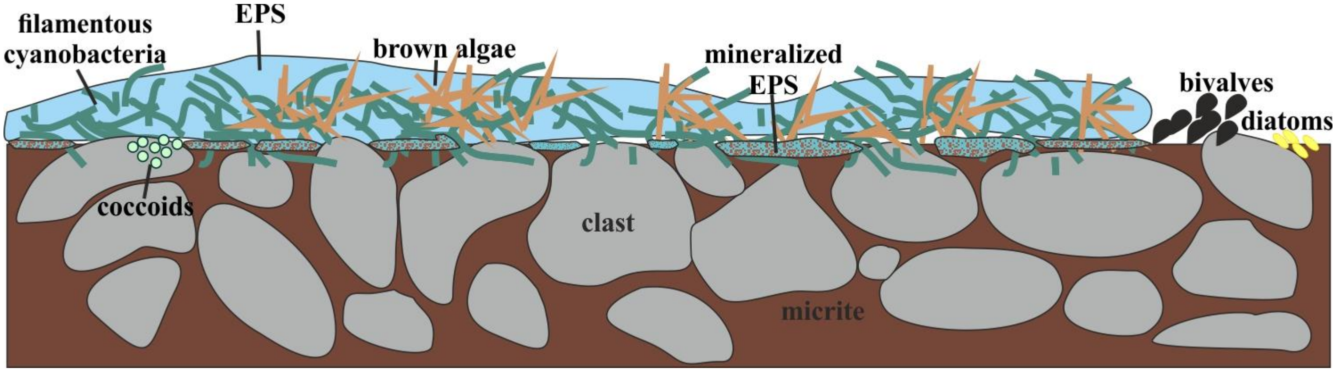

Fig. 5.5 - Ideal cross-section of a Santa Maria di Ricadi rock sample showing organisms and products that form biofilm.

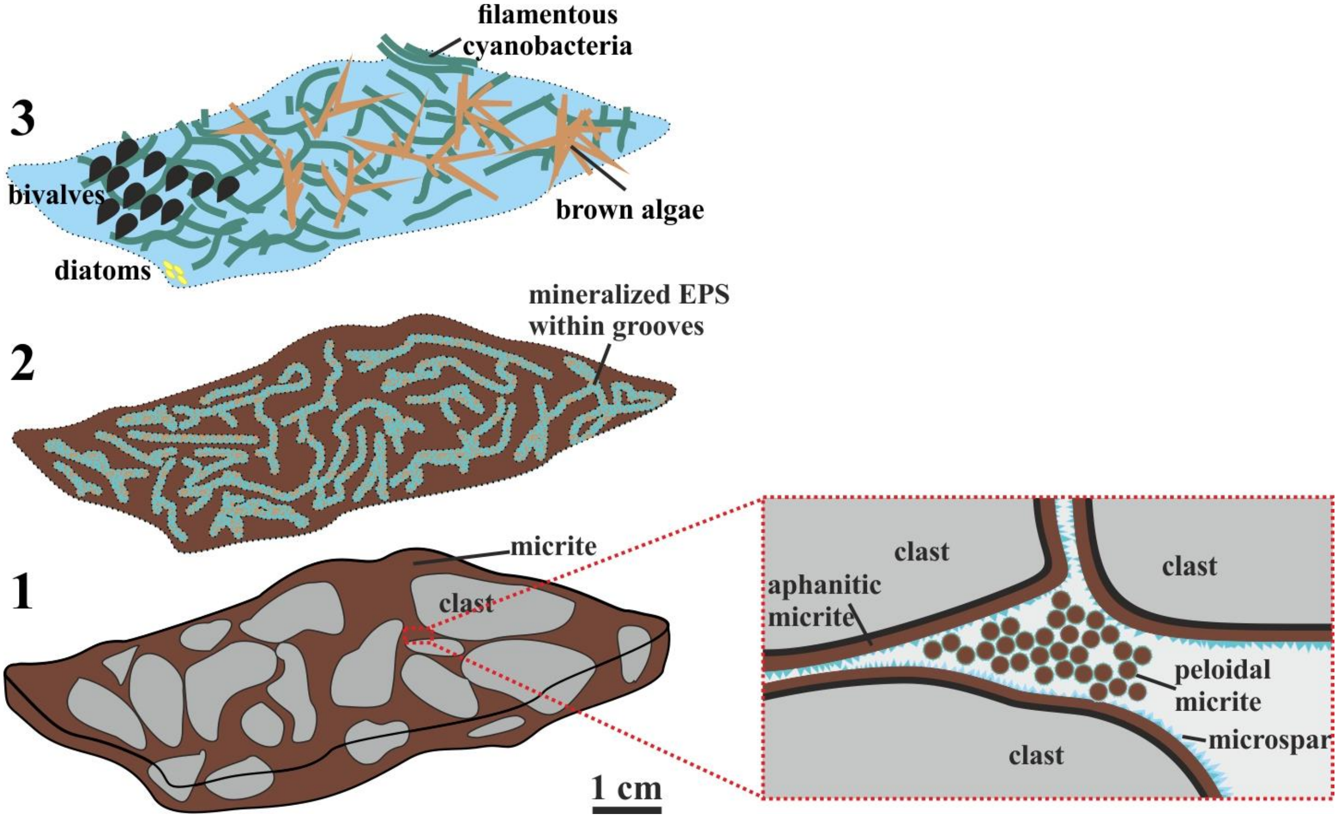

Fig. 5.6 – Formational model of Santa Maria di Ricadi biofilm. (1) Rock. Composed of clasts and neo-formed micrite deposits.(2) Micritic deposit furrowed by an intricate net of grooves almost completely filled by EPS which results strongly mineralized by Ca-carbonate nanospheres. (3) Skeletal organisms, cyanobacteria and brown algae. These elements are covered by thick layer of EPS.

The rock is clast-supported and clasts are surrounded by an isopachous coating, up to 20 μm in thickness, made up by aphanitic micritic cements. Voids among clasts are partially or totally filled by peloidal micrite that occasionally clot together forming shrub-like or denser structures. Both aphanitic and peloidal micritic deposits are composed of Ca-carbonate with ≈ 4 moles % of Mg and ≈ 1 moles % of S. Nanostructure of the two deposits result formed by nanospheres up to 200 nm in diameter tightly clotted together.

Moreover, Ca-carbonate deposits are rimmed by thin fringe of subhedral crystals of micro-sparite.

Euendolithic cyanobacteria adhere on the uppermost part of the Ca-carbonate deposits cementing rock. Here, they furrow the surface, for about 2 µm in depth, forming an intricate net of grooves. Grooves are partially filled by EPS, which is strongly mineralized by Ca-carbonate nanospheres.

On the top of the biofilm, abundant EPS surround filamentous cyanobacteria, diatoms remains and filamentous, highly branched, brown algae (*Phaeophyceae*). Moreover, bivalves occur frequently on submerged rock surfaces, whereas only bivalves occur on air-exposed rocks surfaces present in the splash zone.

5.2 Biologically mediated mineralization

Calcium carbonate precipitation resulted one of most fundamental processes in the collected samples that goes well beyond the mere cementation of organisms or clasts.

In Monasterace samples surfaces result encrusted by macro-organisms skeletal remains representing the primary Ca-carbonate buildups. Red algae left skeletal crusts up to 5 mm (1 mm in average) in thickness. Equally, bryozoan form widespread and patchy crusts, reaching 300 µm of thickness. These elements belong to the, so called, *biologically controlled mineralization*, in which organisms exert a considerable control over all the aspects of the nucleation and growth stages of the mineral.

Neo-formed Ca-carbonate deposits have been observed both in Monasterace and Santa Maria di Ricadi samples. As regard Monasterace, deposits occur as sheets (Fig. 5.7 A), triads (Fig. 5.7 B) and dumbbell- to spherical- shaped crystals. In Santa Maria di Ricadi, deposits occur as aphanitic and peloidal. Nano-structural analyses showed that all these deposits are made up by an assemblage of nanospheres, varying in diameter from 50 to 200 nm, tightly clotted together (Fig. 5.7 C). Moreover, neo-formed Ca-carbonate crystals occurred strictly interconnected both with bacteria and EPS. This process occurs equally among Santa Maria di Ricadi samples where abundant EPS, left by cyanobacteria, is strongly mineralized by nanospheres.

These nano-spherical structures differ notably from the typical abiotic Ca-carbonate crystal known in literature, formed both in natural environmental and in laboratory conditions (Fig. 5.7 D).

Several authors (e.g. Spadafora et al., 2010; Manzo et al., 2011) indicate nanospheres commonly form by replacing non-living degrading organic matter or at point sites on the external surface of a living cyanobacterial sheath. In the first case, EPS, dead bacterial cells and plant tissues are seen to be replaced by nanocrystals with or without the presence of degrading microorganisms (e.g. heterotrophic bacteria, actinomycetes). Many other authors (Pentecost, 1985; Arp et al., 1999; Neuweiler et al., 1999; Trichet et al., 2001; Gautret et al., 2004), suggested that the degradation of EPS is fundamental, whereas the bacterial bodies themselves are less involved.

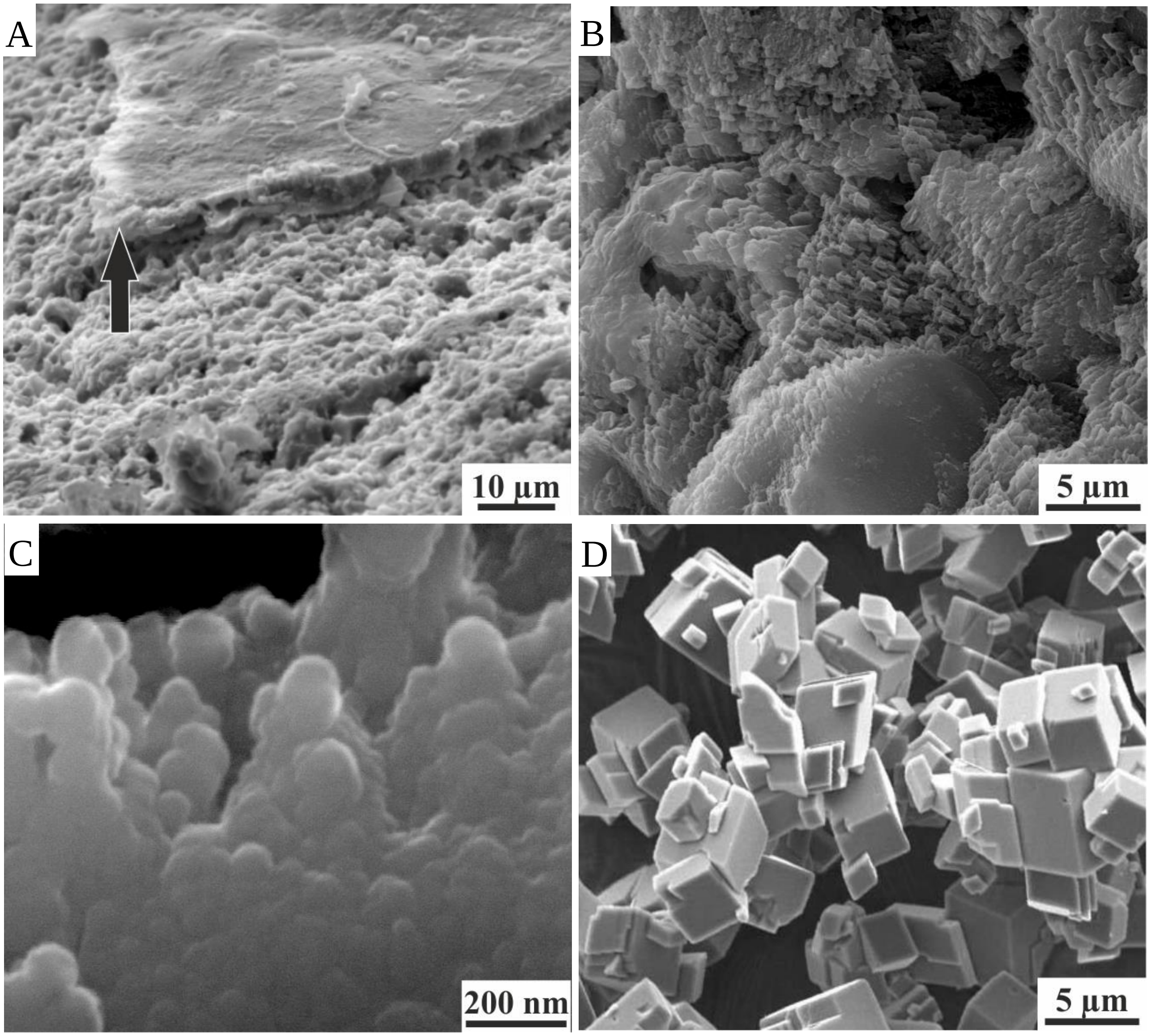

Fig. 5.7 – Micrographs taken under SEM show respectively: (A) Black arrow indicates a Ca-carbonate sheet deposit. (B) Biotic Ca-carbonate crystals formed by small aggregates of triad-shaped crystal; (C) Biotic Ca-carbonate nanospheres; (D) abiotic calcite crystals (mod. From Doner and Lynn, 1989).

This hypothesis is confirmed in this work. On the surface of numerous samples collected in Monasterace, bacteria such as filamentous, coccoids, bacilli and diatoms, surrounded by EPS, form sheet deposits (Fig. 5.8 A, B).

Secondary, early-stage nanospheres gradually replace the degraded EPS (Fig. 5.8 C). In the end of the process, nanospheres replace completely both the EPS and bacterial bodies forming large aggregates as sheet deposits (Fig. 5.8 D).

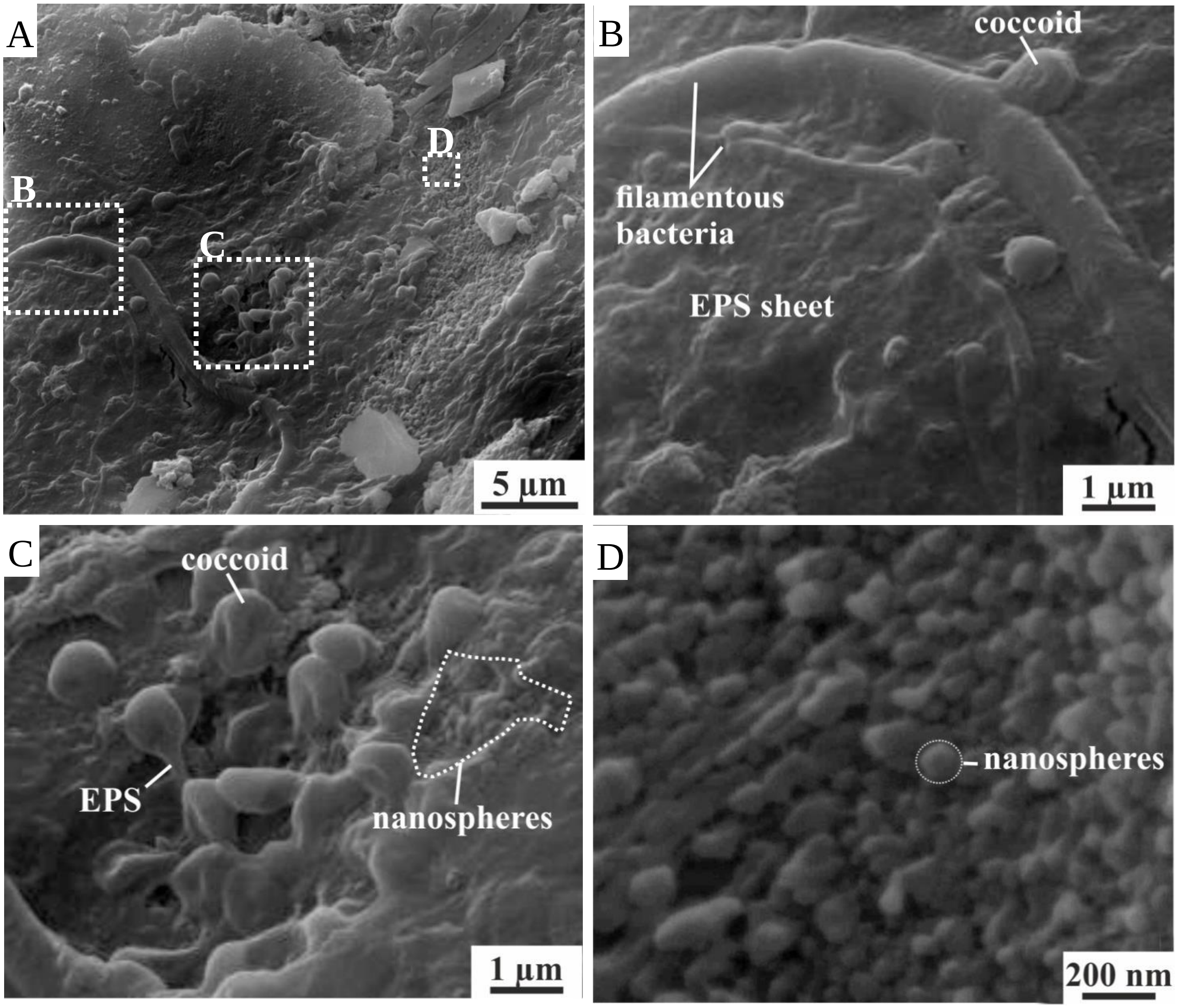

Fig. 5.8 – (A) Micrograph shows a microbial biofilm in which nanospheres formation stages can be distinguished. (B) EPS sheet deposit in which filamentous (f) and cocci cyanobacteria (c) and likely a actinobacterium (a) can be distinguished. (C) Early-stage nanospheres (ns) gradually replace the degraded EPS which form a thin patina and filamentous residues on cocci cyanobacteria (c). (D) White dashed lines circumscribe an area in which nanospheres (ns) replaced completely EPS and bacteria.

Although has been demonstrated that nano-spherical morphologies can be created in laboratory by purely abiotic mechanisms (Bosak et al., 2004), many other works demonstrated that the origin of mineral nanospheres in marine, hypersaline lakes, freshwater and continental environments and laboratory conditions, represent the product of biomineralization (e.g. Pedley et al. 2009; Perri et al., 2012; Dupraz et al., 2013).

Cölfen and Antonietti (2005, 2008) and subsequently Zhou and O'Brien (2012) proposed for this type of crystals a *nonclassical* growth that may involve, self-assembly-based crystallization process, which differs from classical crystallization, where crystal growth occur ion-by-ion or single-molecule attachment to a critical crystal nucleus (Fig. 5.9). Cölfen and Antonietti (2005) suggested that the oriented assemblage of primary nano-particles (nanospheres) produce several, more complex, structures, defined *mesocrystals*, which represent an intermediate in a crystallization reaction, where the final products are single crystals.

Nanospheres can clot together into mesocrystals as triads, varying from 200–500 nm in diameter, with organic matter content. Successively, these latter evolve in a more advanced stage of mineralization forming larger (20–50 µm) super-structures (Cölfen et al., 2005).

Triads morphologies were identified in numerous works, in laboratory by Pedley et al. (2009), and previously by Ben Chekroun et al. (2004), and in natural environment by Turner and Jones (2005) and Manzo et al. (2011).

Several authors showed nanospheres, with diameter ranging from 60 up to 200 nm, occurring in actual lagoonal environment, forming stromatolitic structures (Sprachta et al. 2001), in lake environment forming sub-fossil stromatolites (Perri et al. 2012), in laboratory conditions simulating actual freshwater microbialites (tufa) (Pedley, 2014).

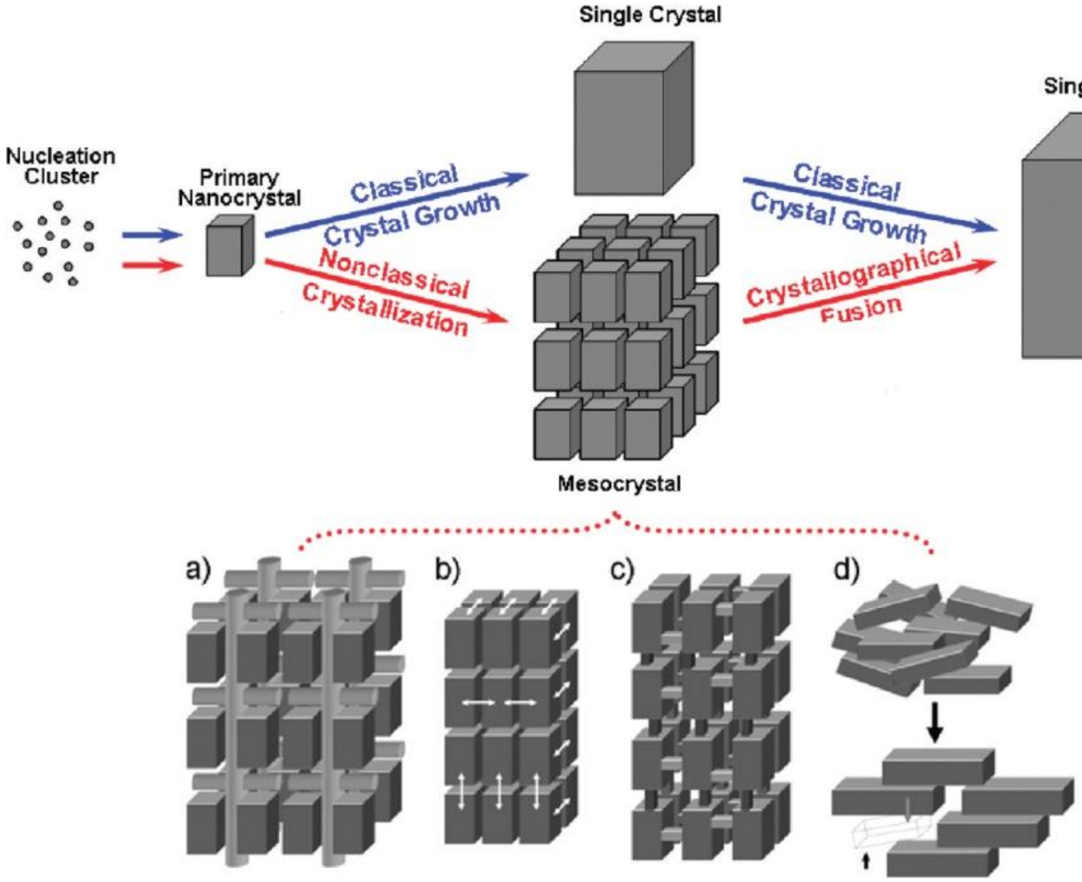

Fig. 5.9 - Classical crystallization (blue pathway) via ion-by-ion addition versus single-crystal formation (red pathway) by a *mesocrystal* intermediate made of nanoparticles. (a−d) Illustrative diagrams of the four principal possibilities that could explain the 3D oriented alignment of nanoparticles, (a) by an oriented organic matrix, where tubes among nanoparticles represent EPS; (b) by physical fields or mutual alignment of identical crystal faces; (c) by epitaxial growth of a nanoparticle employing a mineral bridge connecting the two nanoparticles, and (d) by nanoparticle alignment by spatial constraints. (mod. After Zhou and O’Brien, 2012).

Furthermore, in Santa Maria di Ricadi biofilms, EPS within grooves appear as mineralized by nanospheres.

Calcification is a common process for extracellular polymeric substance, as may contain various acidic residues and sugars that may interact with divalent cations such as Ca^{2+} and Mg^{2+}. When the EPS is degraded, Ca^{2+} may locally reach high concentrations and then precipitate as calcium carbonate (Kramer, 2008).

Furthermore, according to Ramírez-Reinat and Garcia-Pichel (2012), cyanobacteria dissolving calcite, mobilize Calcium ion from the apical cell by cell to cell through the filament and eventually released from the distal cell (the one closest to the mineral’s surface), maintaining internal calcium homeostasis. Thereby, the operation of bio-weathering may leads to Ca-carbonate nanospheres formation in EPS surrounding cyanobacteria or EPS remaining in grooves. Likewise, nanospheres may precipitate in close proximity of cyanobacteria forming micritic deposit.

Dumbbell- to spherical-shaped crystals formed by Ca-carbonate with high content of Mg (≈ 23 moles %) and S (about 4% moles) occurred in Monasterace samples (Fig. 5.10 A). Similar crystal shapes were inferred by Chafetz and Buczynski (1992) as formed around clumps of bacteria. Successively, Van Lith et al. (2003) demonstrated the Ca-dolomite and high-Mg calcite dumb-bell formation is induced by sulphate-reducing bacteria in anoxic environment, both in uppermost sediment, such as Lagoa Vermelha and Brejo do Espinho, and in laboratory culture experiments.

Framboidal pyrite occurred in many samples collected in Monasterace site, both in rock fractures up to ≈ 2 mm in depth (Fig. 5.10 B), and in red algae cavities. These crystals occur either as discrete or in cluster, and are commonly covered by a thin sheet of EPS.

In nature, the formation of pyrite framboids occurs in sediments or waters immediately subjacent to the redox interface separating oxygen-bearing and sulfide-bearing waters. In normal marine sediments with partly oxic bottom waters, the redox interface is located just below, and within centimeters of the sediment–water interface, and the long growth time allows larger pyrite framboids to develop (Wilkin et al., 1996, 1997). According to Cavalazzi et al., (2007) pyrite framboids occurring in sediments covered by microbial mats are interpreted as the product of anoxia in the sediments due to the cover of microbial mats, while the overlying sea water is oxygenated.

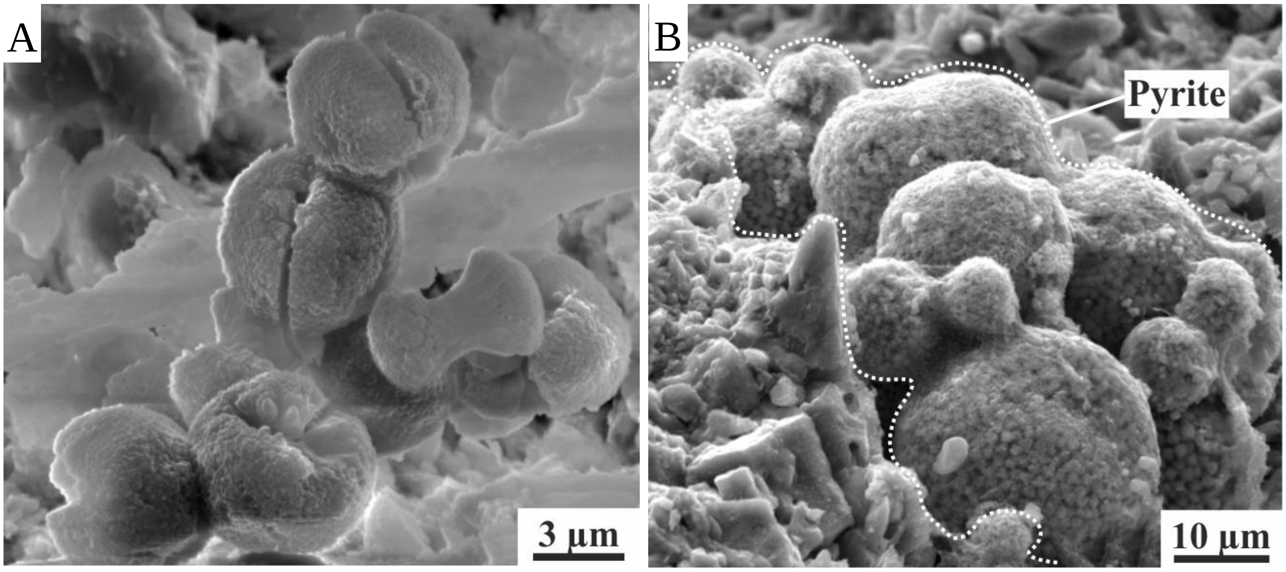

Fig. 5.10 – SEM micrographs showing (A) a cluster of dumbbell and spherical crystals; (B) a cluster of framboidal pyrite.

Currently, the precise mechanisms by which pyrite forms at temperatures below 100°C remains the subject of debate, it is believed to involve a number of Fe sulfide precursors progressively richer in sulfur.

In presence of oxygen, bacteria feed on organic carbon in sediment and respire aerobically. With increasing organic content and/or depth of burial, the diffusion of oxygen into the sediment is impeded and the bacteria are forced to respire anaerobically.

Microbial sulphate reducers play a major role in the degradation of organic matter in anoxic marine sediments (Schenau et al., 2002). This process can be represented by the following reaction:

$$2CH_2O + SO_4^{2-} \longleftrightarrow H_2S + 2HCO_3^{-}$$

This reaction actually progresses through several stages, including the breakdown of organic polymers (by bacterial fermentation) to simpler organic molecules that can fuel sulphate-reducing reactions.

The principal by-product of this reaction is hydrogen sulphide (H_2S) which can be released into the porewater during early diagenesis is, either re-oxidised by oxygen, nitrate, Fe/Mn oxides, incorporated into organic matter by several pathways, or reacts with reactive iron compounds producing finely-particulate iron monosulphide (FeS), such as greigite and mackinawite. Bacterial breakdown of H_2S liberates sulphur (S^0) to solution, which can react with FeS to produce pyrite (Schippers and Jørgensen, 2002).

In addition, Popa et al. (2004) suggested that sphericity of framboids might reflects pseudomorphism of a pre-existing spheroidal body, as an organism or microcolony of bacteria.

Few calcified filamentous cyanobacteria have been observed both on S. Maria di Ricadi and Monasterace biofilms.

Two factors directly influence cyanobacterial calcification are the saturation state of ambient waters and the mechanism of photosynthetic uptake of inorganic carbon (Riding, 2006).

Current literature does not recognize confidently calcified cyanobacteria in present-day marine environments, and indicates them rare to absent throughout the Cenozoic (e.g. Arp et al., 2001). In contrast, cyanobacterial calcification is locally well-developed in present-day calcareous streams and lakes, and is often significantly involved in the formation of tufa and oncoids (Riding, 2011).

5.3 Bioerosion

Agents of bioerosion comprise essentially epilithic and endolithic bioeroders of different sizes. Epilithic macro-organisms (> 100 μm), such as bryozoans, red algae, bivalves and serpulids (polychaetes) that anchor themselves to the substrate, are the responsible for the formation of macro-scale bioerosion products as grooves and irregularly extended areas.

These organisms produce evident bores on carbonate rocks, especially on Monasterace samples, where numerous grooves up to 2 mm in width, were formed by serpulids (Fig. 5.11 A).

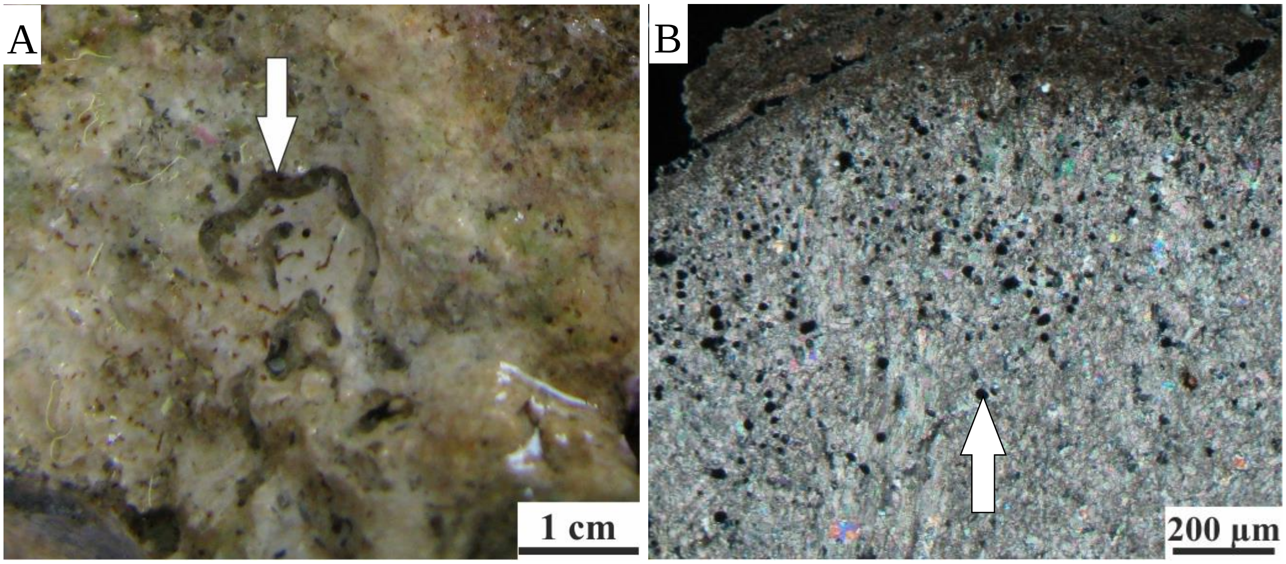

Fig. 5.11 – (A) Top view of a Monasterace sample. White arrows indicates the furrow left by a serpulid. (B) Boreholes (white arrow) produced by endolithic filamentous cyanobacteria.

Same bio-weathering features occurred in S. Maria di Ricadi, where bivalves fix on rock substrate produce boreholes. Endolithic microorganisms (< 100 μm) contribute greatly to the alteration of the original aspect and properties of the rock, as either primary producers or bio-destroyers, or both.

Boreholes, produced by filamentous cyanobacteria, occur below the rock surface up 2 mm in depth of both for Monasterace samples (Fig. 5.11 B) and Santa Maria di Ricadi samples. These bioerosion products increase notably micro-porosity of the rock. Microorganisms take numerous advantages for boring, including availability of nutrients, avoidance of competition, protection from extreme environments and prevention of detachment (Ramirez et al., 2010).

Boring of carbonate rocks has been widely observed in both terrestrial and marine environments (Pohl and Schneider, 2002; Golubic et al., 2005), believed to involves the secretion of acids and/or the sequestration of calcium ions from the rock (Garcia-Pichel, 2006).

Numerous bio-alteration products occur both on skeletal organisms and Ca-carbonate rock, the average diameter of boreholes (4 µm) and average width of grooves (5 µm), thereby suggested that these products are comparable to those formed by euendolithic cyanobacteria. In particular, coccoid cyanobacteria, where present, are implicated into forming boreholes on Ca-carbonate skeletal parts of organisms.

Filamentous cyanobacteria formed an intricate texture of grooves, mostly in Santa Maria di Ricadi samples (Fig. 5.12). The texture corresponds to ichnotaxon *Eurygonum nodosum* (Schmidt, 1992). This permitted to indicate the cyanobacterium *Mastigocoleum testarum* (Lagerheim, 1886) as trace maker. This is characterized by diagnostic trichomes, bifurcated with irregular branching, terminal or lateral heterocysts, and diameters of the thalli between 6 and10 µm. The ends of the trichomes are sometimes whip-shaped (Wisshak et al., 2005).

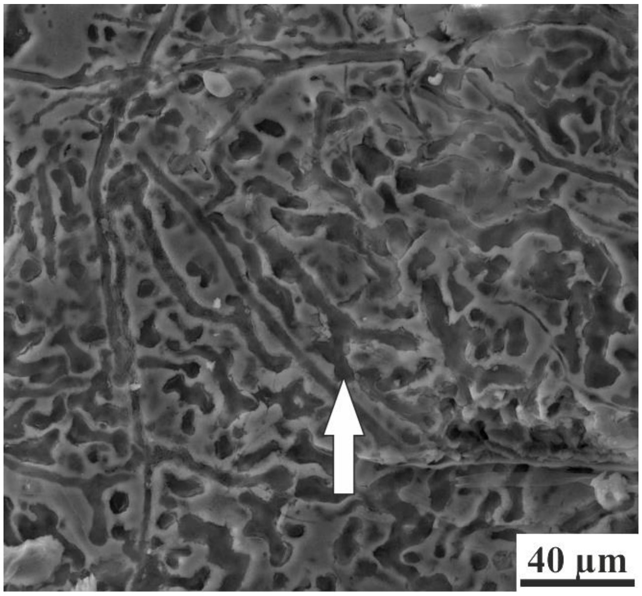

Fig. 5.12- Bifurcated traces of *Eurygonum nodosum* (Schmidt, 1992) produced by filamentous cyanobacterium *Mastigocoleum testarum*, show characteristic lateral swellings, which harbor heterocysts (black arrow).

When euendolithic filaments adhere to the carbonate deposit the cell in closest proximity to the mineral, initiates the boring process by mobilizing Ca^{2+} from the water in the interstitial space between the cell and the mineral. The Ca^{2+} activity is lowered, and the thermodynamic equilibrium is thus shifted towards dissolution. The mineral dissolves, giving off free Ca^{2+} to compensate for the lower ion activity. Once a small amount of the mineral is dissolved, the apical cell will grow to fill the void, and the process of dissolution is resumed. The Ca^{2+} taken is mobilized, from cell to cell, and eventually released from the distal cell (the one closest to the mineral's surface) maintaining Ca^{2+} homeostasis in the cytoplasm. Dissolution will free CO_3^{2-} in solution, and under normal seawater pH conditions, buffering will shift CO_3^{2-} to HCO_3^-, which can be taken by the cells as source of inorganic carbon, achieving charge balance in the process (Ramírez-Reinat and Garcia-Pichel, 2012).

Although the recognized high resistance to weathering process, quartz clasts occurring in the beachrock of S. Maria di Ricadi, present numerous biologically altered areas, irregular in shape and extension that go to enlarge preexistent mechanical fractures (Fig. 5.13 A). The presence of EPS filaments and Ca-carbonate nanospheres on the bioeroded area suggest the past occurrence of microorganisms.

Contrariwise, the rest quartz fresh-cutting surfaces are only interested by mechanical fractures showing sharp and linear edges, without any bacteria or neo-formed crystal of Ca-carbonate (Fig. 5.13 B).

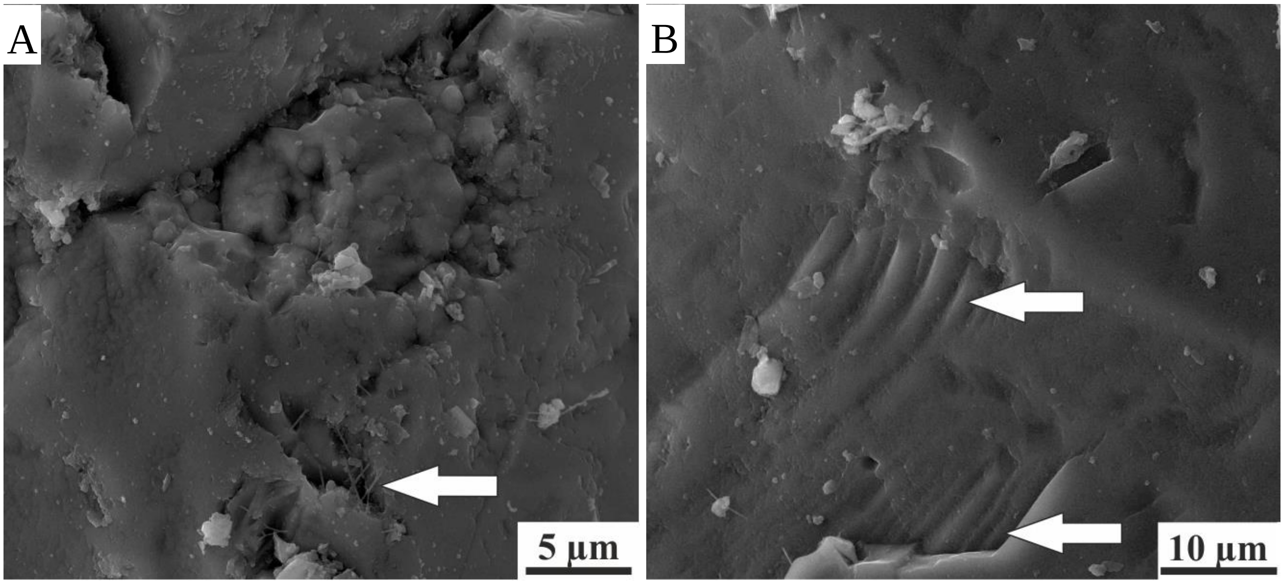

Fig. 5.13 - (A) White arrow indicates EPS filaments associated with Ca-carbonate nanospheres in a bioalteration area on a quartz clast (B) SEM micrograph show a quartz clast surface interested by typical mechanical fractures (white arrows).

Quartz solubility significantly increases in alkaline conditions of pH 9 or higher (e.g. Dove and Rimstidt, 1994). Homogeneous environments with such high alkaline pH values are rare but local variations in physicochemical conditions are possible because of the microorganisms.

In fact, in most environments local changes can occur, for instance in the close proximity of microorganisms that conduct photosynthesis. Photosynthetic activity and associated CO_2 consumption locally raise pH in selected microenvironments around the cell. At pH values lower than 3.5, no quartz dissolution is possible, although mechanical processes may continue (Brehm, 2004).

Extracellular polymeric substance creates a complex microenvironment at the mineral–water interface, where metabolic reactions and generation of acids lead to variation in pH and concentration gradients markedly different from the bulk solution, leading an increases rates of chemical weathering.

CHAPTER 6

CONCLUSIONS

In this study, optical and scanning electron microscopy (SEM) have been used to figure out, through micro- and nano-scale investigation, the role of microorganisms in the precipitation and alteration of minerals on the external surface of natural and artefact rocks collected in the shallow marine water of the archaeological submerged park of Monasterace and along the bay of Santa Maria di Ricadi, respectively sited on the Tyrrhenian and Ionian coast of Southern Calabria.

As regards Monasterace samples, biofilms can be divided in different layers, which reflect a formational order. Rock surface results characterized by the presence of boreholes up to 2 mm below and framboidal pyrite within fractures.

First layer is formed by bacteria, organic substance remains and neo-formed minerals. Here bacilli, cocci and filamentous cyanobacteria occur with EPS, arranged in sheet deposits. Differently, neo-formed Ca-carbonate crystals occur both as sheet deposits, more or less extended, with average thickness of 10 µm, and irregular-shaped deposits made up by triads of 300 nm in diameter; rarely as dumbbell- to spherical- Ca-carbonate crystals.

Second layer is formed skeletal organisms, such as encrusting red algae, bryozoan, diatoms, coccolithophores, polychaete. In addition, some crystals of framboidal pyrite are present within conceptacles of red algae.

This ideal subdivision of the biofilm in different layers has been elaborated also for Santa Maria di Ricadi samples.

Rock samples are made up by quartz, K-feldspar and biotite clasts; differently, neo-formed Ca-carbonate occurs as aphanitic micritic deposits, surrounding clasts, and peloidal micritic deposits, among clasts. Both aphanitic and peloidal micritic deposits are composed of Ca-carbonate with ≈ 4 moles % of Mg and ≈ 1 moles % of S. Nanostructure of the two deposits result formed by nanospheres up to 200 nm in diameter tightly clotted together.

Moreover, Ca-carbonate deposits are rimmed by thin fringe of subhedral crystals of micro-sparite.

First layer is characterized by euendolithic cyanobacteria adhere on the uppermost part of the Ca-carbonate deposits cementing rock. Here, they furrow the surface, for about 2 µm in depth, forming an intricate tangle of grooves. Grooves are partially filled by EPS, which is strongly mineralized by Ca-carbonate nanospheres.

On the top of the biofilm, abundant EPS surround filamentous cyanobacteria, diatoms remains and filamentous, highly branched, brown algae (*Phaeophyceae*). Moreover, bivalves occur frequently on submerged rock surfaces, whereas only bivalves occur on air-exposed rocks surfaces present in the splash zone.

Red algae and bryozoans are responsible of Ca-carbonate crusts formation, and though scattered, they represent the dominant Ca-carbonate formation on Monasterace rock surfaces. These encrusting organisms result absent on Santa Maria di Ricadi rock samples.

The co-existence of degraded EPS and bacteria, strictly associated with the Ca-carbonate nanospheres, implies that the organic matter and the microbial metabolism played a fundamental role in the precipitation of these minerals. In addition, many other works demonstrated that the origin of mineral nanospheres in marine, hypersaline lakes, freshwater, continental environments and laboratory conditions, represent the product of biologically induced mineralization.

These primitive nanocrystals are involved in the non-classical crystallization proposed by Cölfen and Antonietti (2005), in which the oriented assemblage of primary nano-particles (nanospheres) produce several, more complex, structures, defined *mesocrystals*, which represent an intermediate in a crystallization reaction. In fact, they clot together to obtain single crystals as final products.

In addition, dumbbell- to spherical- Ca-carbonate crystals and framboidal pyrite have been found in Monasterace samples. These two different mineral species form in anoxic environment, and are strictly connected with sulphate reducers bacteria which play a major role in their production.

The presence of framboidal pyrite both within fractures, 2 mm below the rock surface, and inside conceptacles of red algae suggest a complex formational history of the Monasterace biofilms.

At first, anoxic conditions favor the formation pyrite within fractures, after that establishment of oxic condition associated with solar irradiation permitted the formation of encrusting red algae on the top of the biofilm. Subsequently, a return to anoxic condition led to the precipitation framboidal pyrite within conceptacles.

Bioerosion comprise different agents, essentially epilithic and endolithic bioeroders of different sizes. Epilithic macro-organisms (> 100 µm), such as bryozoans, red algae, bivalves and serpulids produce grooves and irregularly extended bioaltered areas.

Endolithic microorganisms (< 100 µm), such as filamentous cyanobacteria, produce boreholes both in Monasterace and Santa Maria di Ricadi samples.

Euendolithic bacteria produce boreholes and grooves, both on skeletal organisms and Ca-carbonate rock, the average diameter of boreholes (4 µm) and average width of grooves (5 µm). Boreholes are the result of coccoid activity, whereas grooves of filamentous cyanobacteria.

In particular, in Santa Maria di Ricadi samples, grooves are widespread on Ca-carbonate deposit surfaces. The intricate texture of grooves corresponds to ichnotaxon *Eurygonum nodosum* (Schmidt, 1992). This permitted to indicate as trace maker the cyanobacterium *Mastigocoleum testarum* (Lagerheim, 1886).

Quartz clasts, occurring in Santa Maria di Ricadi samples, show numerous bioalterations along preexistent mechanical fractures due to photosynthetic processes of overlying cyanobacteria colonies.

Nano-structured microbially mediated minerals find numerous applications in engineering (see 1.8 for further details) and in the recognition of the abiotic versus biotic nature of carbonate minerals in several fields, such as in the reconstruction of depositional palaeoenvironments, in astrobiology, in medicine, etc.

Additional, more focused, directed to better understanding electrokinetic properties and mutual interaction of nano-sized particles, are needed. Studies directed toward the very early stage of particle formation would clarify the role of bacteria in the processes of nucleation and aggregation, leading to the formation of nano-structured minerals.

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