# BIOLOGICAL DECAY AND ITS CONTROL BY BIOMINERALISATION IN CALCAREOUS STONES

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## ABSTRACT

### BIOLOGICAL DECAY AND ITS CONTROL BY BIOMINERALISATION IN CALCAREOUS STONES

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Biodeterioration has an important role in weathering of historical materials. Natural stone materials become vulnerable to physical and chemical changes in outdoor conditions, favouring the biological growth.

In this study, biodeterioration on calcareous stones and its control by biomineralisation were studied on limestones from Nemrut Mount Monument and marbles from Pessinous Archaeological Site. For qualitative and quantitative detection of biological activity fluorescein diacetate (FDA) method that was developed for soil microbial activity was applied to stones of historic monuments. Qualitative FDA analysis was used on cross sections of the samples in order to observe the depth of penetration and effects of biomineralisation using a light microscope with fluorescent light source. Quantitative FDA analysis was done by spectrophotometric determination of fluorescence formed by FDA treatment. X-Ray Diffraction (XRD) analyses were used in determining mineralogical

structure of patinas and stone base. Light microscopy was used to investigate changes in morphological structure of historic stone in cross and thin sections of the samples. The control of biodeterioration on stone surfaces was studied by biomineralisation treatments using *Bacillus cereus*. The results of biomineralisation were evaluated by XRD, light microscopy, SEM-EDX and FDA analyses.

The results of this study showed that the biodeterioration was an important decay factor in stone materials. It started from the surface and penetrated through the microstructure of the stone up to about four cm depth. Biodeterioration also contributed to the growth of microcracks. Results of biomineralisation using *B.cereus* to form a protective coating on limestone and marble were also discussed.

**Keywords:** Biodeterioration, historic limestone, historic marble, fluorescence, FDA analyses.

# ÖZ

## TARİHİ YAPI KiREÇTAŞLARINDAKİ BİYOLOJİK BOZULMALAR VE BİYOLOJİK BOZULMANIN BİYOMİNERALLEŞTİRME İLE KONTROLÜ

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Biyolojik aktivite tarihi malzemelerin önemli bozulma nedenlerinden biridir. Açık hava şartlarında, tarihi yapılarda kullanılan doğal yapı taşları fiziksel ve kimyasal değişimlere uğramaktadırlar. Bu şartlar biyolojik bozulmaların oluşmasında da etkilidirler.

Bu çalışmada, Nemrut Dağı kireçtaşları ve Pessinus Arkeolojik Alanı mermerleri üzerindeki biyolojik bozulmalar incelenmiş ve biyomineralleştirme ile yüzey bozulmalarının kontrol altına alınması çalışmaları yapılmıştır. Topraktaki biyolojik aktivitenin ölçülmesi için geliştirilmiş olan Fluorescein diasetat (FDA) analizleri tarihi yapı taşlarındaki biyolojik bozulmaları incelemek üzere uyarlanmıştır.

Biyolojik aktivitenin nitel olarak izlenmesi için taş kesitleri FDA ile muamele edilmiş, örneklerde oluşan floresanlık mikroskopta floresan lamba kullanarak

incelenmiştir. FDA analizleri, biyolojik aktivitenin spektrofotometrik yöntemlerle nicel olarak tayininde de kullanılmıştır. X ışınları toz difraksiyonu (XRD) analizleri taş yüzeyindeki bozulma bölgelerinin ve taşın mineralojik yapısının tanımlanmasında kullanılmıştır. Taş yüzeyindeki biyolojik aktivite sonucu oluşan mikro değişimler ince ve kalın kesitlerde ışık mikroskobu ile incelenmiştir. Biyolojik bozulmanın kontrolü çalışmaları taş yüzeyine kalker bakterilerinin uygulanması ile oluşan biyomineralleştirme ile çalışılmıştır. Bu uygulamanın sonuçları XRD, ışık mikroskobu, SEM-EDXve FDA analizleri ile değerlendirilmiştir.

Çalışmanın sonuçları biyolojik aktivitenin tarihi yapı taşları için önemli bir bozulma faktörü olduğunu göstermiştir. Biyolojik aktivitenin taş yüzeyinden başlayarak mikro çatlaklardan içeriye doğru dört santimetreye kadar ilerlediği görülmüştür. Kireçtaşı ve mermerlerin yüzey bozulmalarının *Bacillus cereus* bakterisi kullanılarak biyomineralleştirme yöntemi ile kontrol altına alınması sonuçları da tartışılmıştır.

**Anahtar Kelimeler**: Biyolojik bozulma, tarihi kireçtaşı, tarihi mermer, floresanlık, FDA analizleri.

**To My Father,**

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# CHAPTER 1

# INTRODUCTION

Weathering is the breakdown and alteration of materials near and on the earth's surface to products that are in equilibrium with newly imposed physico-chemical conditions (Ollier, 1969). The stones used in the construction of monuments are also prone to decay over time as a result of exposure to weathering. Weathering agents taking role in the weathering of natural stone materials can lead to physical, chemical and/or biological processes (Schaffer, 1972). No weathering agent acts alone; the relative importance of each is influenced by the concurrent effect of other agents, or exposure to the action of one may render the material more susceptible to the subsequent action of another (Schaffer, 1972).

Physical weathering is the breakdown of the material by entirely mechanical methods, with little or no chemical change in the rock itself (Ollier, 1969; Carroll, 1970). Fluctuations of temperature and physical changes associated with the presence of water are the main physical weathering agents (Schaffer, 1972). Changes in temperature give two different results in the deterioration of stone: stresses may be set up either by the unequal expansion and contraction of the component minerals or by the expansion and contraction of surface layers relative to the underlying stone (Schaffer, 1972). Physical changes associated with the presence of water and affected by temperature changes are frost formation, crystallisation of soluble salts within the material, and occurrence of efflorescence (Ollier, 1969; Schaffer, 1972). Frost damage happens with expansion of freezing water in a confined space such as the pores and capillaries

of natural stones (Schaffer, 1972). The degree of damage through freezing is affected by temperature changes as well as the physical properties of the stone used in the construction of the structure (Schaffer, 1972). Florescence happens due to the movement of soluble salts with water through the pores, capillaries and recyrstallization of those salts after evaporation of water (Schaffer, 1972). Salt crystals formed inside capillaries and pores result in cracking and powdering of stone (Ollier, 1969). Physical weathering agents: water, temperature and salt crystallization; are involved together or subsequent to the effects of each other. The physical weathering produces the damage; it also exposes the stone to other weathering agents (Schaffer, 1972).

Chemical weathering is a series of surface chemical reactions between rocks, the atmosphere, and water (Ollier, 1969). During chemical weathering oxidation and reduction, hydrolysis, hydration and chelating reactions may occur in mineral structure of stone (Ollier, 1969; Carroll, 1970). Chemical weathering of stone material can be initiated with physical, chemical or biological weathering agents. Water, atmospheric gases and biological growth that largely take part in chemical weathering are the chemical weathering agents (Ollier, 1969; Carroll, 1970; Schaffer, 1972). Acid gases resulted from air pollution cause erosion, skin formation, and exfoliation in the stone material (Schaffer, 1972; Caner *et al.*, 1988; Ngoc Lan *et al.*, 2005).

Biological activity plays an important role in the deterioration of stone. The breakdown of rocks and minerals is largely controlled by plants, animals and microorganisms (Ollier, 1969). The climatic conditions of any region cause changes in the flora that can grow there, the growth and decay of flora provide the raw organic matters which, through the action of microflora and chemical processes produce the soil humus (Carroll, 1970). Biological weathering is a complex process involving several factors of deterioration together with it (Caneva *et al.*, 1991). All those decay agents cause physical and chemical damages to the material (Schaffer, 1972; Fitzner *et al.*, 1995, Warscheid and Braams, 2000).

Although chemical, physical and biological decay agents are responsible for the weathering of materials, in the case of building stones that were used in the construction of monuments, there are additional factors affecting the rate of weathering (Schaffer, 1972). Natural defects in stone materials, faulty craftsmanship during the construction, and wrong choice of material accelerate the weathering of building stones (Schaffer, 1972). In most of the cases all those decay factors act simultaneously in the deterioration process of stones.

Understanding the mechanisms of deterioration is important in order to take measures against weathering (Price, 1996). It is also essential to understand the type and degree of weathering and evaluate the rate of deterioration in structures for taking proper steps for their conservation. Mapping of visual weathering forms is a first step to study the deterioration problems of monumental stones. Fitzner *et al.* (1992, 1995) studied the visual weathering forms of stones in historic monuments constructed with different natural stones in several climatic conditions. The results of Fitzner *et al.* (1992, 1995) showed that the classification of the visual weathering forms can be gathered under four main branches as; loss of material, discoloration/deposits, detachments and fissures/deformations. Their mapping is useful for diagnostic and conservation studies of monuments. The maps of visual weathering forms showing their distribution indicate sources of weathering and ease representative sampling and further studies.

The biodeterioration types such as coloured patina formations, encrustations, biofilm formations, pitting etc. (Tiano, 2002) caused by the activity of microorganisms can be found in all four main branches of weathering types explained above (Fitzner *et al.*, 1995). Activities of microorganisms and organisms interact with the physical and chemical agents make the biodeterioration one of the major processes damaging the natural stones (Caneva *et al.*, 1991; Flores *et al.,* 1997). The importance of biological activity on the weathering of stone have led to many studies on the mechanisms of biodeterioration and its control (Paine *et al.*, 1933; Pochon and Jaton, 1968; Strelczyk, 1981; Caneva and Salvadori, 1988; Griffin *et al.,* 1991; Caneva *et al.*,

1991; May *et al.*, 1993; Bock and Sand, 1993; Urzi and Krumbein, 1994; Warscheid and Krumbein, 1996; Koestler *et al.*, 1997).

## 1.1 Biodeterioration

The biodeterioration of materials in nature cannot be considered as an isolated phenomenon; it always occurs together with other physical, chemical or physicochemical deterioration processes (Caneva *et al.*, 1991). The term biodeterioration refers to any undesired change in material properties due to the activity of microorganisms and/or organisms belonging to various systematic groups (Caneva *et al.*, 1991). They normally play an important role in the weathering mechanisms of organic and inorganic materials (Caneva *et al.*, 1991). Biological activity is a complex process influenced by various factors such as: climatic effects, nutrition, light, etc. (Caneva *et al.*, 1991).

All the factors that promote biodeterioration process can be limiting factors if their values are near (above or below) the limits of tolerance of a species (Caneva *et al.,* 1991). Limiting factors for biological growth are those that inhibit the presence of a biological species (Caneva *et al.*, 1991). The humidity, pH, temperature, light, salinity, pollution are examples of limiting factors (Caneva *et al.,* 1991).

The main consequences of metabolic activity, such as the excretion of enzymes, inorganic and organic acids, are the dissolution of minerals of the substratum and the precipitation of new ones (Caneva *et al.*, 1991). Moreover, the growth and the swelling of some vegetative structures (e.g. roots and lichenic thalli) produce physical stresses and mechanical breaks (Tiano, 2002). The biological alterations also differ according to ecological peculiarities such as the kind of microorganisms and organisms involved and the characteristics of the environment where the object is located (micro- and macro-environment, atmospheric pollution, etc.) (Caneva *et al.*, 1991).

The material properties are another thing affecting the degree and type of biodeterioration. In fact; different kinds of organisms can grow using the mineral

components of a stone and its superficial deposits (Tiano, 2002). In biological weathering the decay process usually starts by the interaction of the stone material with water (rain, dampness, freezing-thawing, etc.) (Tiano, 2002). The contents of air, especially the ratio of pollutants, have also an important role in the biodeterioration of stone (Herrera and Videla, 2004, Nuhoglu *et al.*, 2006). In Figure1. 1 the relationship of several factors in biological deterioration process can be seen.

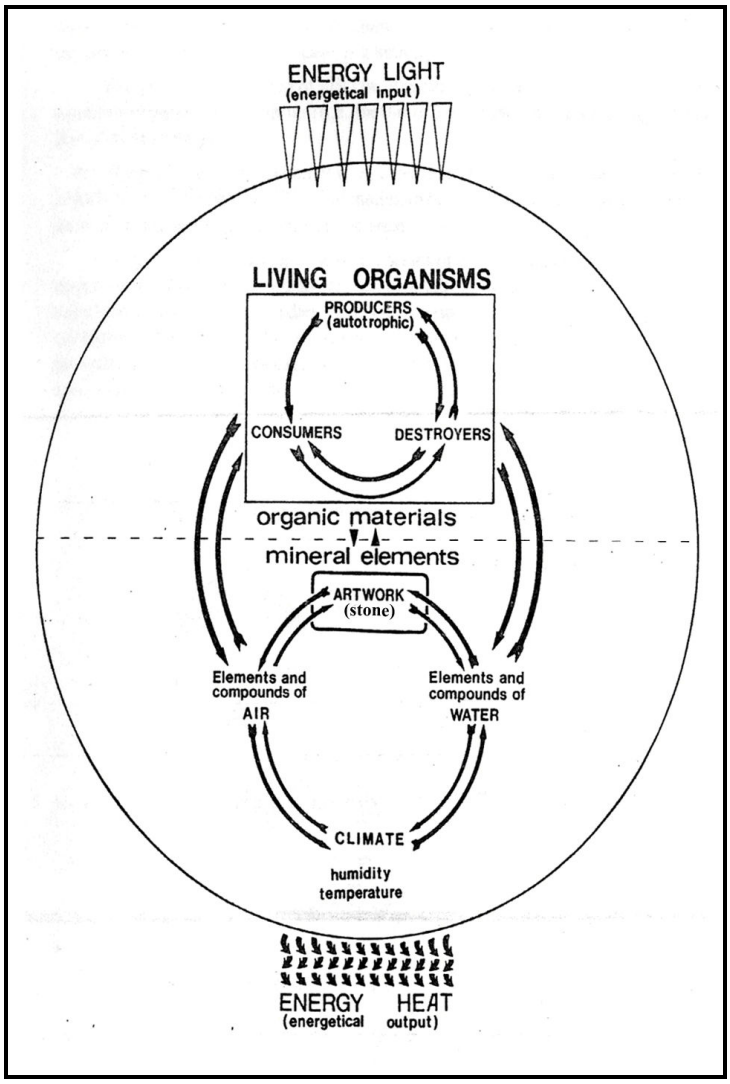



**Figure1. 1 Interaction of artwork (stone) with its environment (Caneva *et al.*, 1991, p.4).**

There is a wide range of organisms causing decay of stone materials. Decay mechanisms caused by different organisms have to be understood in order to take measures for the conservation of deteriorated stone. There are several methods for classification and identification of organisms acting in the deterioration of

stone (Tiano, 2002). New methods are still being developed for the conservation of stone affected by biodeterioration.

In the following section, biodeterioration agents, limiting factors of biological activity, formation of biological patina, methods for the investigation of biodeterioration and control of biodeterioration are briefly reviewed.

### 1.1.1 Biodeterioration Agents

Biodeterioration of the stone monuments has been affected by both micro- and macro-organisms. The organisms taking role in biodeterioration introduce different mechanisms, end products and visual changes on the stone material. Microorganisms play a fundamental role in mineral transformations in the natural environment, most notably in the formation of soils from rock and the cycling of elements (May, 2003; Gadd, 2006). Microbial communities on and in rocks also contribute to the formation of various patinas, films, varnishes, crusts and stromatolites in rock substrates (Gorbushina and Krumbein, 2000). They also prepare the medium for the development of macro organisms like plants and animals.

#### *Bacteria*

Bacteria attack stone by chemical action (Caneva *et al.*, 1991). The bacteria playing role in the microbial weathering of stones are mostly autotrophic, but can also be heterotrophic or chemolithotrophic (which use inorganic and organic substances indifferently) (Krumbein, 1972; Caneva *et al.*, 1991; Tiano, 2002) (Fig. 1. 2). Microbial solubilisation process is always coupled with acidification of the medium and a weight loss of the stone (Caneva *et al.*, 1991).

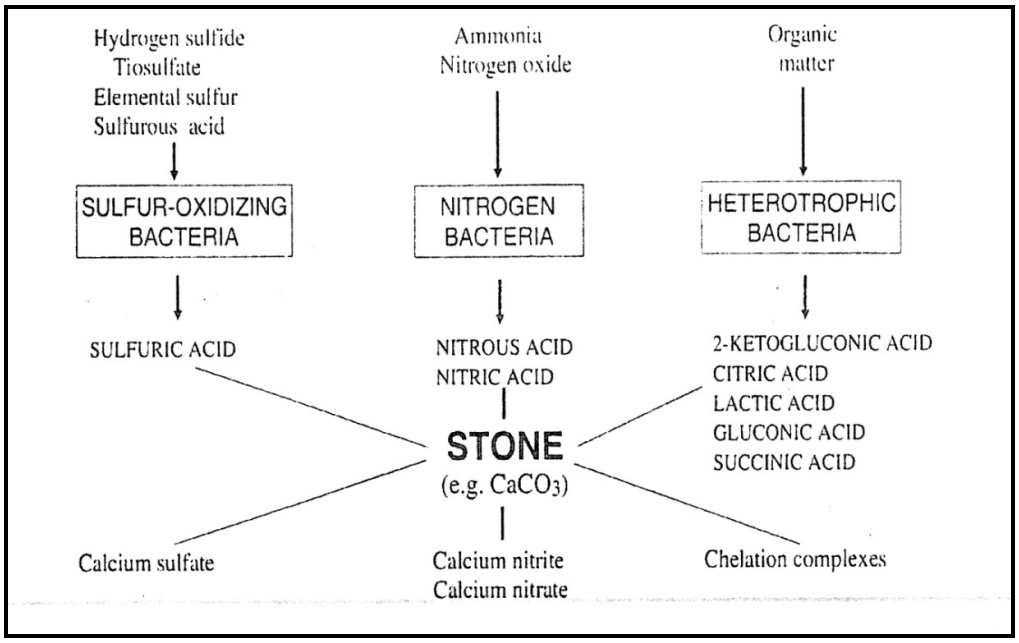



**Figure1. 2 The action of bacteria on stone (Caneva *et al.*,1991, p.89).**

The alterations resulted from bacteria are no different from purely chemical origin that are visually observed as black crusts, powdering, exfoliation (Caneva *et al.*, 1991). It is not possible to separate abiotic and microbiological processes and it is believed that completely sterile weathering is impossible (Polynov, 1937).

The ability of many bacteria (sulphur-oxidizing, nitrifying and heterotrophic) to decay stone has been demonstrated in Figure 1. 2.

Sulphur-oxidizing bacteria have most of the attention as it has a strong acidic end product (Pochon and Coppier, 1950; Gugliandolo and Mangieri, 1988; Gomez-Alarcon *et al.,* 1995). Biological formation of gypsum, nearly always found among weathering products of limestone, is due to the action of sulphur-oxidizing bacteria (Pochon and Jaton, 1968).

The importance of nitrifying bacteria in weathering of stone monuments was first demonstrated by Kauffman (1960).

Iron bacteria obtain energy from the oxidation of ferrous ions to ferric ions (Caneva *et al.*, 1991).

Heterotrophic bacteria also can be found on stone surfaces (Lewis *et al.*, 1986; Flores *et al.*, 1997). They act mostly by means of chelating agents, organic and inorganic acids, bases (ammonia or amines) (Caneva *et al.*, 1991).

Some bacteria can mobilize silica and silicates (Webley *et al.*, 1963). By the same type of chemical action insoluble or inorganic source of phosphorus can be solubilised (Caneva *et al.*, 1991). Some bacteria can attack haematite, goethite, limonite, etc. (Ehrlich, 1981).

***Algae and Cyanobacteria***

Two different systematic groups of microscopic algae can be found on or within the stone are cyanobacteria (blue-green algae) and chlorophytes (green-algae) (Caneva *et al.*, 1991).

The algae and cyanobacteria can be considered the pioneering inhabitants of a stone surface as they can grow whenever the dampness, temperature and light factors are suitable (Tiano, 2002).The most important factors conditioning the establishment of algae are light intensity, humidity, temperature and pH (Caneva *et al.*, 1991). The relationship of the first two factors with different algal associations is given in Figure 1. 3 (Caneva *et al.*, 1991).

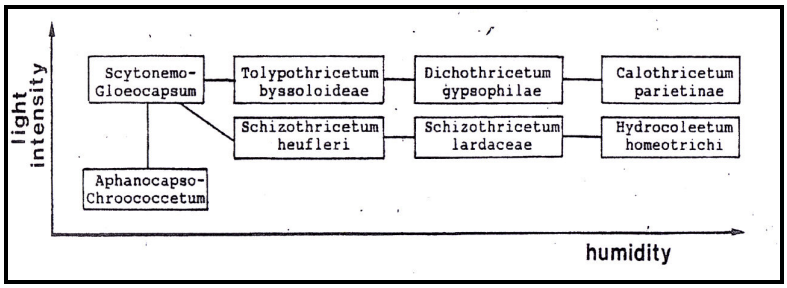



**Figure1. 3 Ecological succession of cyanobacteria associations with regard to light and humidity (Caneva *et al.*, 1991, p.94).**

These organisms can adapt themselves to very particular substrata, changing the colour and morphology. They usually participate in numerous lichenic associations (Tomaselli *et al.,* 1979). Limestone is more often colonized than other kinds of stone (Caneva *et al.*, 1991; Gaylarde *et al.*, 2006).

***Fungi***

Fungi are heterotrophic organisms that cannot grow on inorganic stone material unless there are organic residues (Caneva *et al.*, 1991). Fungi are important decomposers, animal and plant mutualistic symbionts and pathogens, and spoilage organisms of naturally manufactured materials (Gadd, 1993, 1999, 2006; Burford *et al.*, 2003). They also have an important role in the maintenance of soil structure (Gadd, 2007).

Fungi are major biodeterioration agents of stone, wood, plaster and other building materials and they are important components of rock-inhabiting microbial communities with significant roles in mineral dissolution and secondary mineral formation (Hughes and Lawley, 2003; Burford *et al.*, 2003). Recent studies have shown that several fungi can dissolve minerals and mobilize metals at higher pH

values, and over a wide redox range, faster and more efficiently than bacteria (Gu *et al.*, 1998; Castro *et al.*, 2000; Burford *et al.*, 2003).

Fungi have been found on a wide range of rock types including limestone, granite, marble, sandstone, andesite, basalt, gneiss, amphibolite, dolerite, soapstone and quartz in a variety of environments (Staley *et al.*, 1982; Gorbushina *et al.*, 1993; Sterflinger, 2000; Burford *et al.*, 2003).

The damage to the stone substrata can be biomechanical and/or biochemical (Gadd, 2007). Direct biomechanical damage may occur through hyphal penetration (Caneva *et al.*, 1991; Gadd, 2007). Indirect biomechanical weathering is particularly associated with the action of extracellular mucilaginous substances produced by fungi (Gadd, 2007). The most important deterioration aspect is the biochemical action caused by fungi (Caneva *et al.*, 1991; Gadd, 2007).

Biochemical weathering of rocks results in changes in the microtopography of minerals through pitting and etching, mineral displacement reactions, and even complete dissolution of mineral grains (Leyval *et al.*, 1993; Ehrlich, 1998; Adeyemi and Gadd, 2005). Fungi can solubilise minerals and metal compounds through several mechanisms like acidolysis, complexolysis and redoxolysis (Burgstaller and Schinner, 1993). The primary fungal impact on mineral dissolution is the acidolysis effect due to the production of organic acids (Caneva *et al.*, 1991; Fomina *et al.*, 2005).

Fungi also cause patina formation and discoloration of the stone surface due to the release of melanine (Leznicka *et al.*, 1988). This phenomenon causes both biomechanical and biochemical damage to the stone. Melanines are very stable indochinone derivatives that are produced from amino acid tyrosine, causing black coloured patina formation (Caneva *et al.*, 1991).

### *Lichens*

Lichens are formed as a result of the symbiotic association of algaea and/or cyanobacterium and fungi (Gadd, 2007). Their poikilohydric nature (to function and survive at varying water content) allows them to occur in extreme environments (Hawksworth and Hill, 1984). Together with cyanobacteria, they play an important role as pioneer organisms in the colonization on rocks and can colonize man-made substrate in a relatively few years (Caneva *et al.*, 1991).

The metabolic substances produced by the lichens can cause compositional change in the stone surface layer immediately beneath the lichen structure, with depletion of its main chemical elements (Al, Mg, Mn, Zn, Si, Ca, K, and Fe) and the accumulation of some, especially Ca, inside the thallus (Jones and Wilson, 1985).

Moreover, the application of microscopy techniques on the stone conservation showed that the lichenic growths on stone surfaces cause physical damage as a result of deep penetration of hyphae (Bech-Andersen and Christensen, 1983; Jones *et al.,* 1987). Because of their hyphal growths and high growth rate lichenic growth is one of the most unwanted and hard-to-remove biological deterioration types (Caneva *et al.*, 1991).

### *Plants*

Variations in climate in different regions cause changes in plant species growing in that particular area. Still, plants and their accompanying macrofauna and microfauna are abundant species all through the Earth.

They also grow largely on archaeological sites and buildings when the substrates and environmental conditions are favourable (Caneva *et al.*, 1991). Sufficient water content, adequate light for permitting photosynthetic activity and good

porosity of the substrate are the environmental conditions favouring the growth of plants (Caneva *et al.*, 1991).

Besides the problems of physical and visual obstruction caused by vegetation, the role carried out in the weathering processes is of a mechanical and chemical nature (Fisher, 1972; Allsopp and Drayton, 1975).

Plants vary in their ability to cause mechanical or chemical damage to the structure (Caneva *et al.*, 1991). The mechanical damage due to the growth and radial thickening of root tips is of great harm (Winkler, 1975). Other than the physical stress from the roots, the acidity of the root tips and the acidity and chelation ability of exudates cause the chemical action in the structure (Williams and Coleman, 1950; Keller and Frederickson, 1952). Some plants can also cause an undesirable coloration of stone due to the release of organic compounds into the pores of the substrate (Lewin and Charola, 1981). Trees in archaeological sites cause some problems due to the expansion of their root systems that can develop many meters under soil (Caneva *et al.*, 1991).

The presence of plants also induces variations of both macro and micro climatic parameters: an increase of relative humidity (RH) and water stagnation, a reduction of insolation, windiness and pollutants in the air. The last is due to absorption by leaves (Caneva *et al.*, 1991). From the standpoint of conservation, the effects can be either negative (favouring the growth of algae and mosses) or positive (reducing aeolian erosion, hydric exchanges and consequently the migration of salts) so the correct planning of conservation in archaeological areas must also bear these aspects in mind (Caneva *et al.*, 1991)

### *Animals*

Effects of animals of any size to the historical monuments and archaeological sites cannot be underestimated. Although, softer materials are affected more rapidly and severely, stone materials are also affected by animals in time.

Birds and especially urban avifauna seriously damage outdoor stone, in addition to creating problems of aesthetic nature (Caneva *et al.*, 1991). Avifauna damages the stone material directly or indirectly (Tiano, 2002). Direct destructive action occurs through physical damage such as trampling and grazing, or chemical damage caused by dropping of acid excrement containing high amounts of nitrate and phosphate compounds (Tiano, 2002). Indirect damage is made by organic substances accumulated on stone surfaces and serve as nutritive substrata for heterotrophic microflora (bacteria and fungi) (Winkler, 1975; Bassi and Chiatante, 1976; Nimis *et al.*, 1987).

Apart from birds, the ecosystem of insects and artropodes are the groups mostly involved in rock dwelling and rock decay (Urzì and De Leo, 2001). Black spots due to the dense deposits of mosquitoes excretes have been found on white marble surfaces (De Silva, 1975). Artropodes communities such as spiders were observed on stone building supporting algal and lichenic growth (Tiano, 2002) are examples of stone weathering by insects.

The grazing of ship, goat or other animals can also cause physical damage to the structure by injuring the walls and floors of the site (Caneva *et al.*, 1991).

Animals are also deteriorative for submerged historical monuments through the action of stone borers like molluscs, mussels, clams, and sea urchins that can bore up to 10-15 cm of the wall by means of mechanical and chemical actions (Pearson, 1987; Caneva *et al.*, 1991).

### 1.1.2. Limiting Factors of Biological Activity

Two laws of nature have been used in explanation of the interactions between limiting factors (see p.4 for description) and the biological populations (Caneva *et al.,* 1991).

Liebeg's law of minimum says that growth is controlled not by the total amount of resources available, but by the scarcest resource (Caneva *et al.*, 1991).

Shelford's law states that organisms have also an ecological maximum determining an interval representing the limits of tolerance (Caneva *et al.*, 1991).

In Figure 1.4 the behaviour of various species of different taxonomical groups with respect to the environmental parameter 'temperature' shows that each species has specific values under and over which it dies for opposite reasons (values too low or too high). The optimality value obviously lies somewhere between those values, but not necessarily in the arithmetical middle (Caneva *et al.*, 1991). Generally, the optimality value for biological species is nearer to the maximum limit of tolerance (Fig. 1. 4) (Caneva *et al.*, 1991).

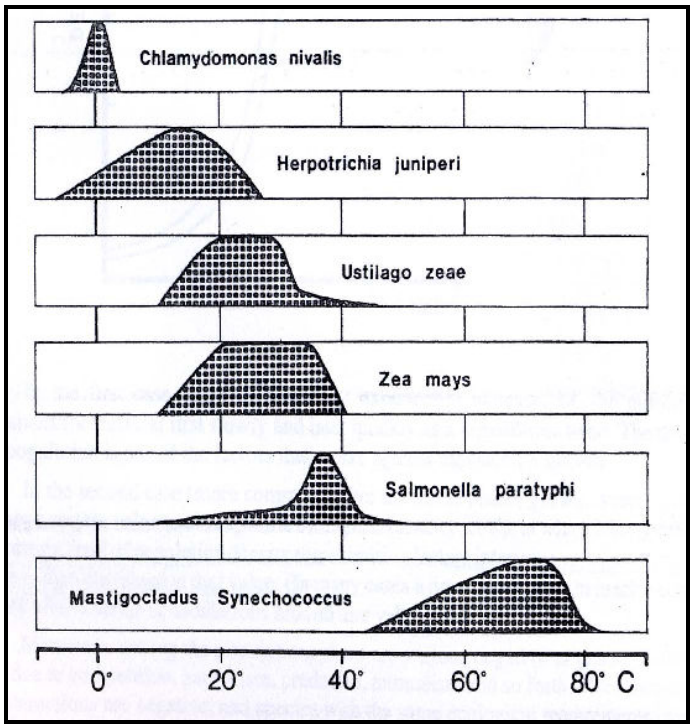



**Figure1. 4 Some organisms living range for temperature (Larcher, 1976; Caneva *et al.*,1991, p.6).**

Organisms may have a wide range of tolerance for one factor and a narrow one for another (Caneva *et al.*, 1991). Those with narrow limits are linked to precise conditions making it possible to interpret the values of environmental parameters to which they are sensitive (Caneva *et al.*, 1991). Thus, when present they can be used as bioindicators that is the biological species or communities that indicate certain values of an environmental parameter. The water content of the environment being high or low values, or the effect of pH on species is some of the limiting factors that can be used as bioindicators (Caneva *et al.*, 1991). Sometimes the absence of a species can also be used as a bioindicator of certain

negative parameters such as the absence or reduction of lichens where there is pollution.

### 1.1.3. Formation of Biological Patina

Biodeterioration can be seen visually by means of patina formation. In the case of calcareous stones the composition of patina is based on the presence of calcium oxalates ($CaC_2O_4 \cdot nH_2O$), gypsum ($CaSO_4 \cdot 2H_2O$), calcite ($CaCO_3$), silicates in various amounts with some accessory minerals (e.g. iron oxide) (Alessandrini and Gianfilippo.; 1988; Rampazzi *et al.*, 2004). The films are often decayed and discontinuous, presenting colours from pale pink to yellow, to ochre or brown (Rampazzi *et al.*, 2004).

There are two main hypotheses for the origin of patina formation: biological or chemical (Rampazzi *et al.*, 2004). In biological origin the characteristic stratigraphy of calcium oxalate films is due to the occurrences of microorganism colonisation on the stone surfaces, oxalic acid being a metabolic product of the same organisms fed by organic compounds of the usual deposition present on the stone (Rampazzi *et al.*, 2004). As in the chemical origin, it is the degredative oxidation products of organic material, deriving, for example, from past conservation treatments present on the substrate (Rampazzi *et al.*, 2004).

In both cases, the reaction between oxalic acid and calcium compounds present on the stone either as constituent mineral (calcite) or as an intentional or accidental deposit (calcium salts), forms calcium oxalate with the mineralogical phase of whewellite ($CaC_2O_4 \cdot H_2O$) and wedellite ($CaC_2O_4 \cdot 2H_2O$) depending on thermodynamic conditions (Rampazzi *et al.*, 2004*).*

### 1.1.4. Methods for Investigation of Biodeterioration

Microorganisms take the lead role in biodeterioration of stone. It is important to identify and quantify the microorganisms affecting the stone deterioration. There

are several analytical methods for identification and quantification of microbial populations.

In order to have reliable results in microbiological study, different experimental methods have to be used. Fluorescence techniques (Adam and Duncan, 2001), microflora total, total carbon, total nitrogen (Diack and Stott, 2001) or total phosphate measurements are helpful for quantification. Microscopic techniques and biological techniques such as growth of the organisms on selective media, DNA extraction and Polymerase Chain Reaction (PCR) techniques are also used for the identification of the species.

***Fluorescence Techniques***

Fluorescence is used in detection of microorganisms in various fields. Fluorescence of Adenosine Tri Phosphate (ATP), in order to detect organisms has been used in many applied fields such as pharmaceutics, wastewater treatments, soil quality, etc. (Jago and Sidorowicz, 1989; Ranalli *et al.,* 1997; Adam and Duncan, 2001; Ranalli*,* 2003). The use of fluorescein esters as a measure of enzyme activity was first noted by Kramer and Guilbalt (1963). Since the total microbial activity is a good general measure of organic matter turnover in natural habitats (Heal and McClean, 1975), the method was adapted to the use of environmental samples in order to use this method as an indication of the total microbial activity.

Detection of fluorescence by colorimetric analyses has been done by using fluorescein diacetat (FDA), 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl tetrazolium chloride (INT) in the detection of microbial activity in soil (Adam and Duncan, 2001; Lundgren, 1981; Taylor and May, 1995). Firefly luciferin-luciferase has been used for the measurement of the microbial activity in the

works of art (Casida *et al.*, 1964; Lundgren, 1981; Harvey and Young, 1980; Bitton and Koopman, 1982; Schnurer and Rosswall, 1982; Ranalli *et al.*, 1997).

The bioluminescent firefly luciferin-luciferase reaction is one of the most sensitive ATP detection systems. The procedure involves the destructive method of extraction of DNA from the samples (Ranalli *et al.*, 1997). According to Taylor and May (1995) INT was a less sensitive measure of activity and it is less suitable to be used in the field study.

In 1980, Swisher and Caroll developed a method and showed that the amount of fluorescein produced by the hydrolysis of fluorescein diacetate (FDA) was directly proportional to the microbial population growing on Douglas Fir foliage. The method developed by Swisher and Caroll (1980) was later evaluated by Shnürer and Rosswall (1982) for determining total microbial activity in soil and straw litter as well as in the determination of cell density in pure microbial cultures. Since 1982, FDA hydrolysis has been used to measure total microbial activity in a range of samples such as: the mould growth on wood and other building materials (Bjurman, 1993), stream sediment biofilms (Battin, 1997), deep sea clay and sand sediment profiles (Gumprecht *et al.*, 1995), and developing a soil quality index (Diack and Stott, 2001).

FDA hydrolyzed by a number of various enzymes, such as proteases, lipases and esterases (Green *et al.,* 2006). It is a colourless compound upon hydrolysis by both free (exoenzymes) and membrane bound enzymes (Stubberfield and Shaw, 1990), and the acid–yellow coloured product fluorescein is released (Fig. 1. 5).

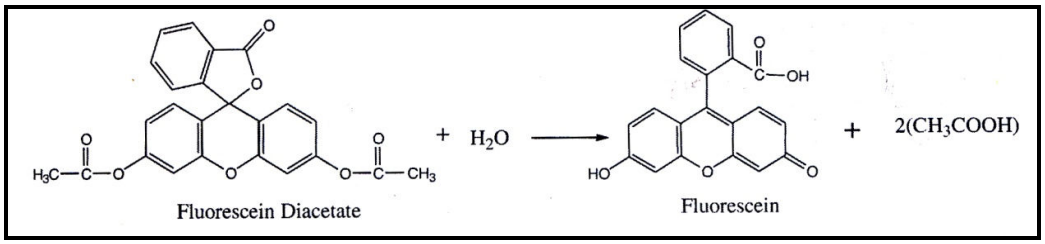



**Figure1. 5The reaction of FDA treatment and the end product (Green *et al.*, 2006)**

The product fluorescein can be measured spectrophotometrically at 490 nm wavelength (Adam and Duncan, 2001). The optimum conditions affecting the FDA reaction with the enzymes include optimum pH (7-8), optimum temperature being 30˚C to 40˚C and time of incubation being 30 minutes to 2 hours (Adam and Duncan, 2001; Green *et al.*, 2006).

### *Microflora Total*

Microflora total is an experimental technique for the quantification and identification of the microbial population. The identification is based on the general groups of microorganisms that can be found on deteriorated stone (Debusschere, 1973).

## 1.2. Control of Biological Growth

Control of biological growth is one of the treatments to be developed in conservation studies. The aim of such control is the elimination of biodeteriogens. The method includes sterilization of microflora, extermination of larvae and insects and eradication of higher plants (Caneva *et al.*, 1991). The efficacy of these treatments depends on the methods and the products chosen, but new growth will inevitably occur if the environmental conditions favouring biological growth are not changed (Caneva *et al.*, 1991). To obtain lasting results, all the necessary interventions other than direct control of biodeterioration have to be established when conservation is being planned (Caneva *et al.*, 1991). A flow chart can be drawn including the questions to be considered before deciding to perform a biodeterioration control treatment (Fig. 1. 6).

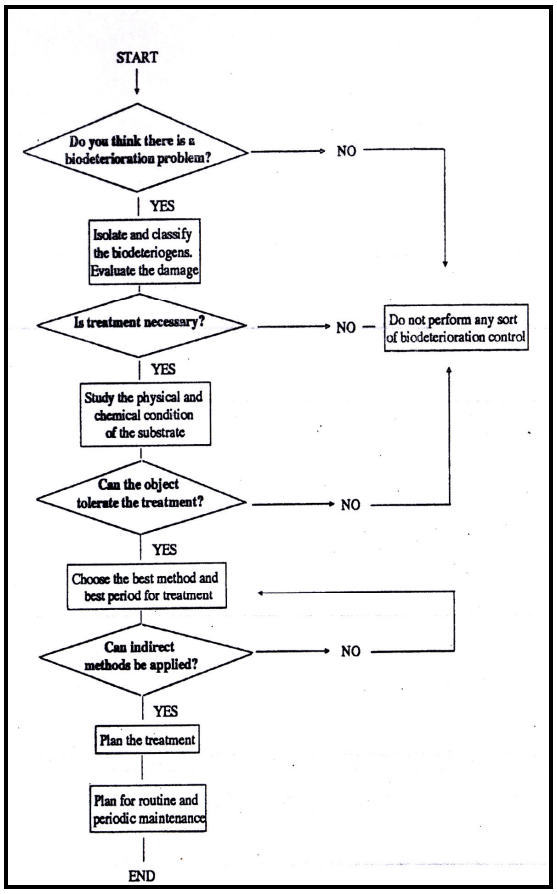



**Figure1. 6 Flow chart showing the questions to be considered before deciding to perform a biodeterioration control treatment (Caneva *et al.*, 1991, p.125).**

With regards to the principles and the nature of the means employed control methods can be classified as mechanical, physical, biological, and chemical or biochemical methods (Caneva *et al*, 1991).

Mechanical technique is a method of displacing the biodeteriogen (Caneva *et al.*, 1991; Tiano, 2002). Those methods do not produce lasting results; moreover they

can damage the substrate during removal of root systems (Caneva *et al.*, 1991). When properly employed by restorers, those methods can be useful especially when coupled with chemical methods (Caneva *et al.*, 1991).

The methods used in physical control of biological activity are: ultraviolet rays (UV), gamma rays, low frequency electrical current systems, heat, deep-freeze temperatures and ultrasonic (Caneva *et al.*, 1991).

Biological combat is based upon exploitation of the parasitic or antagonistic groups of animals and vegetal organisms (Caneva and Salvadori, 1988).

Chemical control of biological activity involves pesticides and disinfectants (Caneva *et al.*, 1991; Tretiach *et al.*, 2007). Pesticides are chemicals used for destroying undesirable biological growth (Caneva *et al.*, 1991). Disinfectants are chemicals that destroy vegetative forms of harmful microorganisms (Caneva *et al.*, 1991).

Biochemical methods involve the use of chemical compounds of biological origin which cannot be considered as pesticides (Caneva *et al.*, 1991). Antibiotics and enzymes are some examples of biochemical compounds. All these methods used in the control of biological growth have side effects on stone monuments and are not long lasting. The use of biotechnology should also be considered as a control method of biological growth.

### 1.2.1. Biomineralisation

Biomineralisation is simply the synthesis of minerals from simple compounds by organisms. It involves the selective extraction and uptake of elements from the local environment and their incorporation into functional structures under strict biological control (Mann, 2001) (Fig. 1. 7). Elements such as calcium, iron, carbon, phosphorus and silicon are cycled over millions of years through complex pathways that at some critical stage involve biomineralisation (Mann, 2001).

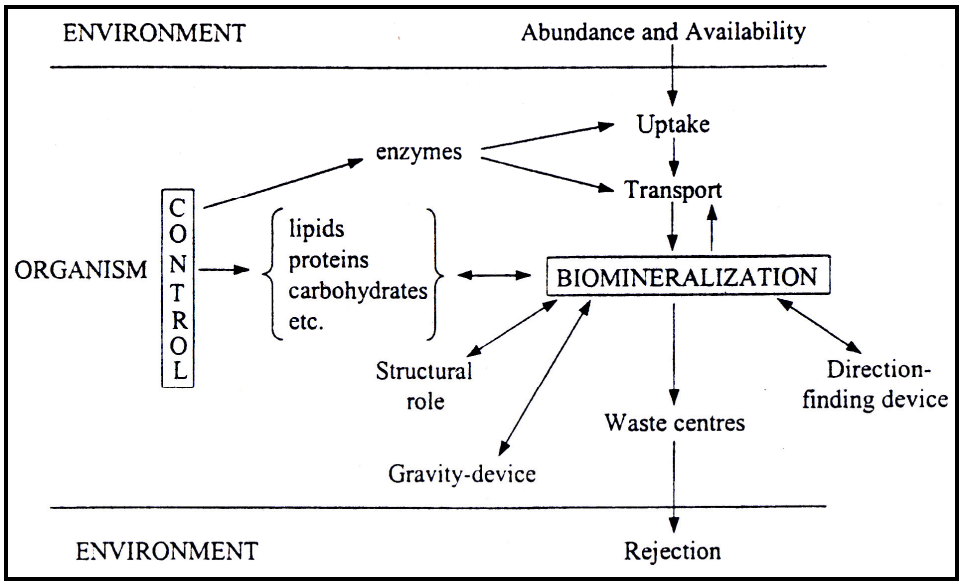



**Figure1. 7 Biomineralisation and the environment (Mann, 2001, p.1).**

One property of biominerals must be their low solubility under physiological conditions (Mann, 2001). In biologically induced mineralization, inorganic minerals are deposited by precipitation, which arises from secondary interactions between various metabolic processes and the surrounding environment (Mann, 2001). For example, in certain types of green algae, calcium carbonates are precipitated from saturated calcium bicarbonate solutions by metabolic removal of carbon dioxide during photosynthesis, according to the equilibrium in Figure 1. 8 (Mann, 2001).

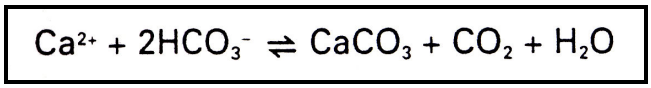


**Figure1. 8 The equation of calcium carbonate precipitation in some types of green algae (Mann, 2001, p.24).**

In a similar way, the extrusion of many metabolic products across or into the cell wall of bacteria can result in the biologically induced precipitation of various inorganic materials by subsequent reaction with metal ions (Mann, 2001).

The biologically controlled mineralization is a highly regulated process that produces materials such as bones, shells and teeth (Mann, 2001). These biominerals are distinguished by reproducible and species-specific crystallochemical properties such as; uniform particle size, well-defined structure and composition, high level of spatial organization, and complex morphologies (Mann, 2001). Biologically controlled mineralization is widespread in unicellular creatures such as algae and protozoa, and extremely common in multicellular organisms (Mann, 2001). One of the well documented cases for biologically controlled biomineralisation is the formation of magnetite in bacteria so called *magnetotactic bacteria* (Mann, 2001).

Calcium based biominerals are found in a very large number of organisms (Crichton, 2001). The shells of molluscs are among the most abundant biogenic minerals, and are composed of 95-99% calcium carbonate crystal and less than 5% organic matrix (Crichton, 2001). In nature carbonate precipitation theoretically occur following several known processes: abiotic chemical precipitation from saturated solutions by evaporation, temperature increase and/or pressure decrease; external or internal skeleton production by eukaryotes

(Crichton, 2001); lowering of $CO_2$ pressure under effect of autotrophic processes (photosynthesis); fungal mediation (Callot *et al.*, 1985; Verracchia and Loisy, 1997); heterotrophic bacterial mediation (Caneva *et al.*, 1991).

The production of calcium carbonate particles through bacterial mediation follows different ways (Castanier *et al.*, 1999). In autotrophy, three metabolic pathways are involved: non-methylotrophic methanogenesis (Marty, 1983), anoxygenic photosynthesis and oxygenic photosynthesis (Castanier *et al.*, 1999) (Fig. 1. 9). All three pathways use $CO_2$ as carbon source to produce organic matter (Castanier *et al.*, 1999).

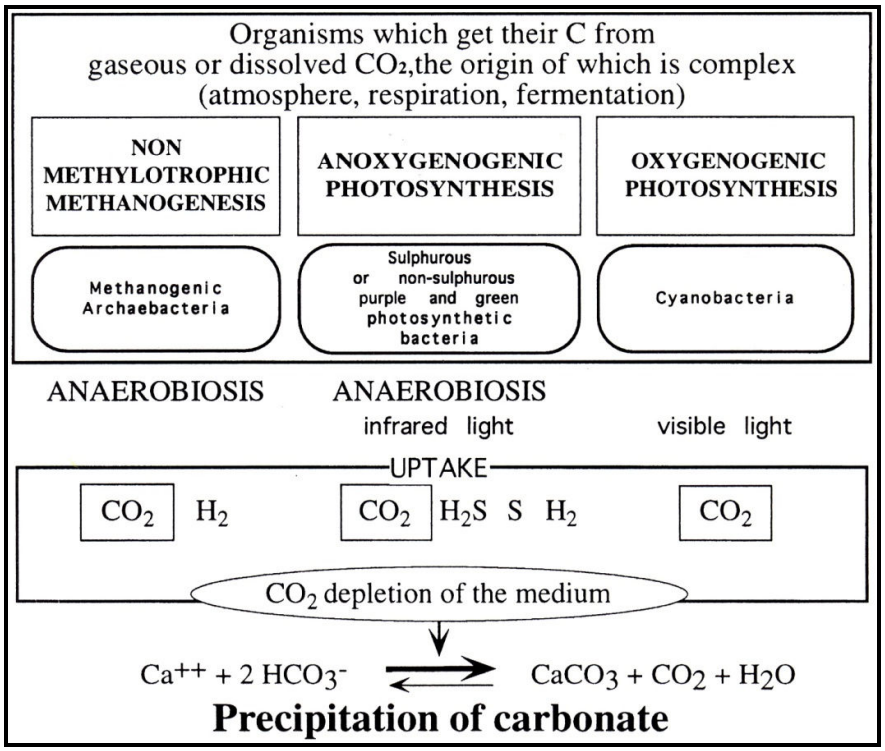



**Figure1. 9 Pathways for the production of calcium carbonate by bacterial mediation (Castanier *et al.*, 1999, p.11).**

Heterotrophic pathways have two bacterial processes as passive and active precipitation (Castanier *et al.*, 1999). Passive precipitation operates by producing carbonate and bicarbonate ions and inducing various chemical modifications in the medium that lead to the precipitation of calcium carbonate by two different metabolic cycles as; nitrogen cycle and the sulphur cycle (Castanier *et al.*, 1999) (Fig. 1. 10, 1. 11).

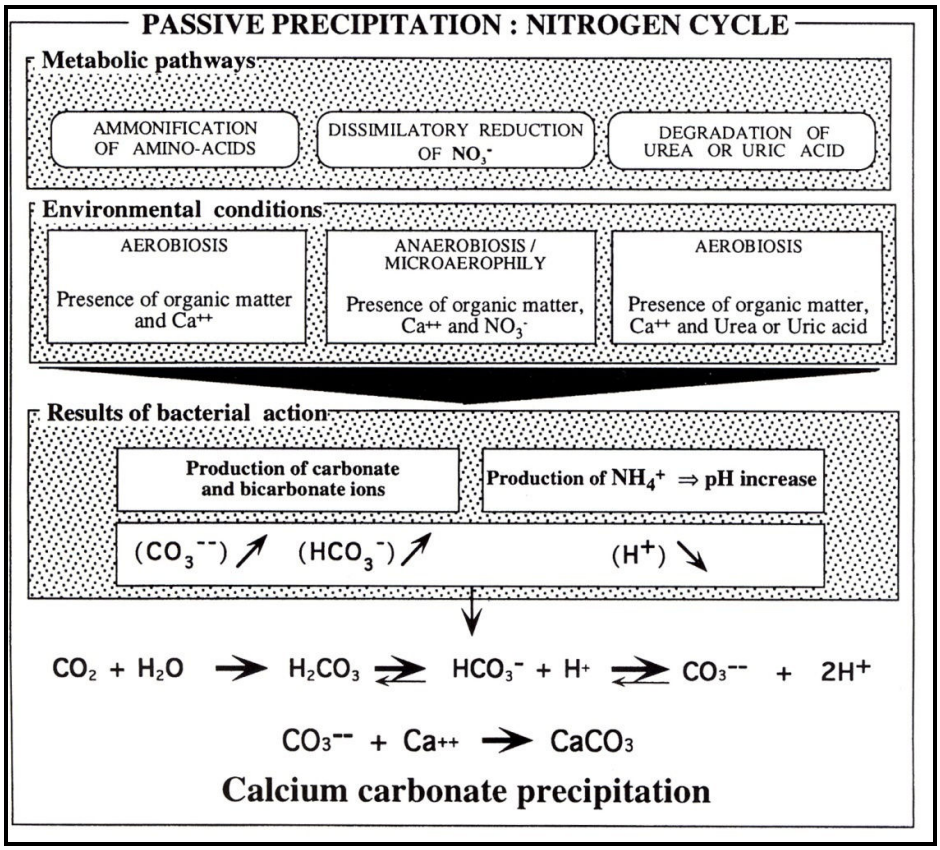



**Figure1. 10 Passive calcium carbonate precipitation by nitrogen cycle (Castanier *et al.*, 1999, p.12).**

Active precipitation is independent of the other previously mentioned metabolic pathways (Castanier *et al.*, 1999). The carbonate particles are produced by ionic exchanges through the cell membrane by activation of calcium and/or magnesium ionic pumps or channels, probably coupled with carbonate ion production (Castanier *et al.*, 1999). Numerous bacterial groups are able to operate such processes (Castanier *et al.*, 1999).

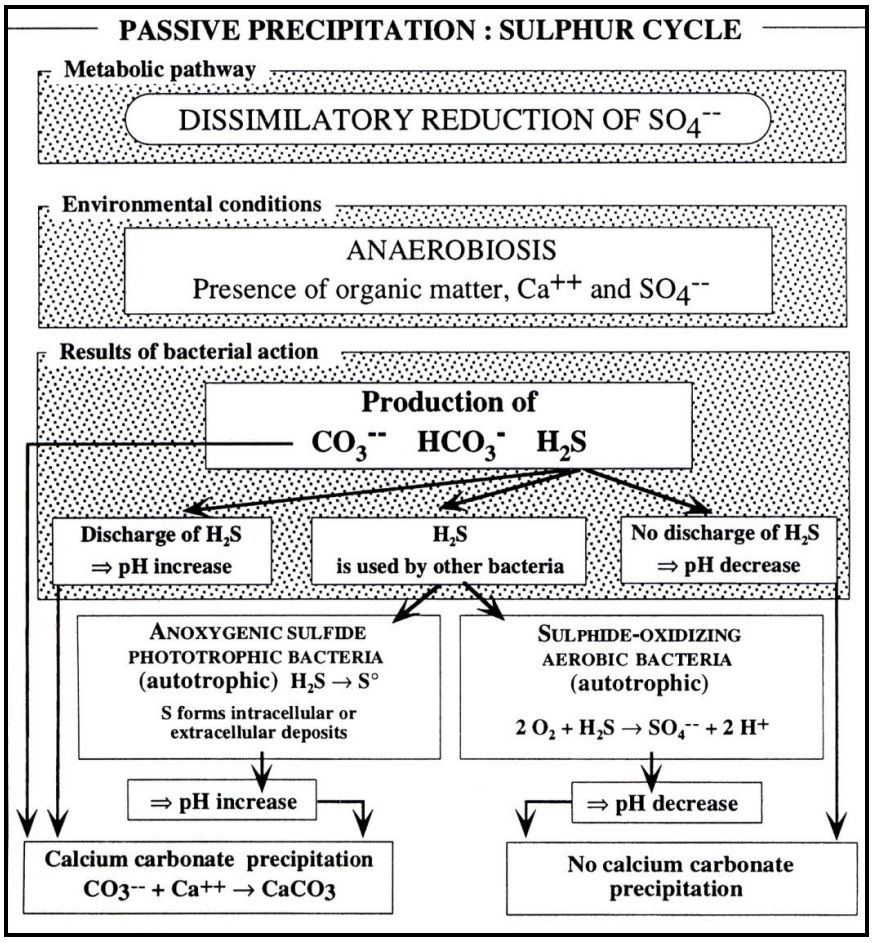



**Figure1. 11 Passive calcium carbonate precipitation by sulphur cycle (Castanier *et al.*, 1999, p.13).**

In nature, carbonatogenesis is generally carried out by plurispecific populations so that sediment organic matter may be totally mineralised into carbonates (Castanier *et al.*, 1999). The production of solid carbonate depends upon the strains in the bacterial population, the environmental conditions, the quality and

quantity of available nutrients and time (Castanier *et al.*, 1999). Biomineralisation studies carried under laboratory conditions have considered those aspects. Heterotrophic *Bacillus cereus* strains were found to be useful for calcification procedure under laboratory conditions (Castanier *et al.*, 1999; Le Métayer-Levrel *et al.*, 1999). The procedure of biomineralisation used in this study was taken from the study of Le Métayer-Levrel *et al.* (1999).

### 1.3 Aim of the Study

This study aimed to examine biological deterioration and its effects on some stone monuments and to test a method for controlling their biodeterioration problem. It was desired to analyse the damage and the compositional characteristics of the biofilms present on calcareous stone surfaces such as marble and limestone. For that purpose, analyses of representative stone samples from deteriorated monuments and natural stones showing similar structure and deterioration that were in the vicinity of those monuments were studied; namely limestones of Nemrut Mount Monument and marbles of Pessinous Archaeological Site. Time, space and the effect of environmental conditions were not taken into consideration in this study. The aim of this study was to find a practical and easy method to examine biological decay qualitatively and quantitatively, and also to test a biomineralisation process for controlling biodeterioration.

The problem of defining the extent to which alterations can be ascribed to chemical or to microbiological processes has been studied at length but has not been solved yet. It is necessary not only to have a deeper knowledge of the microbial ecology of stone but also to establish common standardized laboratory procedures so that the results obtained by different authors can be compared for a better understanding of the microbial decay of stone. Therefore, this study aimed at composing practical and reliable methods for the examination of

biodeterioration in historic stone monuments. It is necessary to use qualitative and quantitative methods to see how the biological activity is distributed in the stone and estimate the amount of biological activity that is responsible from biodeterioration.

In this study, biological activity was examined by using FDA analyses, light microscopy, SEM-EDX, XRD and spectrophotometric analyses. Biomineralisation method was used in order to control biological growth.

# CHAPTER 2

# MATERIALS AND METHODS

In this study the representative stone samples associated with biological deterioration zones and patinas, coming from two important monuments namely; limestones of Nemrut Mount Monument and marbles of Pessinous Archaeological Site were analysed.

Biologically deteriorated zones and relatively undeteriorated interiors of the samples were investigated by using several analytical techniques.

Determination of material composition and mineralogical properties of selected samples were done by the help of several analyses: petrographic analyses of thin and cross sections under light microscope and stereo microscope, respectively; X-Ray Powder Diffraction analyses (XRD) were used to determine inorganic and organic minerals. In addition, scanning electron microscope (SEM) coupled with EDX analyses were used for the samples before and after the biomineralisation procedure.

Biological measurement methods were also applied to the samples to get the degree of biological deterioration. Fluorescence techniques were used on selected samples using fluorescein diacetate (FDA). Cross section images of samples were taken by light microscope with fluorescent light source. Visible absorption spectrophotometer was used to determine the fluorescence of biological deterioration in the samples, quantitatively.

*Bacillus cereus* bacteria were applied to the stone surfaces together with nutritive solution to form artificial biomineralisation. After sufficient time passed the changes on the surfaces were investigated by various analytical techniques. In

addition, colorimetric measurements were done before and after the application of bacteria.

### 2.1. Nomenclature of the Samples

In order to simplify the presentation of experimental results, samples were coded. The nomenclature of the samples is given below.

First, two or three capital letters correspond to the location where the samples were taken and/or type of stone studied (e.g. NEL shows Nemrut East Terrace Limestone, PM shows Pessinous Marble). First number following the letters corresponds to the sample number. Samples having significant biological deposition on their surface were named as P (patina) (e.g. NEL1P shows Nemrut East Terrace Limestone sample 1 Patina). To signify untreated, treated samples with lyophilized and fresh bacteria applied U, L and F capital letters were used after sample number indicating untreated, lyophilized bacteria, and fresh bacteria applied conditions (e.g. NEL2U shows Nemrut East Terrace Untreated Limestone sample 2, NES1L shows Nemrut East Terrace Lyophilized bacteria applied Sandstone sample 1). Small letters appeared at the end indicate colour of the patina (e.g. ‘g’ correspond to green, ‘o’: orange,, ‘gr’: gray,, ‘w’: white and ‘bk’: black patina).

### 2.2. Historic Buildings and the Description of Samples

In this section, the samples studied and the monuments from where the samples were taken were described briefly.

#### 2.2.1. Nemrut Mount Monument

Nemrut Mount Monument is located in Kahta, Adıyaman. The monument was dedicated to Kommagene King Antiochus and that of the gods. The tomb of the

king was concealed under the man-made burial mound. The construction of the monument headed back to 40 B.C. (Appendix 1).

Limestone and sandstone samples of Nemrut Mount Monument and their related analyses are given in Table 2. 1 - Table 2. 5.

**Table 2. 1 Nemrut Untreated Limestone Samples.**

| Sample | Photograph | Type of Analysis |
|---|---|---|
| NEL1U |  | Thin/Cross Section<br>XRD<br>SEM-EDX<br>FDA<br>Colorimetric Data (L*a*b) |
| NEL2U |  | Thin/Cross Section<br>Colorimetric Data (L*a*b) |

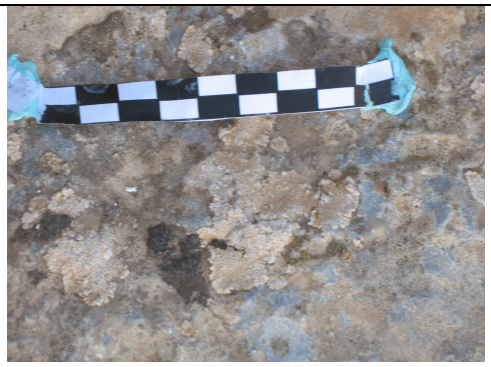
**Table 2. 2 Nemrut Lyophilized Bacteria Treated Limestone Samples.**

| Sample | Photograph | Type of Analysis |
|---|---|---|
| NEL2L |  | SEM-EDX<br>Colorimetric Data (L*a*b) |
| NEL3L | | XRD<br>Colorimetric Data (L*a*b) |
| NEL4L | | FDA<br>Colorimetric Data (L*a*b) |
| NEL5L | | FDA<br>Colorimetric Data (L*a*b) |

**Table 2. 3 Nemrut Fresh Bacteria Treated Limestone Samples.**

| Sample | Photograph | Type of Analysis |
|---|---|---|
| NEL2F |  | SEM-EDX<br>Colorimetric Data (L*a*b) |
| NEL3F | | XRD<br>Colorimetric Data (L*a*b) |
| NEL4F | | FDA<br>Colorimetric Data (L*a*b) |
| NEL5F | | FDA<br>Colorimetric Data (L*a*b) |

**Table 2. 4 Nemrut Limestone Samples with Patina.**

| Sample | Photograph | Type of Analysis |
|---|---|---|
| $NEL1P_w$ |  | XRD |
| $NEL2P_w$ |  | XRD<br>FDA<br>SEM-EDX |
| $NEL3P_g$ |  | XRD<br>FDA |

**Continuation of Table 2.4.**

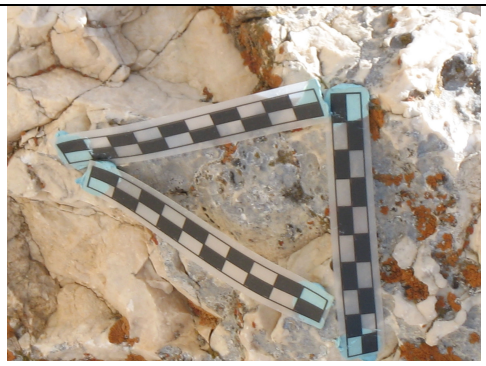
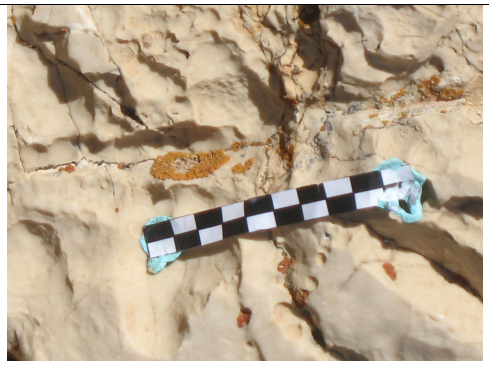
| | | |
|---|---|---|
| $NEL4P_{gr}$ |  | XRD |
| $NEL5P_{o}$ |  | XRD<br>FDA |
| $NEL6P_{bk}$ |  | XRD<br>FDA |

**Table 2. 5 Nemrut Sandstone Samples.**

| Sample | Photograph | Type of Analysis |
|---|---|---|
| NES1U |  | XRD<br>FDA<br>Colorimetric Data (L*a*b) |
| NES1L |  | SEM-EDX<br>FDA<br>Colorimetric Data (L*a*b) |

### 2.1.2. Pessinous Archaeological Site

Pessinous archaeological site has been located at Ballıhisar village, on Ankara-Eskişehir highway. Phyrigian city of Pessinous was dedicated to the mother god of Kybele. It is dated back to 700 B.C. Pessinous is a city that has harboured various cultures in itself. The Phyrigien city of Pessinous was founded by King Midas. It had independence even after the occupation of Galatians. Since Galatians became incorporated by Romans the city of Pessinous became the capital city of Galatians and developed as a major trading centre in central

Anatolia. Late Roman and Byzantine period continued up to 5th century A.D until Seljuk Turks invaded this area. General view and map of Pessinous with its chronological order were given at Appendix 2.

The marble samples were collected from the detached parts of the theatre (from a seat) in Pessinous Archaeological Site (PM2U) and from ancient quarry of Pessinous from Roman period. Those samples and the type of analyses done with the samples are described in Table 2. 6 - Table 2. 9.

**Table 2. 6 Pessinous Marble Samples.**

| Sample | Photograph | Type of Analysis |
|---|---|---|
| PM1U |  | Thin Section<br>XRD<br>FDA<br>Colorimetric Data (L*a*b) |
| PM2U |  | FDA<br>XRD<br>Colorimetric Data (L*a*b) |

**Continuation of Table 2. 6**

| | | |
|---|---|---|
| PM3U |  | Thin Section<br>XRD<br>FDA<br>SEM-EDX<br>Colorimetric Data (L*a*b) |

**Table 2. 7 Pessinous Lyophilized Bacteria Treated Marble Samples.**

| Sample | Photograph | Type of Analysis |
|---|---|---|
| PM2L |  | XRD<br>Colorimetric Data (L*a*b) |
| PM3L | | FDA<br>Colorimetric Data (L*a*b) |
| PM4L | | FDA<br>Colorimetric Data (L*a*b) |

**Table 2. 8 Pessinous Fresh Bacteria Treated Marble Samples.**

| Sample | Photograph | Type of Analysis |
|---|---|---|
| PM2F |  | XRD<br>Colorimetric Data (L*a*b) |
| PM3F | | FDA<br>Colorimetric Data (L*a*b) |
| PM4F | | FDA<br>Colorimetric Data (L*a*b) |

**Table 2. 9 Pessinous Marble Samples with Patina.**

| Sample | Photograph | Type of Analysis |
|---|---|---|
| $PM1P_{bk}$ |  | XRD |
| $PM2P_{bk}$ |  | XRD |

## 2.2 Determination of Petrographical and Mineralogical Properties

Mineralogical properties of samples were examined by using XRD, thin section and SEM-EDX analyses.

### 2.2.1. X-Ray Diffraction (XRD) Analysis

Powdered samples were prepared for X-ray diffraction analyses. The biological patinas were scraped off from the stone surfaces. The scraped pieces of the

samples were grounded in an agate mortar. Deteriorated stone surfaces were also scraped and grounded in an agate mortar. The bulk stone sample separated from patina and deteriorated zones were also grounded in an agate mortar to be analysed by XRD.

Some of the patina samples were treated with 5% HCl in order to investigate, the presence of clay minerals at deterioration zones. $CaCO_3$ was expected to be dissolved in dilute acid. The solution was filtered with Whatman No. 40 filter paper. The residue was dried at 60°C in the oven and grounded in an agate mortar. X-ray diffraction analyses were carried on these powdered samples using unoriented mounts.

The instrument used was X-Ray Diffractometer Rigaku D/MAX2200/PC. Analysis was done using CuKα radiation, adjusted to 40kV and 40 mA. The XRD traces were recorded for the 2θ values from about 3˚ to 70˚. Mineral phases were identified in XRD traces.

### 2.2.2. Thin Section Analysis

For these analyses, the samples were put into plastic moulds having sizes of 1.5x3x1 cm. Araldite D (ESCIL) and Durcisseur HY 956 (ESCIL) were mixed and the samples were covered with the mixture. They were kept in vacuum of 100 torr for 30 minutes. After one day, the samples were cut into half with a low speed saw (BUEHLER, ISOMET). One of the slices was treated with fluorescein diacetate to inspect the depth of the biological deterioration under the fluorescence microscope (Leica DM025) at 490nm wavelength. The other was kept for thin section analysis and reduced to 30μm thickness on a microscope slide. The thin sections of the samples were examined using Leica DM025 light microscope with a photographic attachment.

### 2.2.3. Scanning Electron Microscopy (SEM) Coupled With Energy Dispersive Analyzer (EDX)

SEM analysis aimed to provide complementary information about morphology and microstructure of patinas and stone as well as the product of the biomineralisation process. Samples of about 1 cm dimensions with a flat surface was prepared and coated with gold. The instrument used was a JEOL JSM-5400 Scanning Electron Microscope operated at 22 kV and coupled with Energy Dispersive X-ray analysis (EDX) system by which elemental analysis could be done.

SEM micrographs were taken where possible and elemental analyses were carried out and evaluated together with the micrographs.

## 2.3. Biomineralisation

Biomineralisation treatment was applied to the representative samples from each location (Table 2.2, 2.3, 2.5, 2.7 and 2.8). The sample sets were separated into two groups according to the type of treatment with bacteria being freshly prepared bacteria and lyophilized bacteria.

The procedures followed are given below.

### 2.3.1. Preparation of Bacterial Medium

It is known that bacteria and fungi can grow on various kinds of media which include changing amounts of phosphate, sulphate, chloride, glucose, acetate, amino acids and yeast extract and so on (Madigon *et al.*, 2003).

Nutrient broth was used for growing both lyophilized and fresh *B.cereus.* Medium was prepared by dissolving 8 grams of nutrient broth (Merck, BDH Analar) in 1 litre of demineralised water. In order to prevent the possible contamination occurrences during the preparation of the medium, autoclaving

was performed. The nutrient broth was autoclaved at 121˚C for 15 minutes. The pH of the solution was 7±0.2 at 25˚C.

### 2.3.2. Preparation of Fresh Bacteria

Lyophilized bacteria frozen under high vacuum have been planted on agar plates and left for growth at 28˚C for 48 hours. The bacteria cultured from the lyophilized bacteria have been removed from agar plates to nutrient broth mentioned above. They were left for growth at 28˚C for 48 hours. The growing bacteria in the liquid medium were centrifuged at 5000 rpm for 10 minutes to get the pellet. The pellet contained a dense population of calcitic bacteria. It was kept at 4˚C until application.

### 2.3.3. Preparation of Lyophilized Bacteria

25 grams of nutritive powder (Nutricale) was dissolved in 1 litres of deionised water which was free of $Cl^-$ ions by shaking vigorously. After dissolving all the nutritive powder, 3 grams of lyophilised bacteria were added to the medium. After addition of bacteria the medium was not supposed to be shaken anymore and settled for 12 hours at room temperature before application.

### 2.3.4. Preparation of Nutritive Medium

25 grams of nutritive powder (Nutricale) was dissolved in 1 litre of deionised water which was free of ions by shaking vigorously.

### 2.3.5. Application of Fresh and Lyophilized Bacteria to the Samples

The media prepared with fresh bacteria and with lyophilized bacteria were applied to the surface of stone samples by the aid of a paint brush. Then, the nutritive medium was applied to the same surfaces of the stone samples. This

process was repeated within a period of five days. At the end of those five days the application of nutritive medium was stopped. After a period of two months the samples were examined using different analytic methods in order to observe the changes due to biomineralisation.

### 2.4. Examination of Biological Activity

Qualitative and quantitative analyses of biological activity were done by using fluorescence techniques before and after biomineralisation. The fluorescence were developed by FDA treatment.

#### 2.4.1. Fluorescence of samples with FDA

Fluorescein diacetate (Lipase Substrate, Sigma Aldrich Co. Ltd., USA) was used for determining the depth of biodeterioration and the effects of the biomineralisation on samples. The solutions used were buffer solution, FDA stock solution, and fluorescein stock solution. FDA treatment procedures applied to the samples were adapted from the studies done by Adam and Duncan (2001).

***Preparation of Buffer Solution***

The buffer solution used in the preparation of fluorescein standard solution was prepared from $K_2HPO_4$ (Riedel-de Haën, Sigma-Aldrich Co. Ltd, Analar) and $KH_2PO_4$ (Merck, BDH Analar). According to Adam and Duncan (2001) the rate of hydrolysis of fluorescein compound reaches to maximum between pH 7.0 and 8.0. In this study, a buffer solution with pH 7.5 was prepared as follows.

8.7 grams of $K_2HPO_4$ and 1.3 grams of $KH_2PO_4$ were dissolved in 800 ml distilled water and the volume was completed to one litre with addition of distilled water. The pH of the solutions was measured using pH papers before usage and at some intervals during the usage (Adam and Duncan, 2001).

***Preparation of FDA stock solution***

0.1 grams of FDA was dissolved in approximately 80 ml of acetone and the volume was completed to 100 ml with acetone. The resulting solution had a concentration of 1 mg FDA/ml. Solution was kept in refrigerator below 0˚C until application. The solution was prepared freshly for each application (Adam and Duncan, 2001).

***Preparation of fluorescein stock solution***

0.2265 gram of fluorescein sodium salt (Sigma-Aldrich) was dissolved in approximately 80 ml phosphate buffer of pH 7.5 and the volume was made up to 100 ml with the same buffer. The resulting solution was 2 mg fluorescein/ml (Adam and Duncan, 2001).

***Preparation of 20 µg fluorescein $ml^{-1}$ standard solution***

1 ml of stock solution (2 mg fluorescein/ml) was added to a 100 ml volumetric flask and the volume was made up to the mark with buffer solution of pH 7.5. Then, 1-5 µg/ml standards were prepared from that standard solution by appropriate dilution with buffer solution of pH 7.5 (Adam and Duncan, 2001).

***FDA application to the stone samples***

FDA applications to the samples were done for two different purposes: to measure the depth of the biological deterioration from the surface through the stone by staining the cross sections of the samples and to measure the total microbial activity in the stone before and after biomineralisation in powdered samples.

Cross sections of the samples were prepared as slices from the stone surfaces down to interiors having thickness of 2 mm and 1 cm depth. The cross sections

were stained by FDA solution and analysed by using light microscopy. On the other hand, those type of cross sections were grounded in an agate mortar for quantitative FDA analysis using spectrophotometric measurements.

Samples prepared for both types of FDA analyses were placed in glass flasks and 15 ml of phosphate buffer (pH 7.5) was added to them. Then, FDA stock solution was added to start the reaction. The samples were shaken thoroughly and placed in an oven at 30˚C for 30 minutes. After removing the samples from oven, the powdered samples were centrifuged at 4000 rpm for 3 minutes. The supernatant was filtered with Whatman No 40 filter paper and the filtrate was measured at 490 nm in a spectrophotometer (Optima SP-3000 Plus UV/VIS Spectrophotometer).

Examinations of cross-sections were done under light microscope (Leica DMC 320) with photographic attachment having external light source (Leica EL6000) for fluorescence excitation under blue filter (450-490 nm).

### 2.5. Colorimetric measurements

Colorimetric measurements were done on the surfaces of samples before and after the biomineralisation treatment with a spectrophotometer (Konika Minolta Cmd-2600) using the CIELa*b* standards (1976). Distinct colour changes on the samples before and after the biomineralisation were undesirable.

# CHAPTER 3

# EXPERIMENTAL RESULTS

Nemrut limestones, Pessinous Archaeological Site marbles and Nemrut sandstones were examined for their mineralogical and petrographical characteristics as well as distribution and amount of biological activity present in them. Results of those analyses are given in the following sections of this chapter.

## 3.1. Mineralogical and Petrographical Properties

Mineralogical properties of samples were studied by XRD, light microscopy of thin sections and SEM-EDX analyses to determine the mineralogical phases, their distribution and elemental composition.

### 3.1.1. X-Ray Diffraction Analysis

All the samples were examined for their mineralogical composition at their exterior surface and compared with their composition at relatively undeteriorated interiors. For the determination of minor components, some samples were treated with 5% HCl to dissolve the major component that was calcite mineral. XRD analyses of Nemrut limestone samples were carried out on micro samples of statues, on similar samples from geological formations showing different types of biological patina and on biological patina samples after fresh and lyophilized bacteria applications. Different coloured biological patinas such as: green, orange, black, white and gray on limestones were examined.

XRD analyses of Pessinous marbles were carried out on the biological patina samples, on samples from crack surfaces of the marbles having biological activity and on the biological patina samples after fresh and lyophilized bacteria applications.

XRD analyses of Nemrut sandstones were carried out on the biological patina of samples.

XRD patterns of the samples indicated that the main component of the limestones from Nemrut Mount Monument was calcite as expected (Fig. 3. 1).

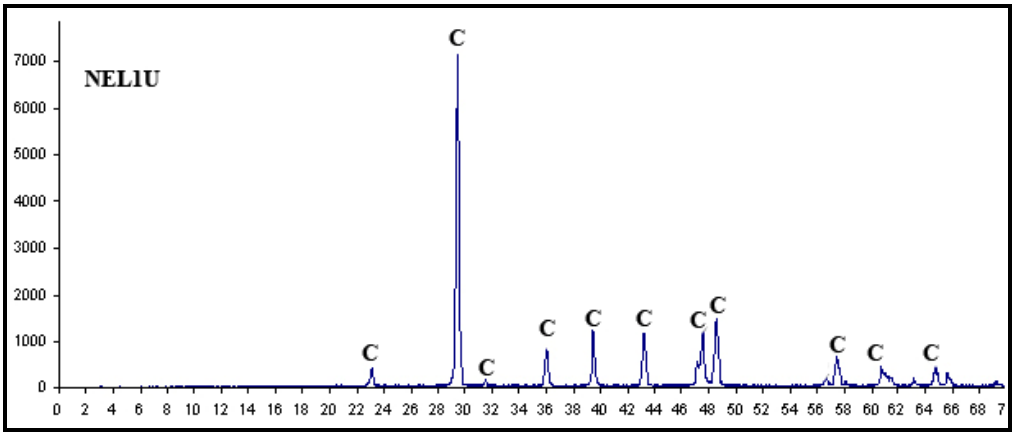
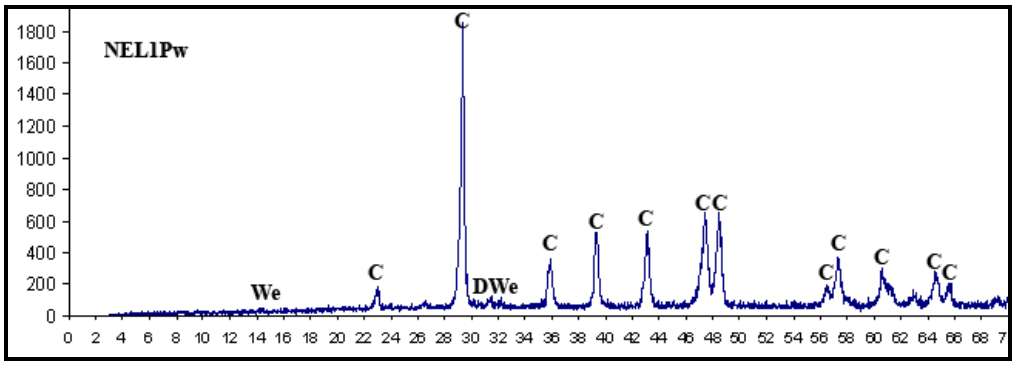



**Figure 3. 1 Interior parts of geological limestone sample from East Terrace, C: Calcite.**

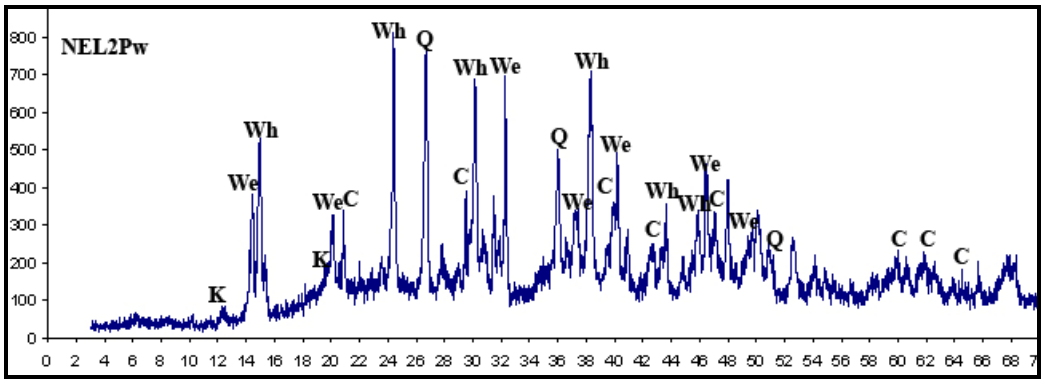
The XRD patterns of white patina formation on the throne at the east terrace revealed that the main component was calcite with little amounts of wedellite and dolomite (Fig. 3. 2).




2θ˚ Cu Kα

**Figure 3. 2 White patina sample (NEL1Pw) from East Terrace, C: Calcite, D: Dolomite, We: Wedellite.**

Another sample with white patina formation from east terrace showed traces of calcite, calcium oxalates: wedellite, and whewellite, quartz and kaolinite (Fig. 3. 3).




2θ˚ Cu Kα

**Figure 3. 3 White patina sample (NEL2Pw) from East Terrace, C: Calcite, K: Kaolinite, Q: Quartz, We: Wedellite, Wh: Whewellite.**

XRD patterns of a sample with green patina formation on the east terrace before and after treatment with HCl revealed that the main components of the patina were calcite, wedellite and whewellite along with small amounts of quartz (Fig. 3. 4).

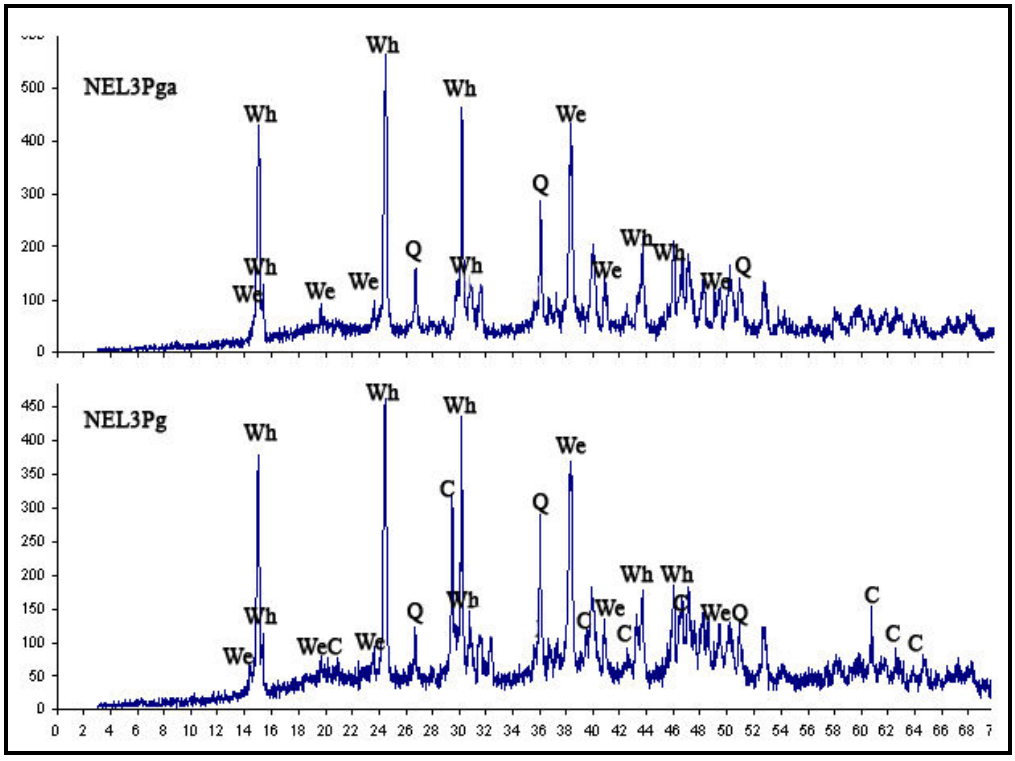



**Figure 3. 4 Green patina sample from East Terrace before (NEL3Pg) and after (NEL3Pga) acid treatment, C: Calcite, Q: Quartz, We: Wedellite, Wh: Whewellite.**

Gray patina formed on eagle statue at the east terrace was mainly composed of calcite and wedellite with traces of quartz mineral (Fig. 3. 5).

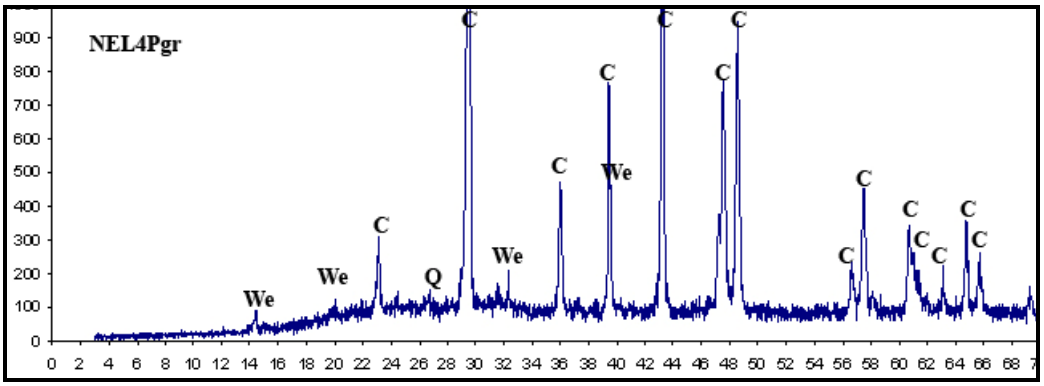



**Figure 3. 5 Gray patina sample from Eagle statue of East Terrace, C: Calcite, Q: Quartz, We: Wedellite.**

The orange coloured patina on Heracles statue from east terrace showed the presence of calcite, quartz, wedellite, and traces of clay minerals. Diffused peaks around 22°, 26° and 41°, seen on the XRD pattern might be due to the presence of amorphous silicate minerals such as opal-A and amorphous clay minerals, etc (Fig. 3. 6).

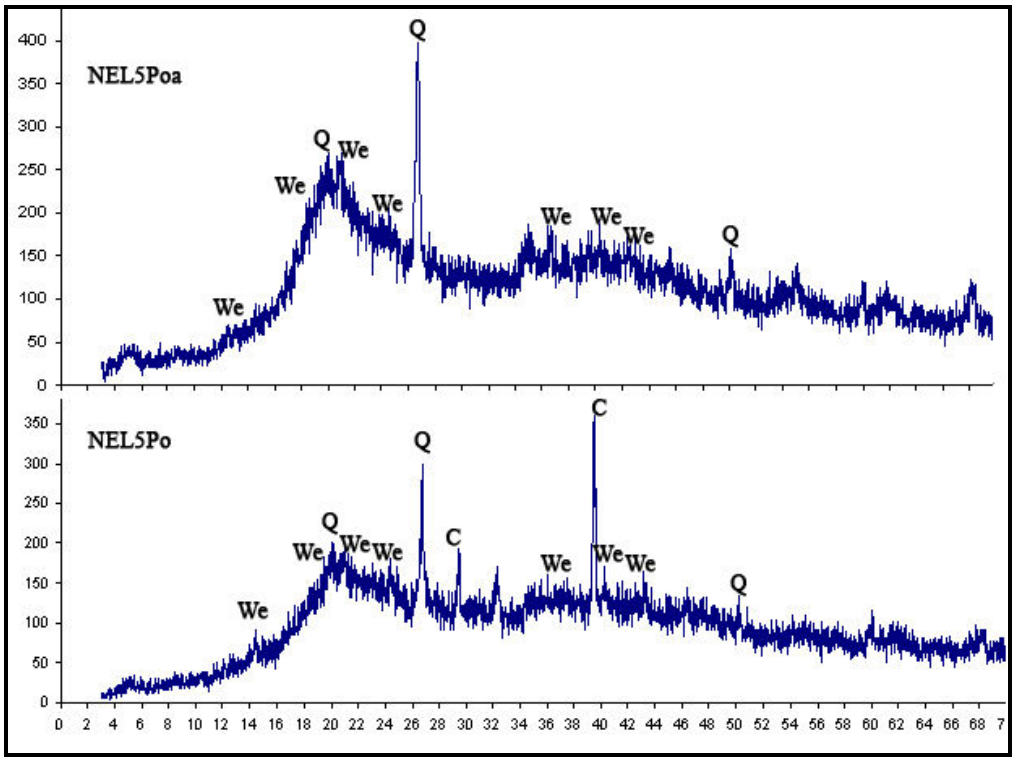



**Figure 3. 6 Orange patina sample from Heracles statue at the East Terrace before (NEL5Po) and after (NEL5Poa) acid treatment, C: Calcite, Q: Quartz, We: Wedellite.**

Black coloured biofilm on the limestones of east terrace presented peaks of calcite, quartz, feldspars and clay minerals mainly kaolinite (Fig. 3. 7).

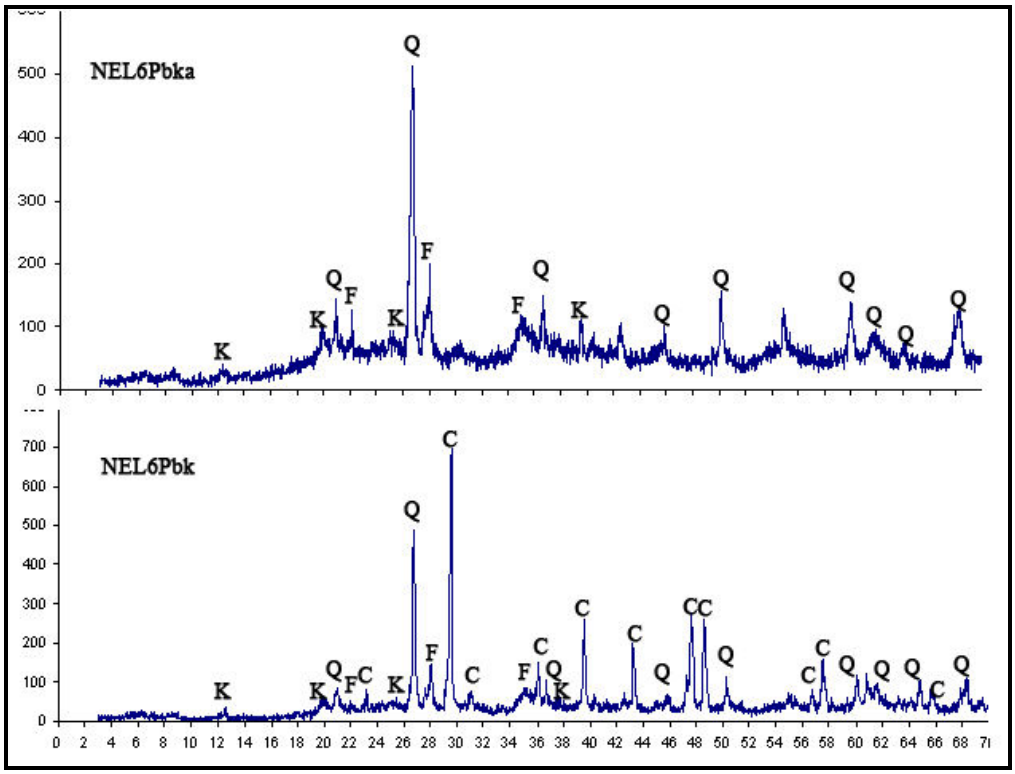



**Figure 3. 7 Black patina sample from East Terrace before (NEL6Pbk) and after (NEL6Pbka) acid treatment, C: Calcite, F: Feldspars, K: Kaolinite, Q: Quartz.**

The XRD patterns of limestone surfaces free of visible biological patina that were treated with lyophilized and fresh bacteria, revealed no biominerals but calcite (Fig. 3. 8).

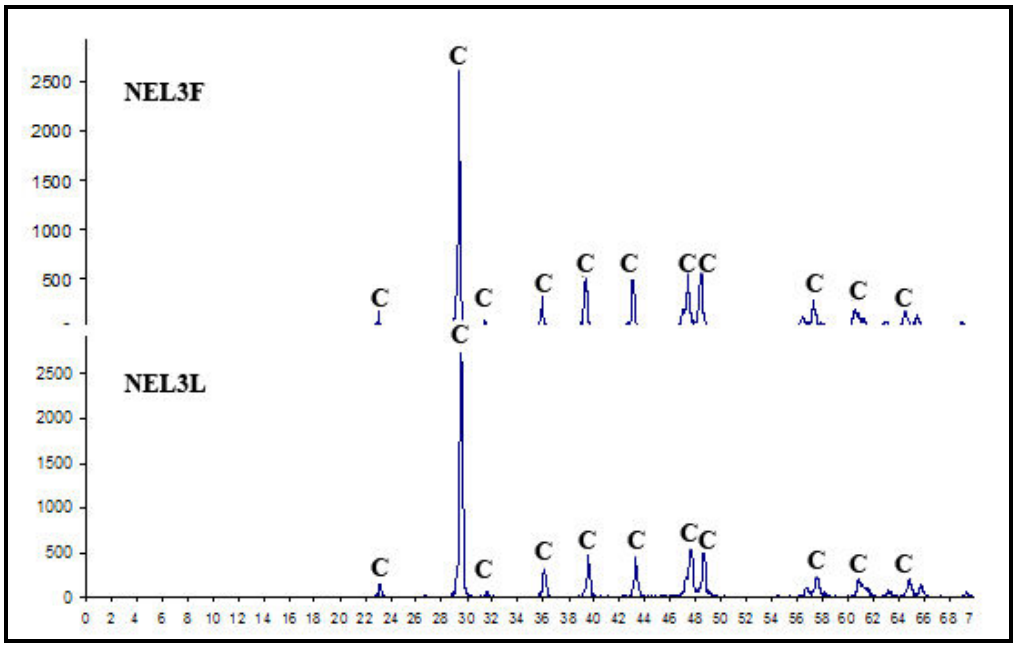



**Figure 3. 8 Limestone sample treated with lyophilized bacteria (NEL3L) and sample treated with fresh bacteria (NEL3F), C: Calcite.**

Pessinous marbles had mainly calcite with small amounts of dolomite in their composition. The exposed surface showed the presence of biomineral wedellite (Fig. 3. 9).

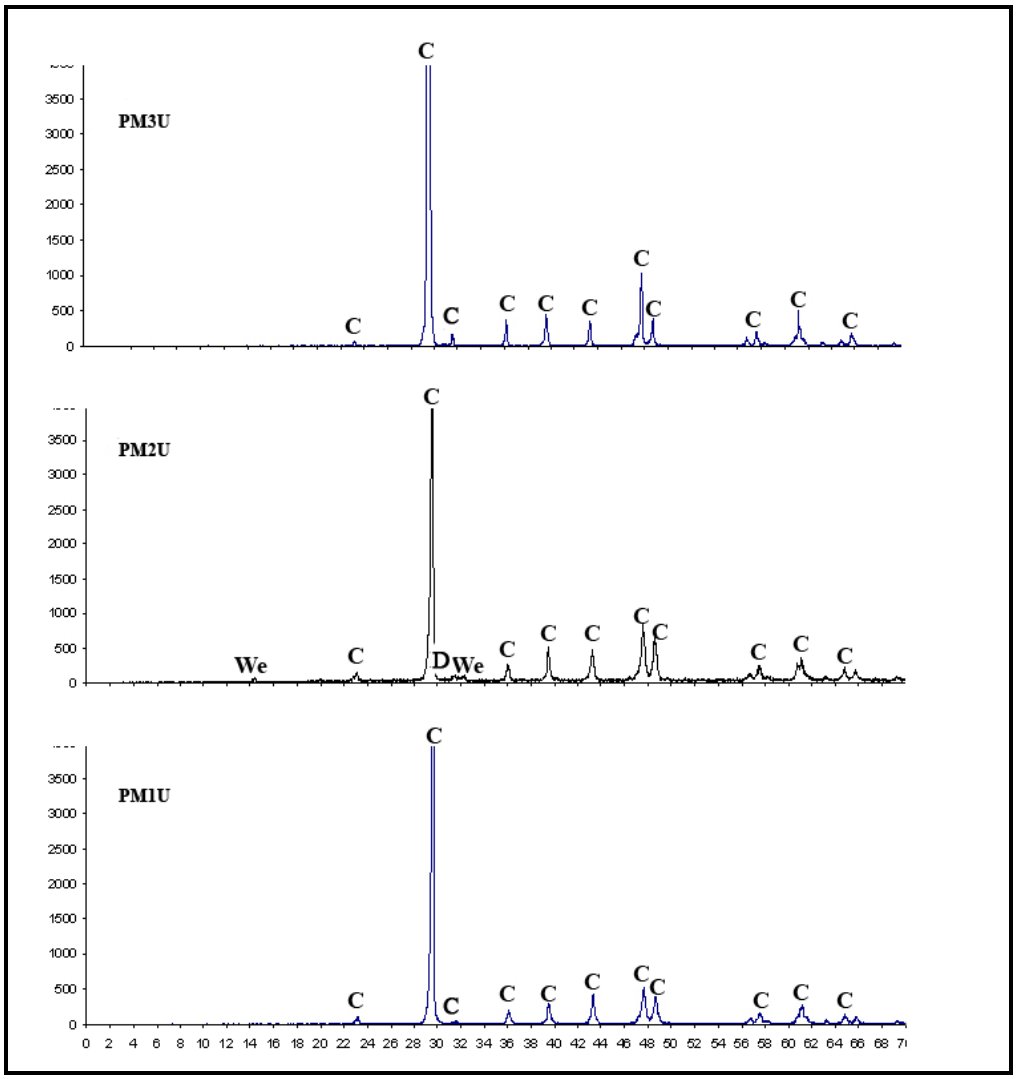



**Figure 3. 9 Deteriorated marble sample from Pessinous Archaeological Site (PM2U) and marble samples from ancient Pessinous quarry (PM1U,PM3U), C: Calcite, D: Dolomite, We: Wedellite.**

XRD patterns of biological patina taken from Pessinous Archaeological Site revealed the presence of wedellite, dolomite and quartz minerals along with the main component calcite (Fig. 3. 10).

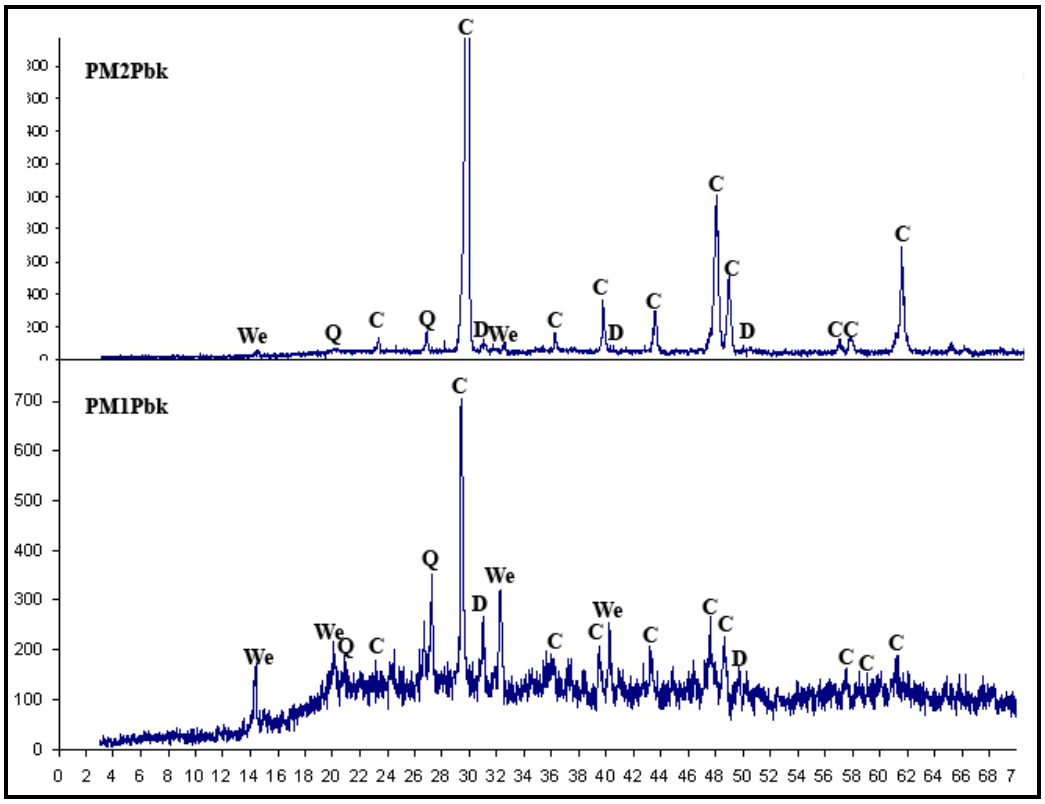



**Figure 3. 10 Black patinas from the surface of Pessinous Archaeological Site, C: Calcite, D: Dolomite, Q: Quartz, We: Wedellite.**

XRD traces of lyophilized and fresh bacteria applied marble samples with no visible biological patina revealed only calcite peaks (Fig. 3. 11).

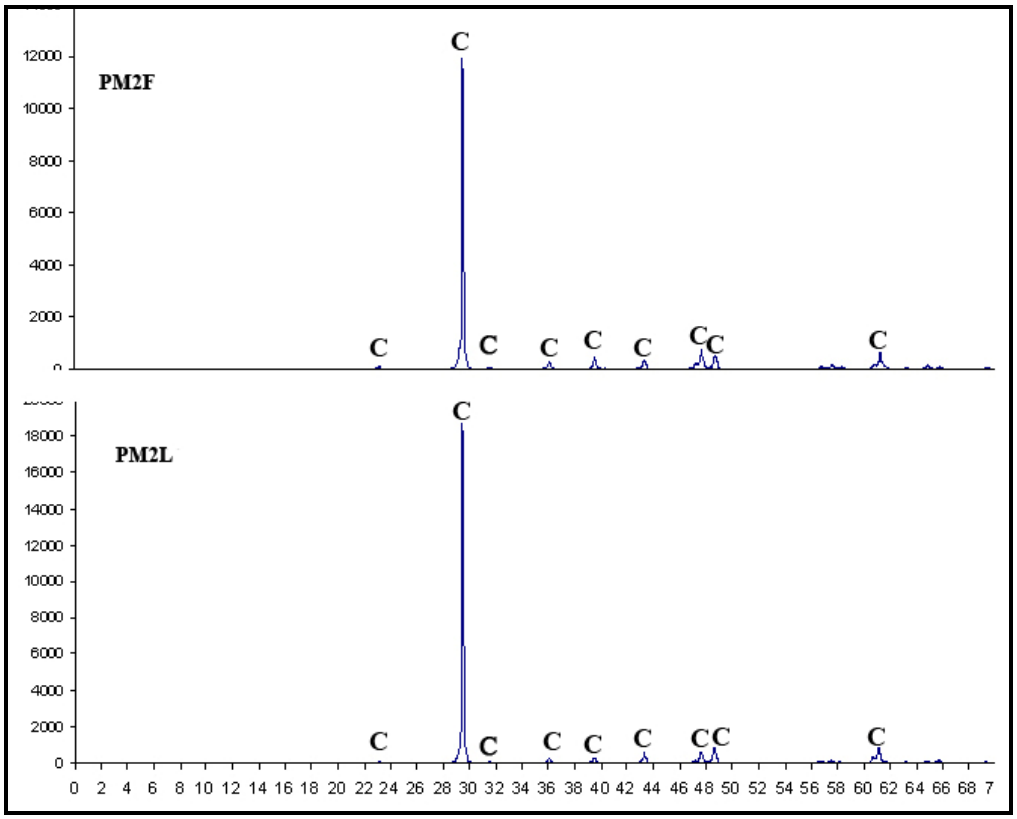



**Figure 3. 11 Marble sample with no visible biological patina treated with lyophilized bacteria (PM2L) and marble sample treated with fresh bacteria (PM2F), C: Calcite.**

Sandstone sample from Nemrut Mount Monument having black patina was mainly composed of quartz, feldspars and with small amounts of calcite, and clay mineral kaolinite (Fig. 3. 12).

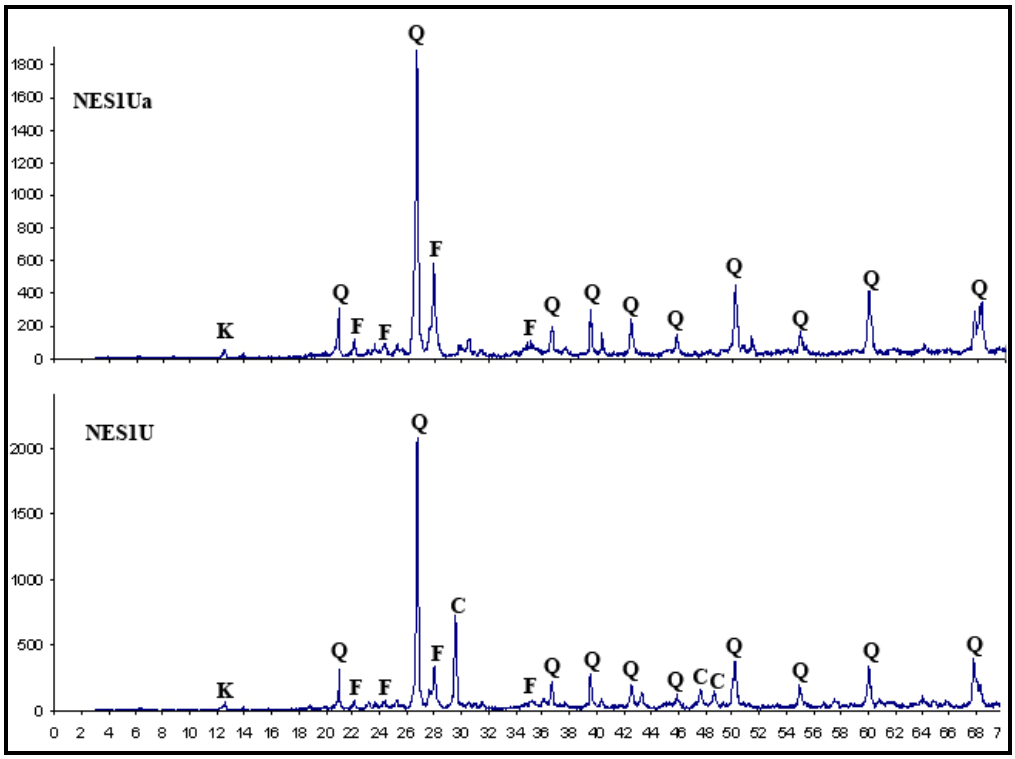



**Figure 3. 12 Sandstone samples from East Terrace before (NES1U) and after (NES1Ua) acid treatment, C: Calcite, F: Feldspars, K: Kaolinite, Q: Quartz.**

### 3.1.2 Thin Section Analysis

Petrographic analyses were carried out on thin sections of limestone samples from Nemrut Mount Monument and marble samples from Pessinous Archaeological Site. Some of the samples had visible biological patina at their surface.

The petrographic analyses carried out on thin sections revealed that Nemrut limestones were composed of micritic calcite with fossil remains (Fig. 3. 13).

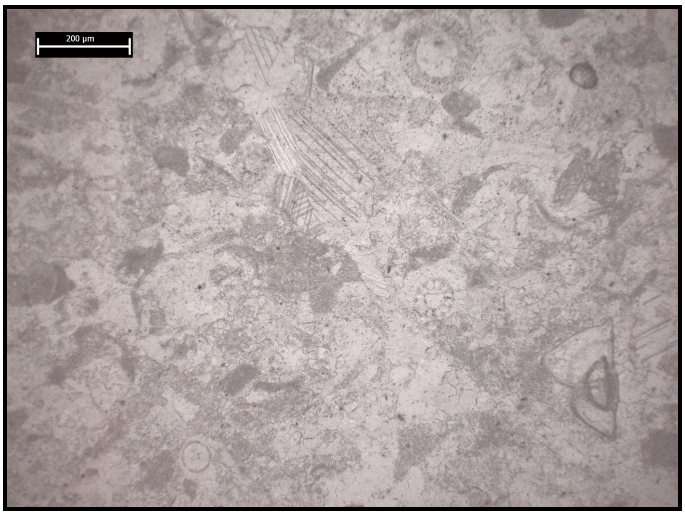
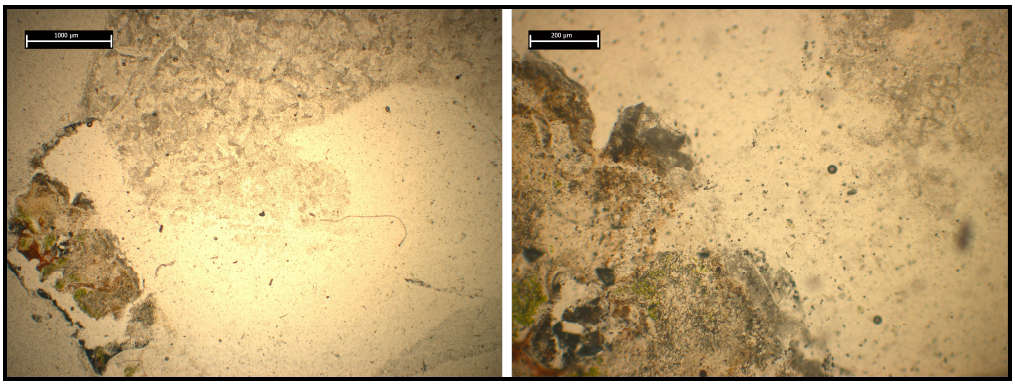



**Figure 3. 13 Thin section view of limestone from Nemrut East Terrace, NEL2U (10X, single nicol).**

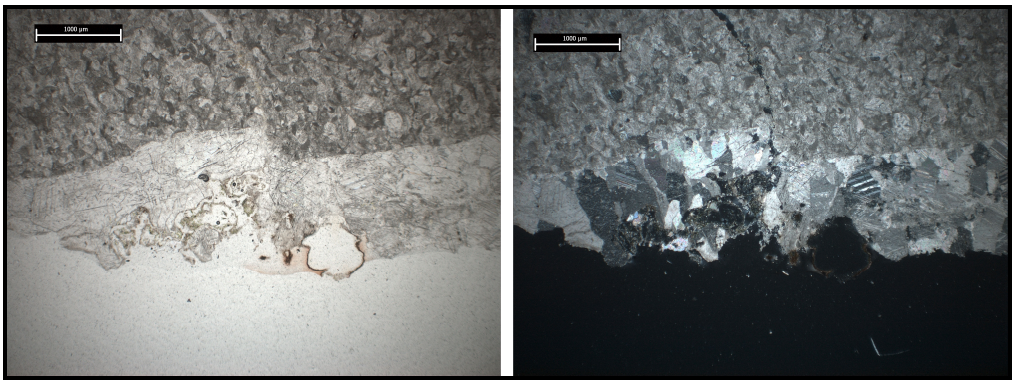
Biological patina formation was visible in some of the samples and the thickness of patina on the surface was variable from 1 mm to 2 mm (Fig. 3. 14).



2.5X, single nicol 10X, single nicol

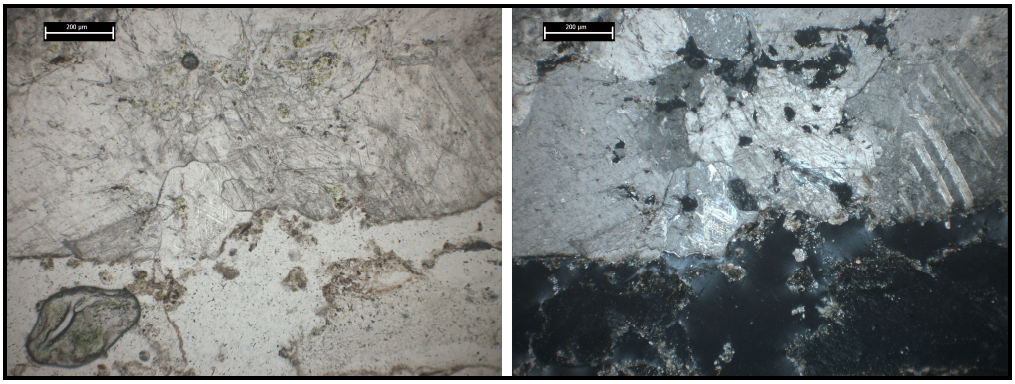
**Figure 3. 14 Thin section views of patina on the limestone surface, NEL1U.**

It was observed that biological activity has penetrated through the microcracks. The depth of penetration of visible biological activity was up to 4 mm from the exterior surface (Fig. 3. 15). The biological activity inside the stone had a deteriorative effect on the stone structure causing detachments in the compact stone structure and increase in porosity (Fig. 3. 16).



single nicol cross nicols

**Figure 3. 15 Thin section view of microcracks through the surface and biological activity inside the microcracks, NEL2U (2.5X).**



single nicol cross nicols

**Figure 3. 16 Thin section view of biological activity inside the stone, NEL2U (10X).**

Thin sections of Pessinous marbles revealed that they were composed of heteroblastic textured calcite grains with twins (Fig. 3. 17). Biological activity was visible especially in intergranular zones (Fig. 3. 17 - 3. 19). The penetration

of visible biological activity was approximately up to 5 mm from the surface of the stone (Fig. 3. 19).

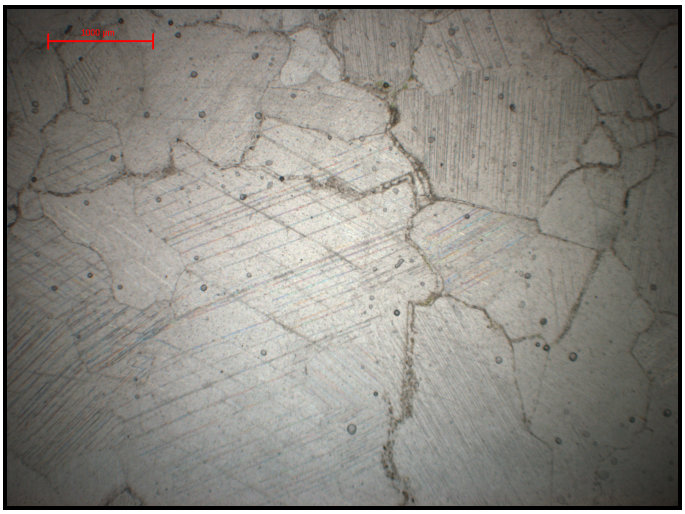


**Figure 3. 17 Thin section view of Pessinous quarry marble (PM1U) showing biological activity in intergranular zones, (2.5X, single nicol).**

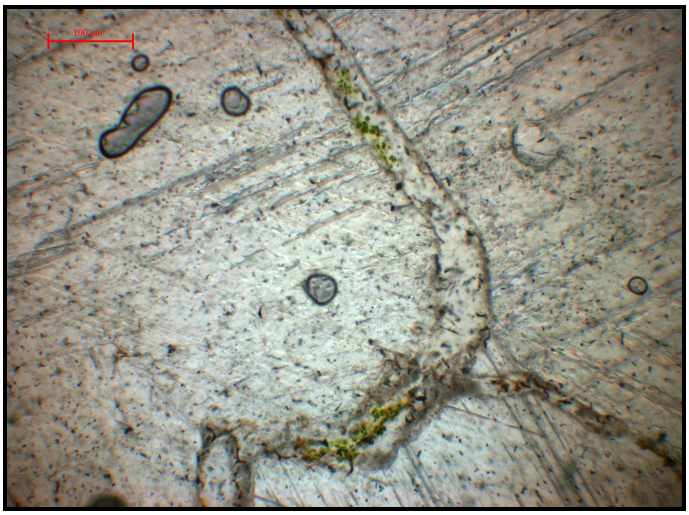
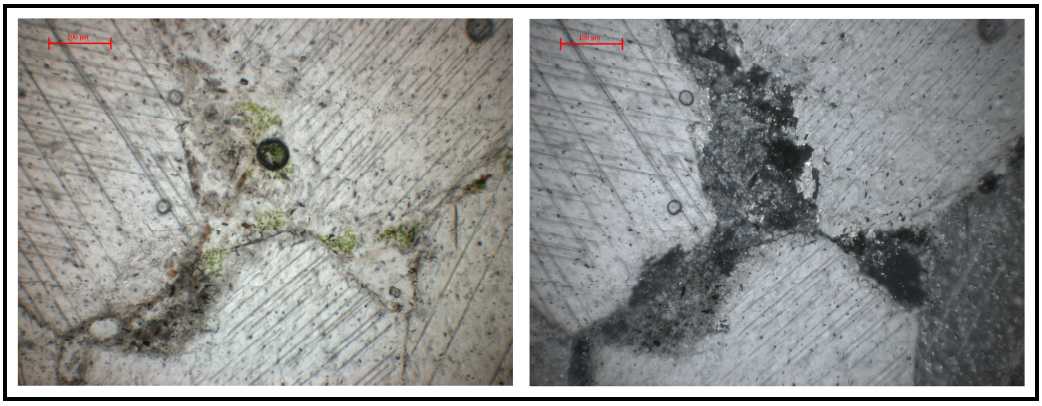


**Figure 3. 18 Thin section view of Pessinous quarry marble (PM3U) showing biological activity in intergranular and intragranular zones of calcite grains, (20X, single nicol).**



single nicol cross nicols

**Figure 3. 19 Thin section view of Pessinous quarry marble (PM3U) showing biological activity in intergranular and intragranular zones of calcite grains, (10X).**

### 3.1.3. SEM Analyses Coupled with EDX

The SEM analyses coupled with EDX were done on limestone and marble samples with biological patina, as well as on limestone and sandstone samples with biological patina that were treated with lyophilized bacteria. Limestone patinas treated with fresh bacteria were also analysed.

The SEM images of limestone cross sections with biological patina showed that there were severely deteriorated zones at the surface. Upper parts of the images showed the biological patina and lower parts showed the deteriorated stone (Fig 3. 20a, 3. 20b). The EDX analysis done at the patina revealed calcium as the main element (Fig. 3. 20c). In the images organic structure of the patina was clearly visible (Fig. 3. 21, 3. 22). The EDX analysis done on several parts of the patina showed that in addition to the main element calcium there were traces of Al and Si elements indicating presence of clay minerals in biological patina (Fig. 3.21b).

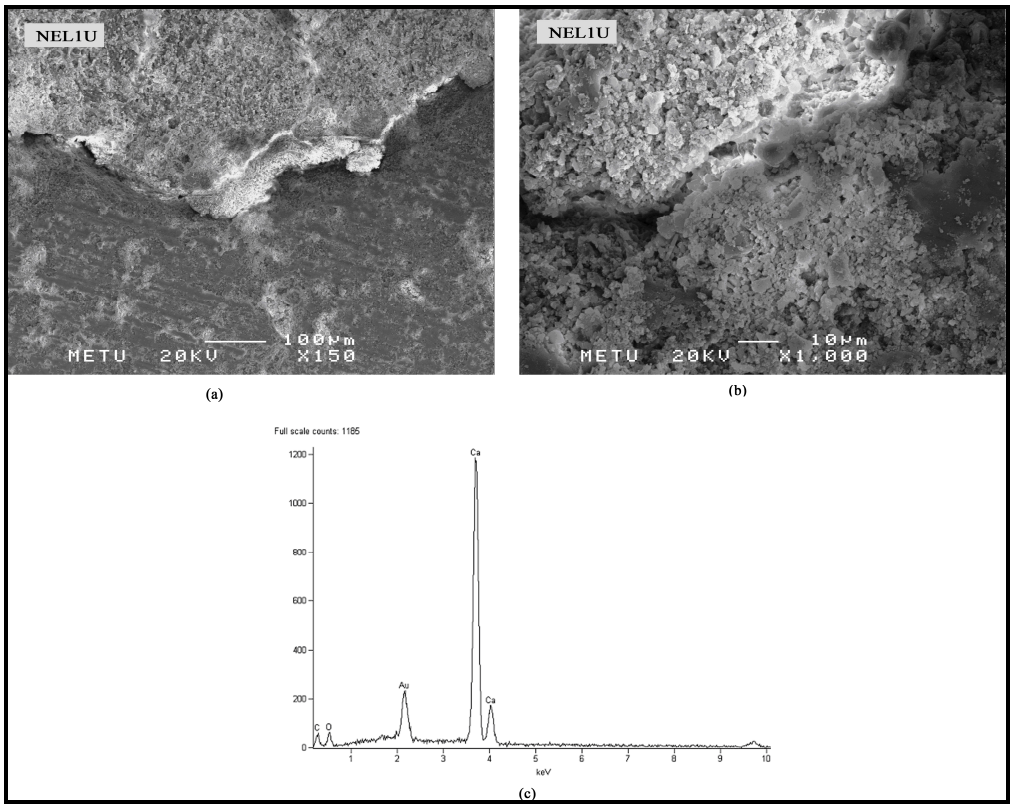



**Figure 3. 20 SEM view (a and b) and EDX analysis (c) of Nemrut limestone with white biological patina (NEL1U).**

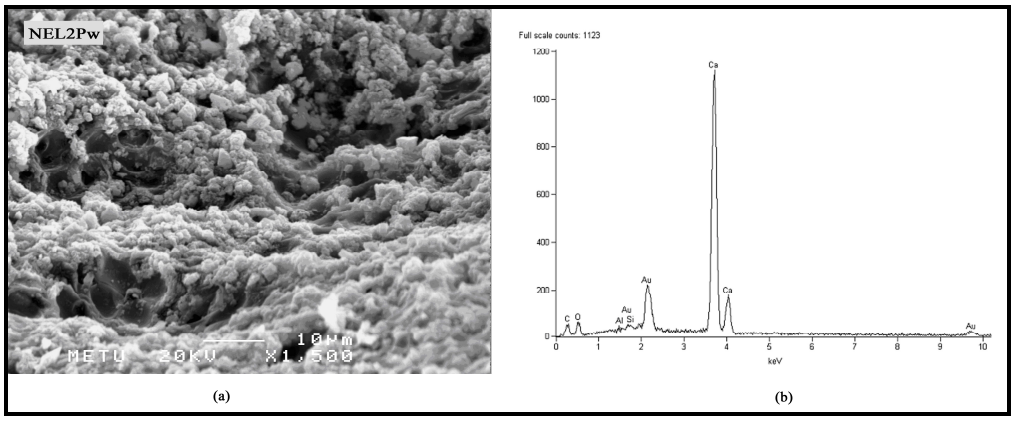



**Figure 3. 21 SEM view (a) and EDX analysis (b) of white patina on Nemrut limestone (NEL2Pw).**

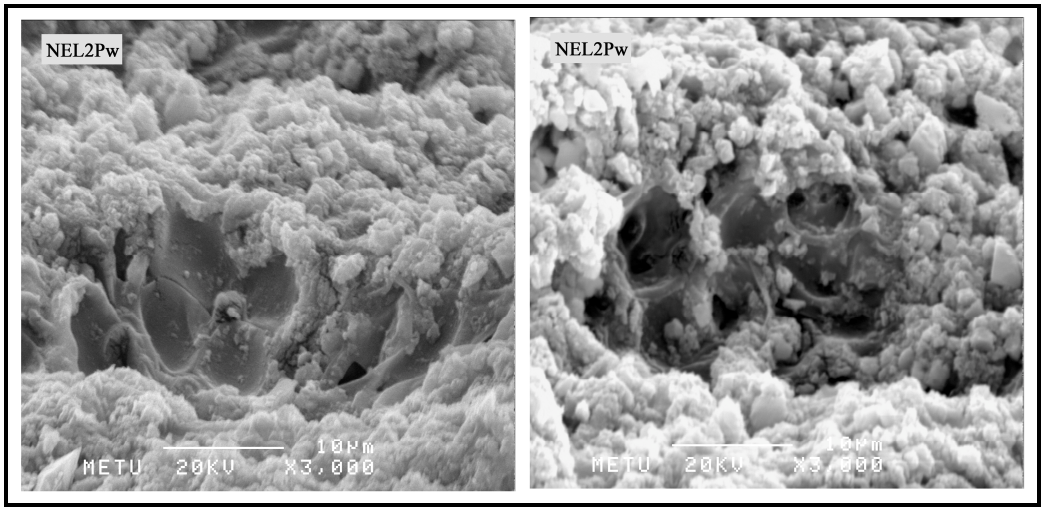



**Figure 3. 22 SEM view of white patina on Nemrut limestone (NEL2Pw) showing organic structures and biominerals.**

Lyophilized bacteria applied samples of Nemrut limestone had originally a biofilm on the surface of about 0.2 mm thickness (Fig. 3. 23a, 3. 23b). The EDX analysis of the biofilm has shown the presence of main element calcium with varying amounts of Si, Mg, Na, Al, K and S (Fig. 3. 23c). These elements may be related with the presence of clay minerals and the metabolic products of organisms. Bacterial bodies with calcite formation were not easy to detect o the sample investigated (Fig. 3. 24).

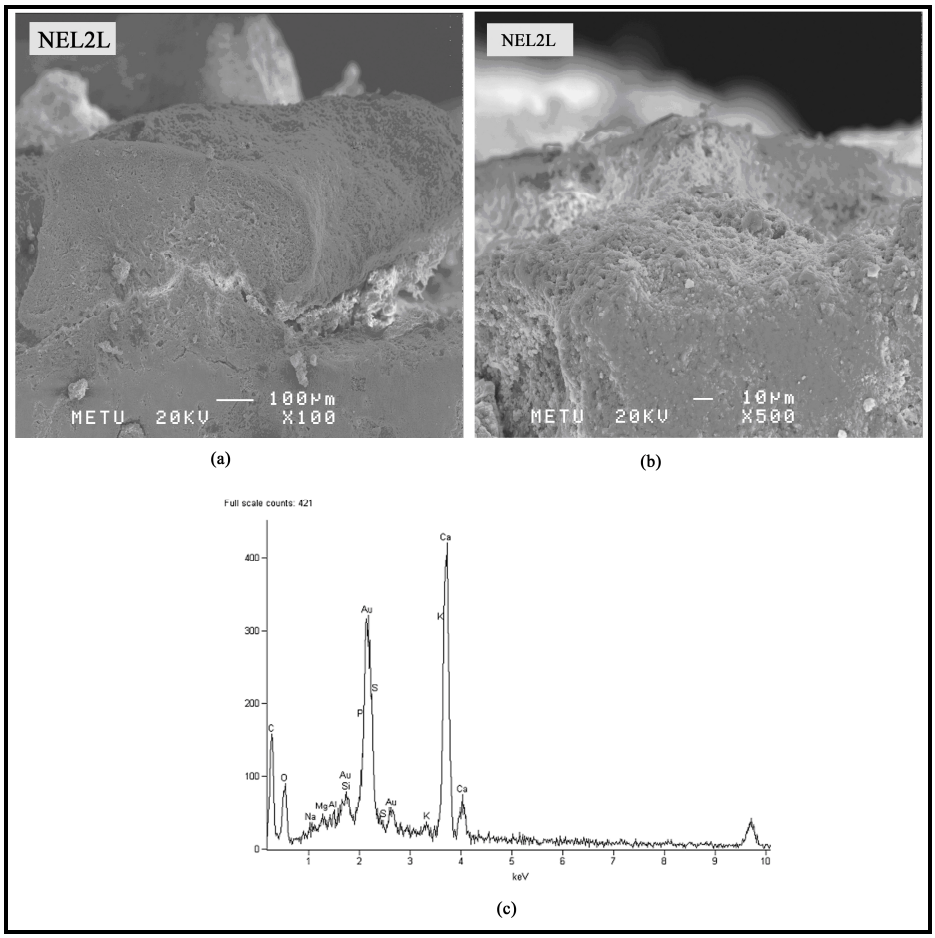



**Figure 3. 23 SEM view (a), (b) and EDX analysis (c) of patina formation after treatment.**

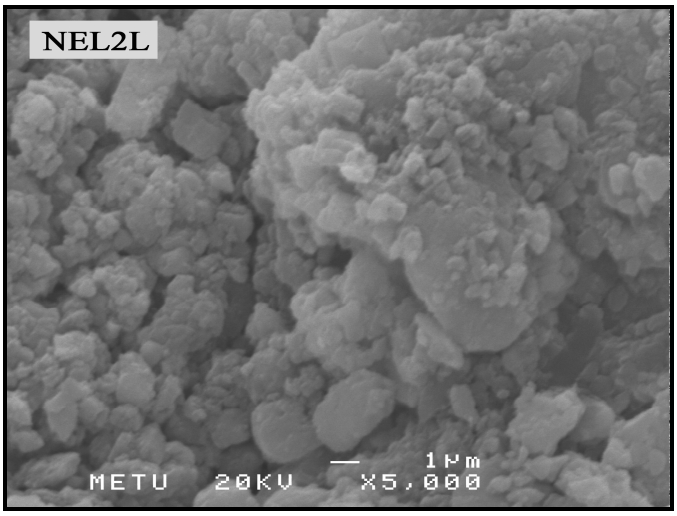



**Figure 3. 24 SEM view of limestone surface treated with lyophilized bacteria showing calcite crytals.**

In fresh bacteria applied samples of Nemrut limestones, the depth of penetration for biological patina was about 0.5 mm before treatment. The stone was severely deteriorated at the surface (Fig. 3. 25a, 3. 25b). The EDX analysis showed that the decay zones consisted of Ca and Si elements (Fig. 3. 25c). In the SEM views of the samples from the surface, corpses of bacteria surrounded with calcite grains resulting from biomineralisation could be seen (Fig. 3. 26, 3. 27). Corpses of *B.cereus* and calcite grains were quite visible (Fig. 3. 26).

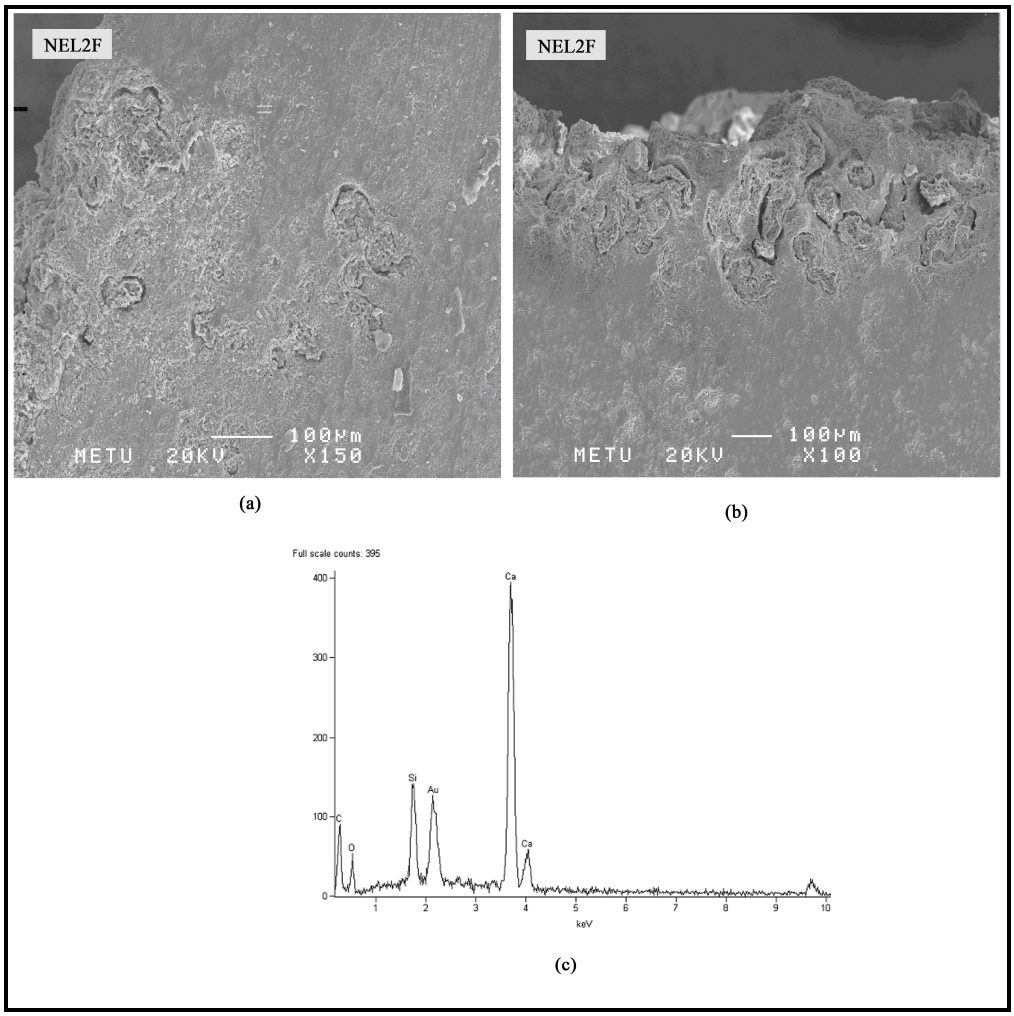



**Figure 3. 25 SEM view (a), (b) and EDX analysis (c) of lyophilized bacteria applied samples of Nemrut limestone with biological patina (NEL2L).**

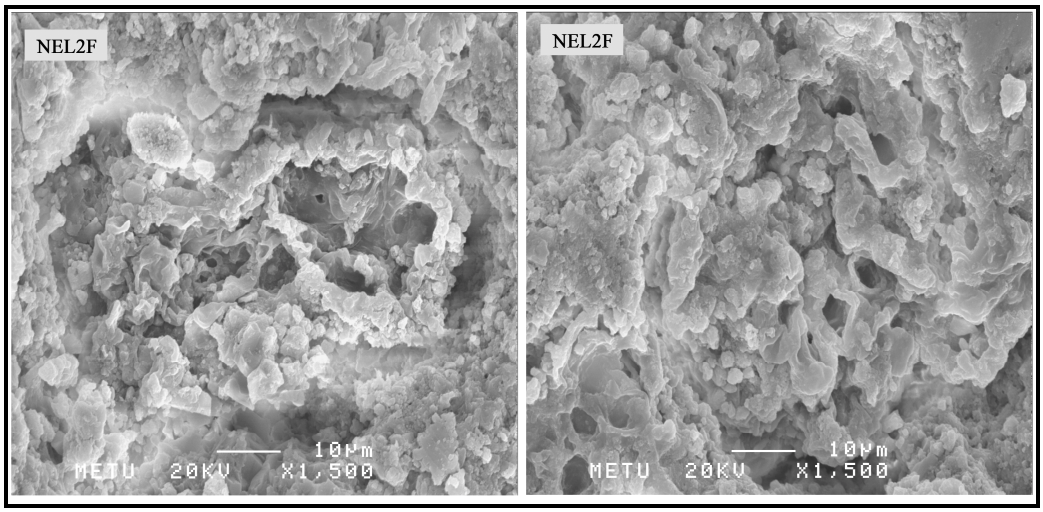



**Figure 3. 26 SEM view of Nemrut limestone treated with fresh bacteria (NEL2F) showing the bacterial bodies surrounded with calcite grains.**

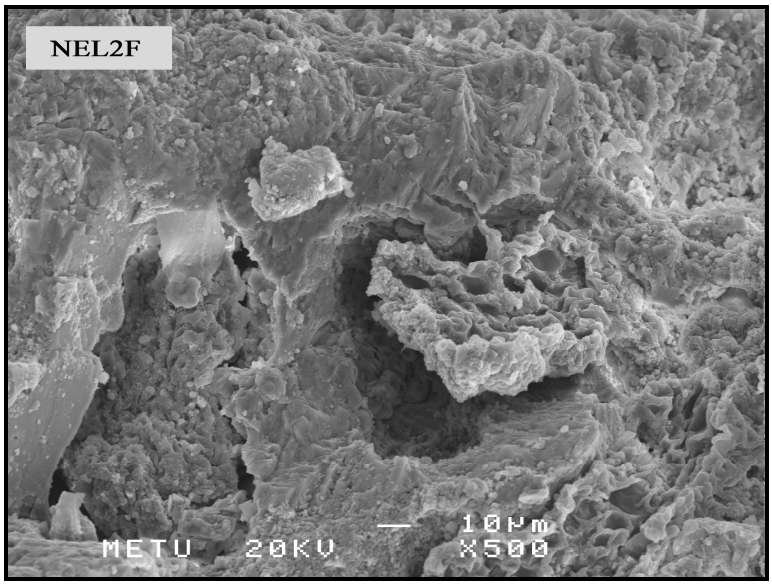



**Figure 3. 27 Another SEM view of biological patina on Nemrut limestone treated with fresh bacteria.**

The SEM views of Pessinous quarry marble showed that it was formed of coarse calcite grains (Fig. 3. 28). The intergranular and intragranular detachments of calcite grains were visible at surface (Fig. 3. 28). Formation of intergranular and intragranular cracks and growth of microorganisms in those decayed zones were visible (Fig. 3. 29).

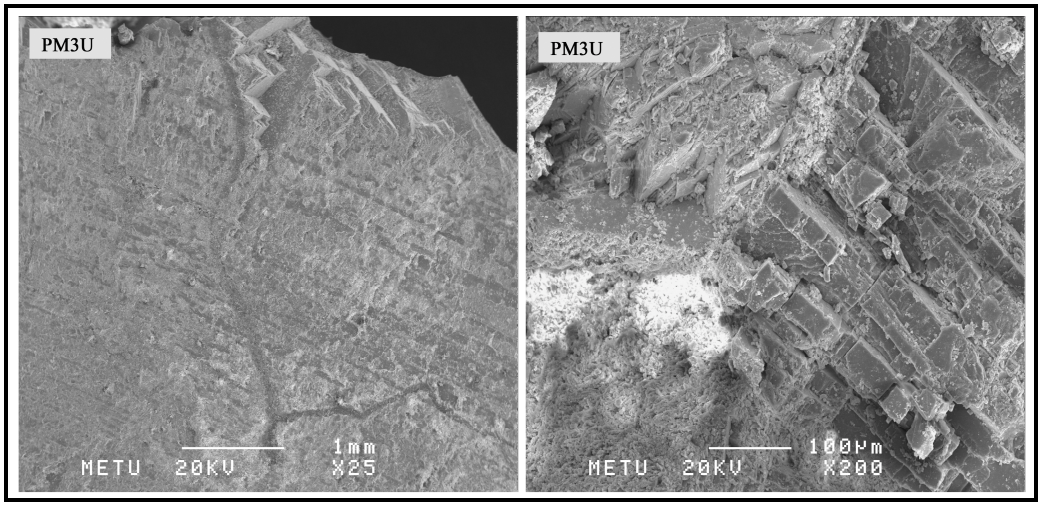



**Figure 3. 28 SEM view of Pessinous quarry marble sample (PM3U) showing intergranular and intragranular cracks**

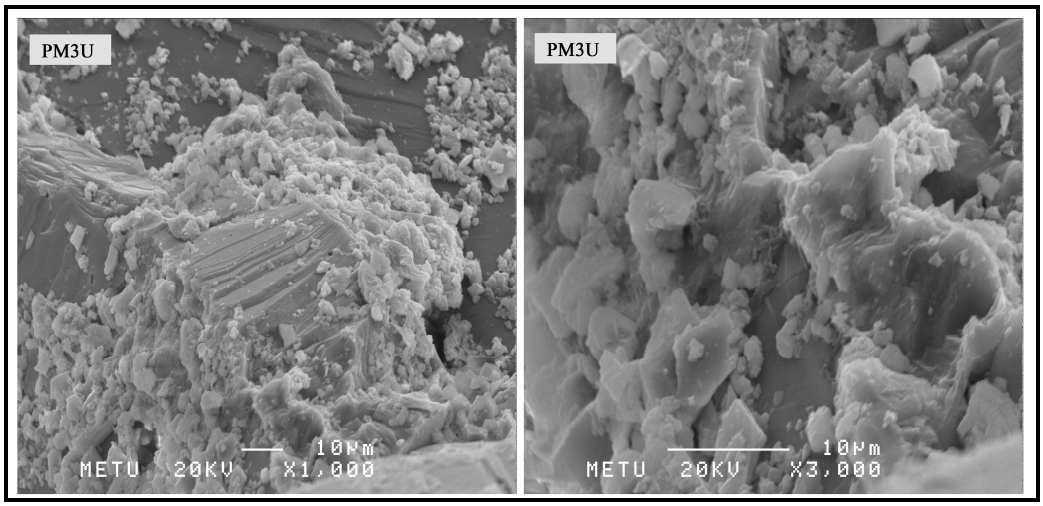



**Figure 3. 29 SEM view of Pessinous quarry marble sample (PM3U) showing biological patina and deteriorated zones at the surface.**

In the SEM view of Nemrut sandstone with no visible biological patina, the treatment with lyophilized bacteria did not reveal any new biological patina formation. However, presence of micritic calcite minerals was observed in SEM view (Fig. 3. 30a). The EDX analysis of the lyophilized bacteria treated surface has shown the presence of Si, Mg, Al, Fe elements together with Ca (Fig. 3. 30b).

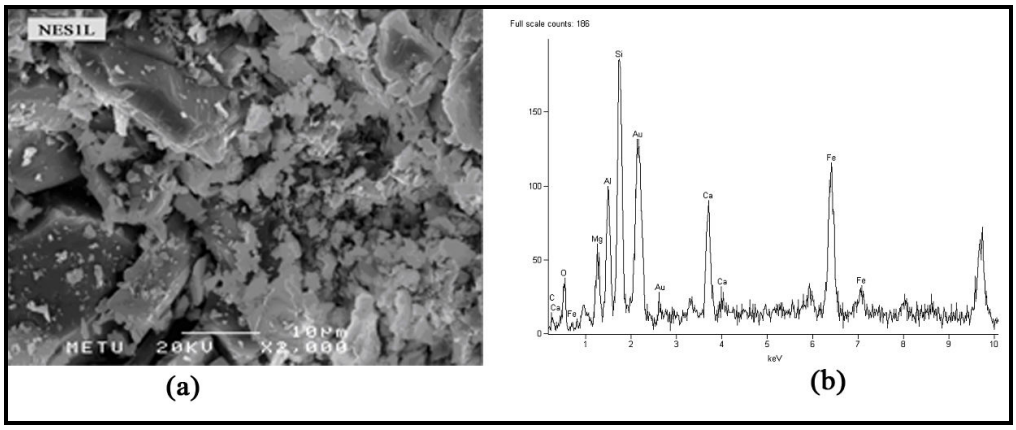



**Figure 3. 30 SEM view (a) and EDX analysis (b) of Nemrut sandstone showing its elemental composition.**

### 3.2. Results of Biological Activity Analyses by FDA

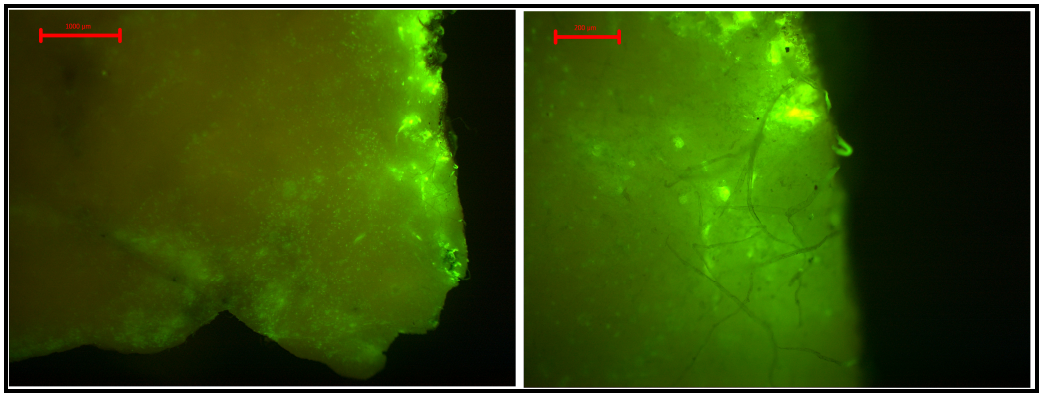
Biological activity was examined by FDA treatment through qualitative and quantitative analyses. The fluorescence developed as a result of biological activity after the FDA treatment of cross sections was observed under light microscope with filter cube I3 (440-490 nm).

Quantitative analyses of biological activity were done by treatment of powdered samples taken from the surface and inner parts of the stone with FDA solution. The fluorescence at 492 nm was quantitatively evaluated. The results of those qualitative and quantitative analyses were summarized in the following sections.

#### 3.2.1. Results of Qualitative FDA Analyses

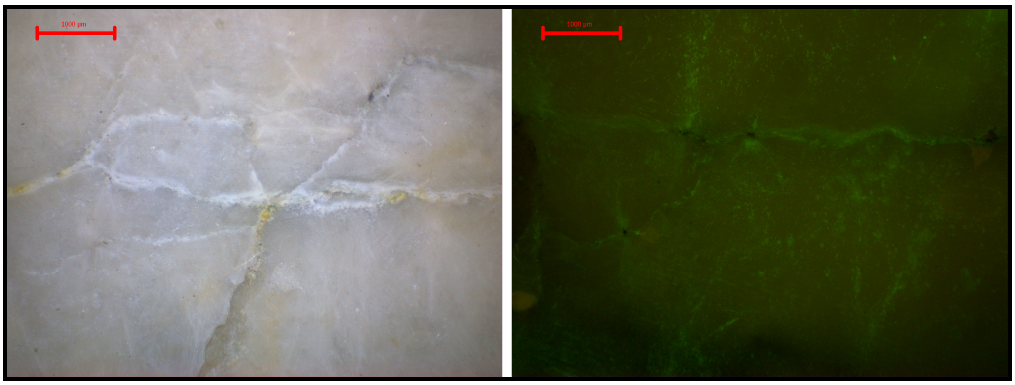
Fluorescence developed by the treatment of Nemrut Mount Monument limestones' cross sections in FDA solution, showed that the depth of penetration of biological activity was up to 5 mm from the surface. Biological activity was

detected around the microcracks towards the interior parts of the stone as well as in the interior cracks (Fig. 3. 32, 3. 33). Root–like structures due to biological activity became visible by fluorescence. The areas of heavy biological activity at the surface were highly fluorescent (Fig. 3. 31Figure 3. 31).



(a) (b)

**Figure 3. 31 Cross section of limestone from Nemrut East Terrace (NEL1U) showing biological activity as fluorescent areas (a: 2.5X, b: 10X).**



Visible light Fluorescent light

**Figure 3. 32 Cross section of limestone from Nemrut East Terrace (NEL1U) showing the interior cracks with biological activity under fluorescent light (2.5X).**

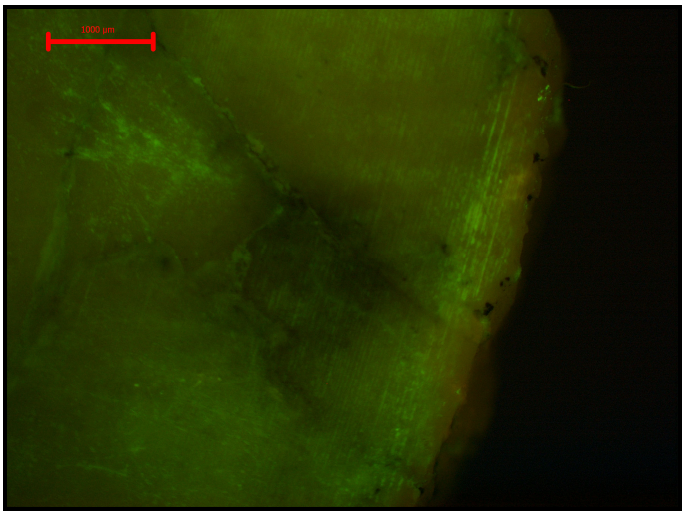
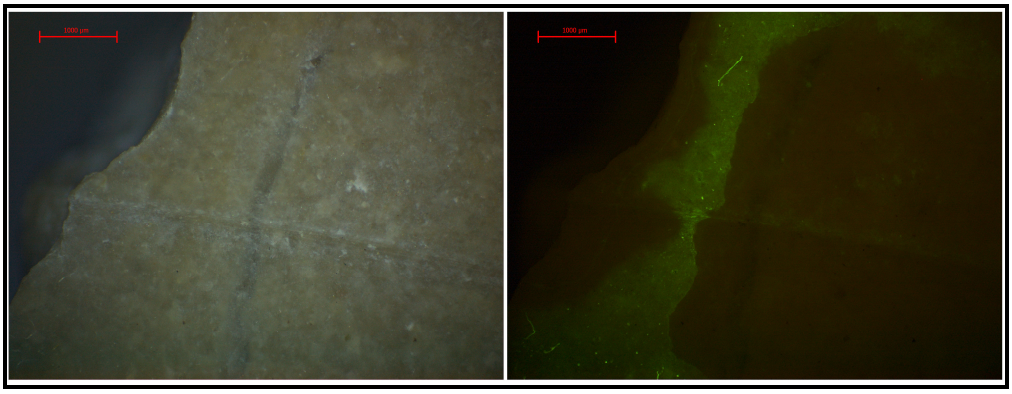


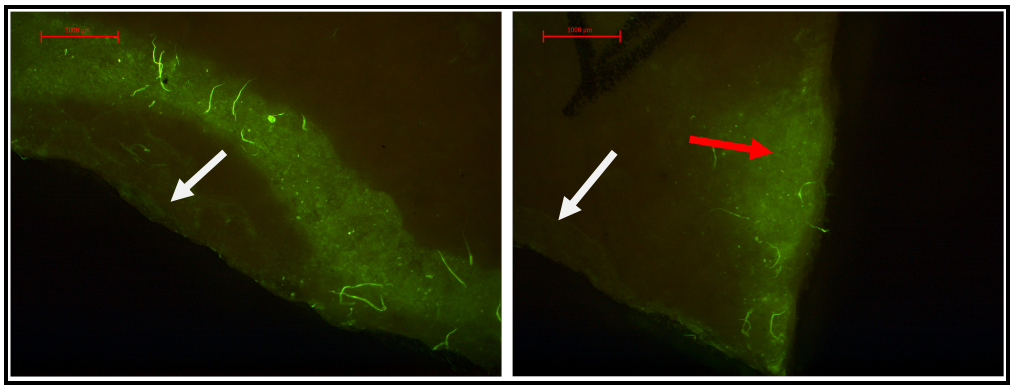
**Figure 3. 33 Cross section of limestone from East Terrace (NEL1U) showing biological activity at the surface as highly fluorescent area (2.5X).**

Lyophilized bacteria were applied to several surfaces of the limestone samples, with no visible biological patina, and the samples were treated with FDA after 6 months. Lyophilized bacteria applied surfaces of those limestone samples, with no visible biological patina, showed low or no biological activity at the surface (Fig. 3. 34 – 3. 36). However, the untreated surface parts of the samples and the interior parts were active zones of biodeterioration (Fig. 3. 34, 3. 35). Depth of biological activity below the lyophilized bacteria applied surfaces was about 2 mm (Fig. 3. 34, 3.35). It showed that the lyophilized bacteria stopped biological activity up to 1 mm depth.



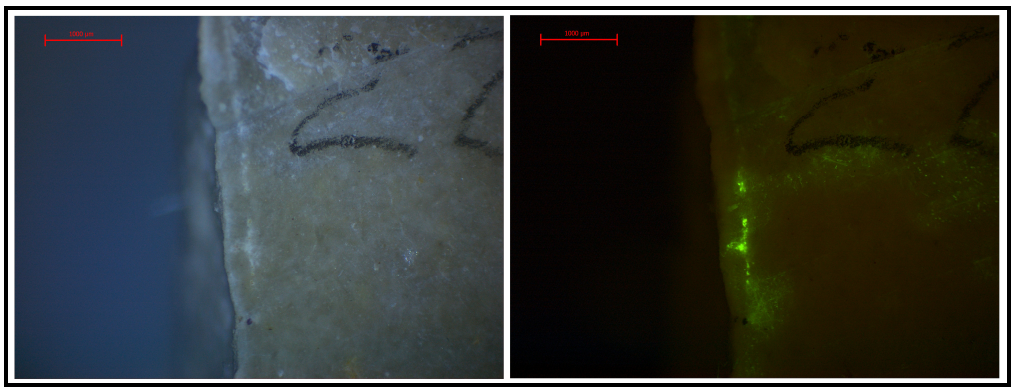
Visible light Fluorescent light

**Figure 3. 34 Cross section of limestone from Nemrut East Terrace (NEL4L) after the treatment with lyophilized bacteria showing no biological activity at the surface but some activity below the surface (2.5X).**



(a) (b)

**Figure 3. 35 Cross section of limestone from Nemrut East Terrace (NEL4L) partly treated with lyophilized bacteria (white arrow: treated area, red arrow: untreated area), treated areas showing no biological activity (2.5X).**



Visible light Fluorescent light

**Figure 3. 36 Cross section of limestone from Nemrut East Terrace (NEL4L) showing no biological activity at the lyophilized bacteria applied surface (2.5X).**

Fresh bacteria applied limestone surfaces revealed identical results to those of lyophilized bacteria applied ones. Fresh bacteria applied surfaces showed low biological activity in comparison to those of untreated ones (Fig. 3. 37, 3. 38). Depth of biological activity on fresh bacteria treated samples were about 1 mm below the surface (Fig. 3. 38). It showed that the fresh bacteria stopped biological activity up to 1 mm depth.

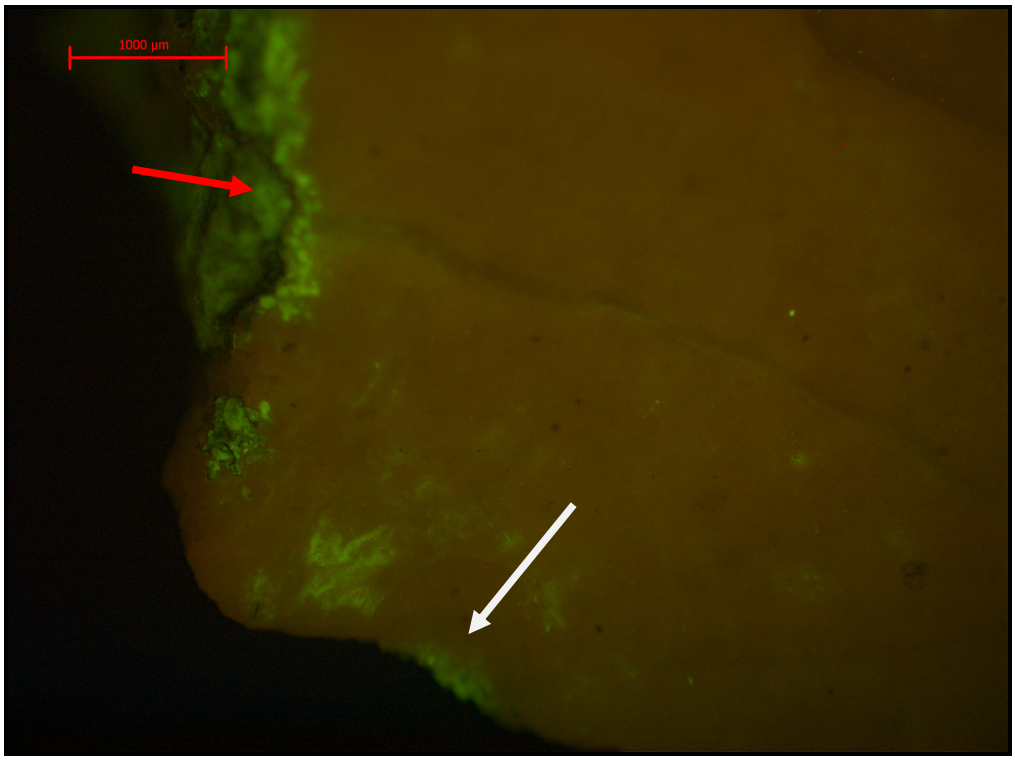



**Figure 3. 37 Cross section of limestone from Nemrut East Terrace (NEL4F) partly treated with fresh bacteria (white arrow: treated part, red arrow: untreated part), treated areas showing less biological activity (2.5X).**

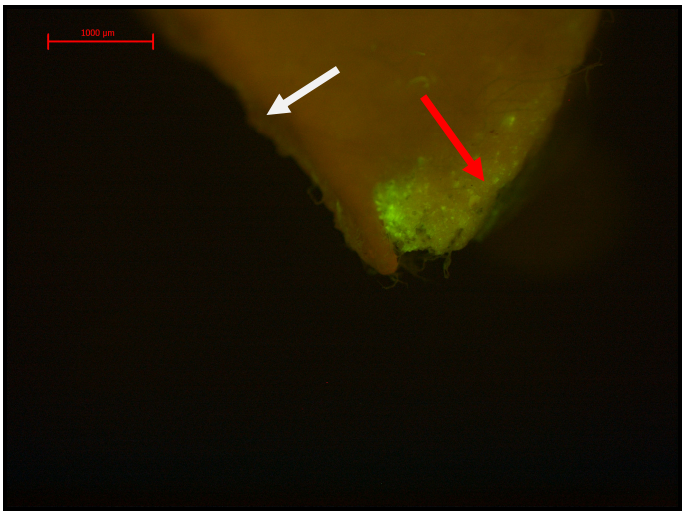



**Figure 3. 38 Cross section of limestone from Nemrut East Terrace (NEL4F) partly treated with fresh bacteria (white arrow: treated part, red arrow: untreated part), treated areas showing no biological activity (2.5X).**

The fluorescence of biological activity was efficiently detected under fluorescent light in marble samples. Examination of the marbles from Pessinous Archaeological Site and Pessinous ancient quarry revealed that the surfaces and intergranular zones around the calcite particles had high biological activity (Fig. 3. 39, 3. 42, 3. 43). The depth of biodeterioration was up to 1 cm in marble samples. The biological activity was heavily formed around intergranular zones and sometimes through the intragranular zones (Fig. 3. 40, 3. 41).

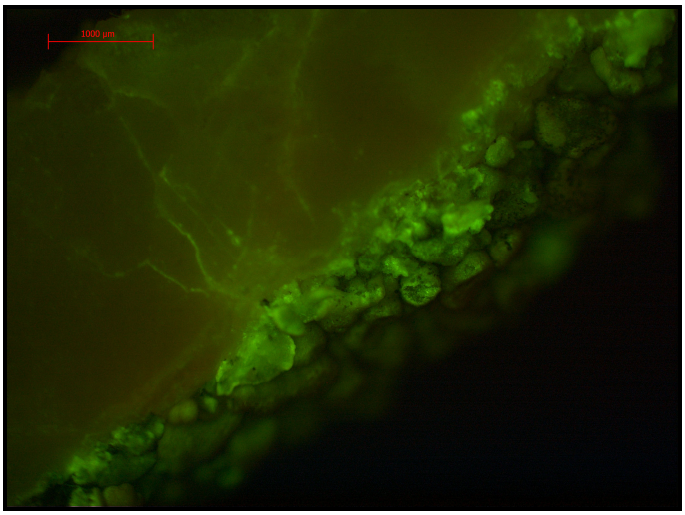
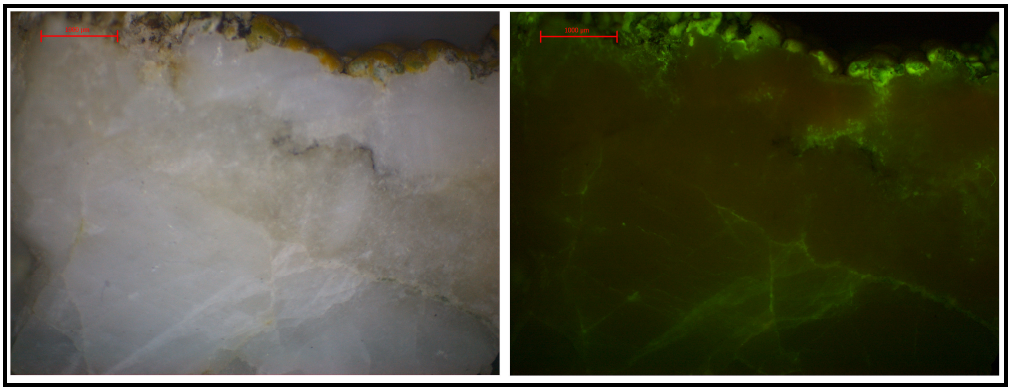
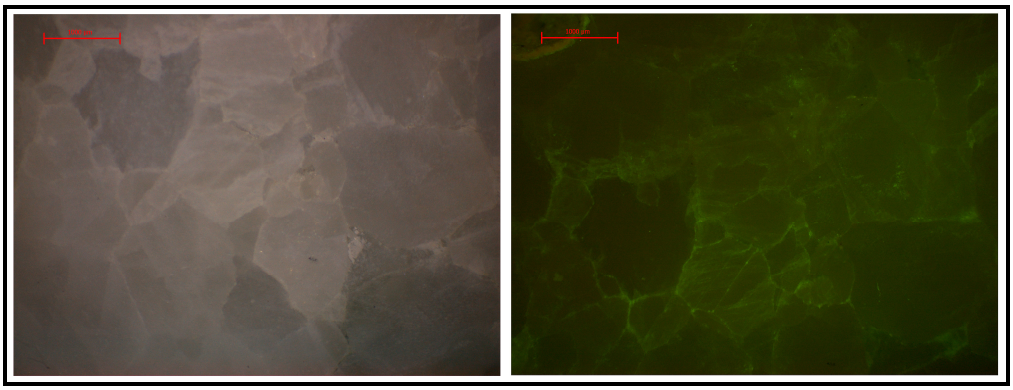



**Figure 3. 39 Cross section of marble from Pessinous ancient quarry (PM1U) showing heavy biological activity at the surface (2.5X).**



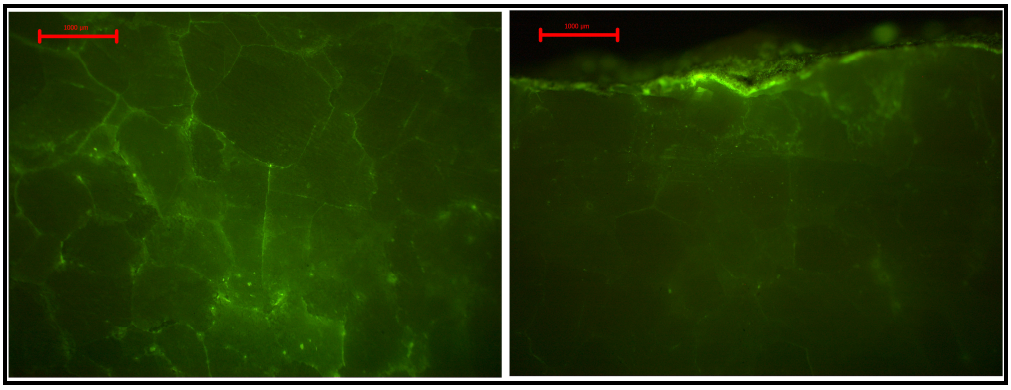
Visible light Fluorescent light

**Figure 3. 40 Cross section of marble from Pessinous ancient quarry (PM1U) showing biological activity at the surface and its penetration to the interior parts (2.5X).**



Visible light Fluorescent light

**Figure 3. 41 Cross section of marble from Pessinous ancient quarry (PM1U) showing biological activity at the interior parts (2.5X).**



(a) (b)

**Figure 3. 42 Cross section of marble from Pessinous Archaeological Site (PM2U) showing biological activity at interior parts (a) and considerable biological activity at the surface (b) (2.5X).**

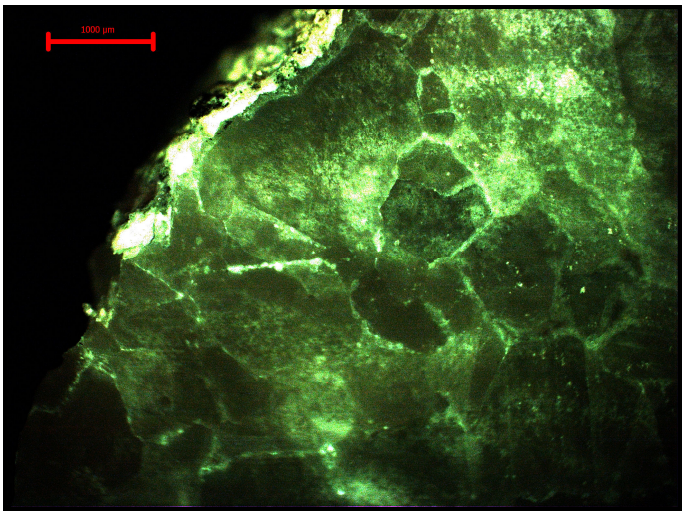
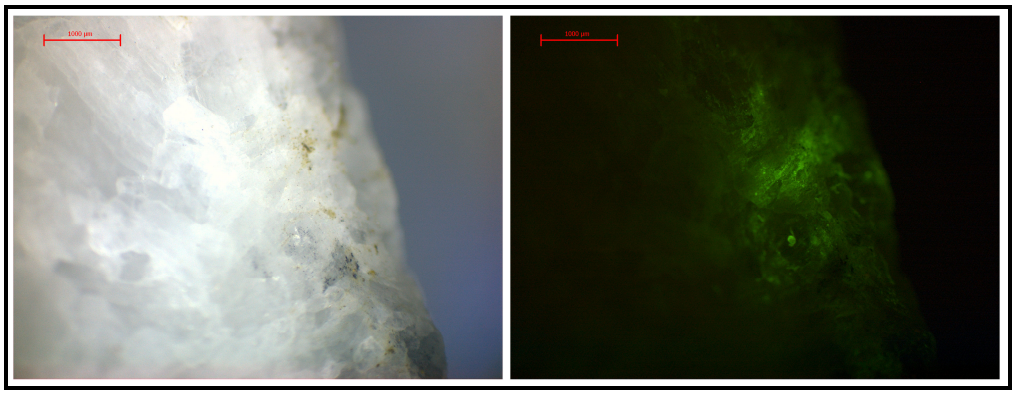


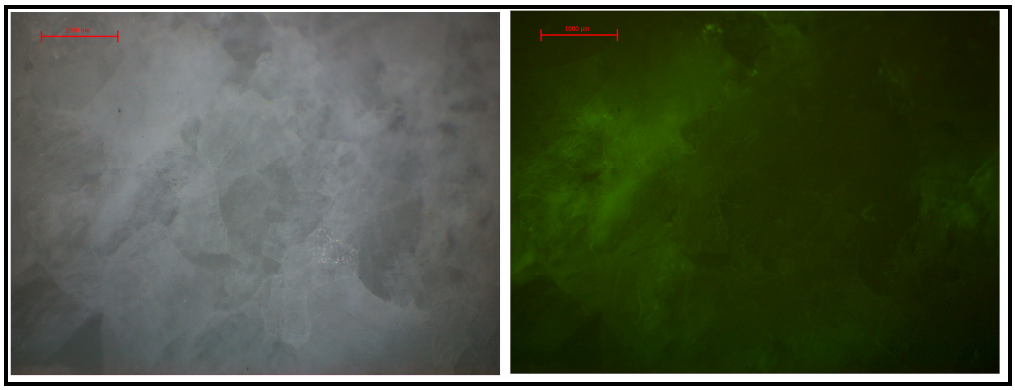

**Figure 3. 43 Cross section of marble from Pessinous ancient quarry (PM3U) showing high biological activity at the surface and at the interior parts (2.5X).**

In marbles, lyophilized and fresh bacteria applied samples showed decreased biological activity at the surface. The depth of biological activity dropped down to 2 mm after the application of fresh and lyophilized bacteria (Fig. 3. 44, 3. 45). Biological activity around the intergranular zones considerably decreased after the treatment with bacteria (Fig. 3. 45).



Visible light Fluorescent light

**Figure 3. 44 Cross section of marble from Pessinous Archaeological Site (PM3L) the surface treated with lyophilized bacteria showing lower biological activity at the surface in comparison to untreated samples (2.5X).**



Visible light Fluorescent light

**Figure 3. 45 Cross section of marble from Pessinous Archaeological Site (PM3F) the surface treated with fresh bacteria showing some biological activity at the interior parts (2.5X).**

The depth of biological activity in sandstones from Nemrut Mount Monument was up to 1 cm from the exterior surface. The activity was high at the surface and decreased through the interior parts (Fig. 3. 46). Sandstones partially treated with lyophilized bacteria showed high biological activity at the untreated areas whereas, biological activity considerably decreased at the treated areas (Fig.3. 47, 3. 48). However, biological activity was still visible below the surface treated with lyophilized bacteria (Fig. 3. 49).

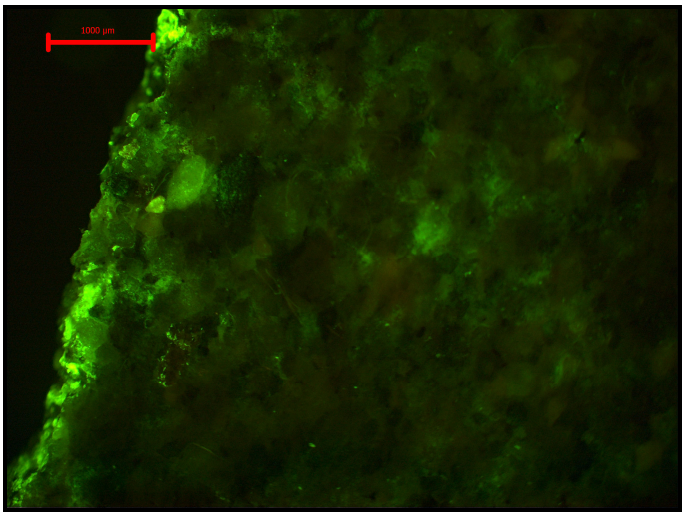
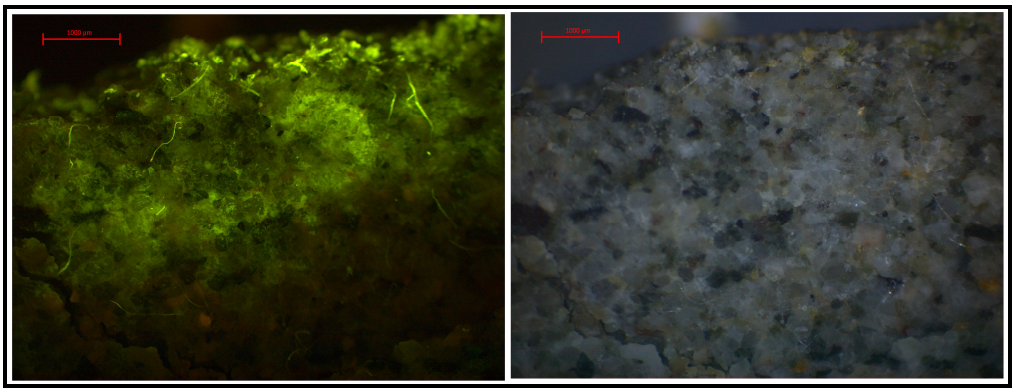
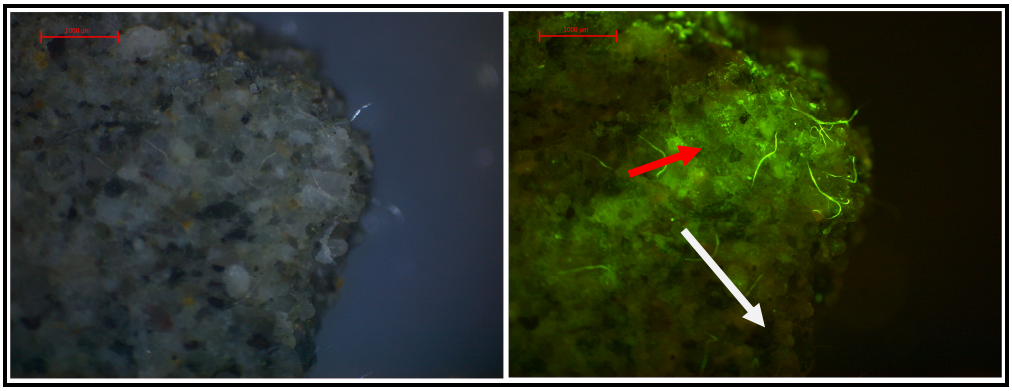



**Figure 3. 46 Cross section of sandstone from Nemrut East Terrace (NES1U) the surface having considerable biological activity (2.5X).**



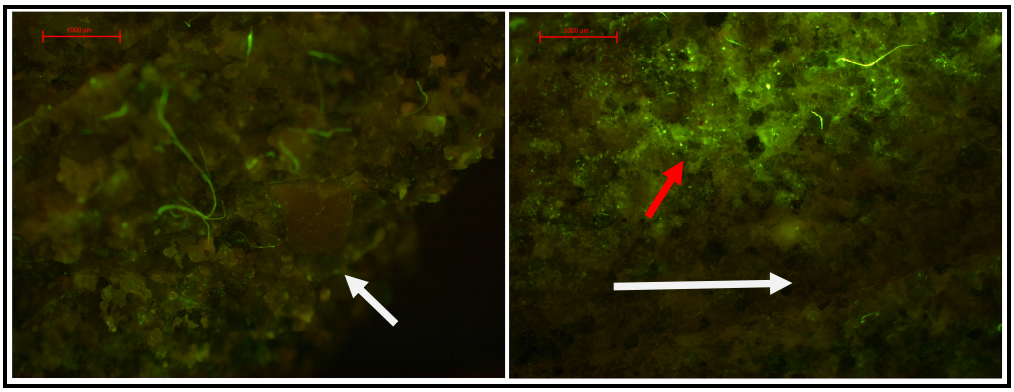
Visible light Fluorescent light

**Figure 3. 47 Cross section of sandstone from Nemrut East Terrace (NES1L) partly treated with lyophilized bacteria the micrographs showing untreated areas with considerable biological activity (2.5X).**



Visible light Fluorescent light

**Figure 3. 48 Cross section of sandstone from Nemrut East Terrace (NES1L) partly treated with lyophilized bacteria (white arrow: treated part, red arrow: untreated part), (2.5X).**



(a) (b)

**Figure 3. 49 Cross section of sandstone from Nemrut East Terrace (NES1L) partly treated with lyophilized bacteria (white arrow: treated part, red arrow: untreated part), treated parts showing no biological activity (2.5X).**

### 3.2.2. FDA Results in Spectrophotometric Measurements

Spectrophotometric measurements were done on limestones and sandstones of Nemrut Mount Monument and marbles of Pessinous Archaeological Site. The samples were prepared by powdering the cross sections cut from the stone surfaces, being slices of 2 mm thickness and 1 cm depth. The fluorescence of those powdered samples and the powdered samples of biological patina scraped from the surface, were measured at 492 nm before and after bacterial treatments. A standard curve was prepared with fluorescein solutions in acetone having a concentration range 1-5 µg/ml fluorescein (Fig. 3.50). Biological activity of the samples was expressed as µg of fluorescein per gram of powdered sample (Table 3.1, 3.2). The fluorescence of the samples was calculated considering their initial weight.

It was seen that untreated limestone samples from Nemrut Mount Monument had about 3.52 µg fluorescein/g. The lyophilized bacteria treated sample had higher biological activity (5.77 µg fluorescein/g) in comparison to fresh bacteria treated

sample (3.65 µg fluorescein/g). The samples of Nemrut limestone with visible biological patina had shown higher biological activity being in the range of 10-15 µg fluorescein/g. Marble samples had much higher biological activity being in the range of 10-22 µg fluorescein/g. Whereas lyophilized and fresh bacteria treated samples showed decreased biological activity being in the range of 1.24-1.46 µg fluorescein/g. Sandstone sample of Nemrut Mount Monument had lower biological activity than limestone (2.86 µg fluorescein/g).

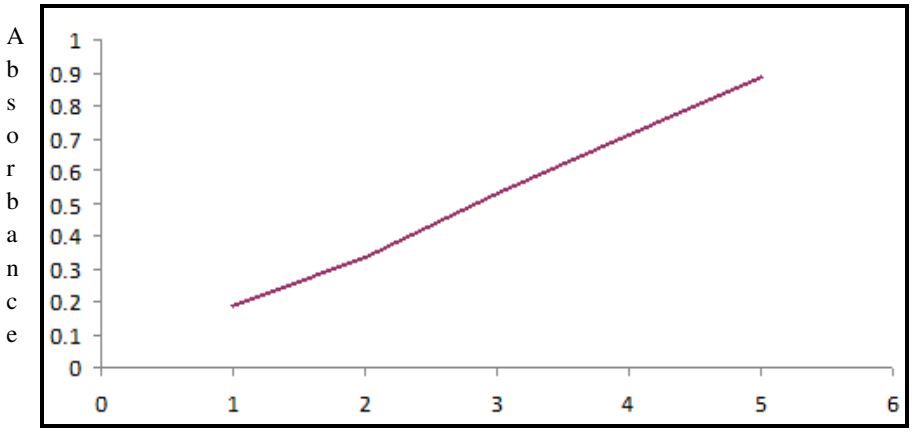



**Figure 3. 50 Standard curve of concentration of fluorescein vs. absorbance.**

**Table 3. 1 Biological activity of patina samples calculated from standard curve and converted to microgram of fluorescein per gram of sample.**

| Sample | Absorbance* | Concentration (ppm) | µg fluorescein/g of Stone |
|---|---|---|---|
| NEL3Pg | 1.379 | 7.82 | 10.25 |
| NEL5Po | 1.747 | 9.91 | 13.37 |
| NEL6Pbk | 1.986 | 11.26 | 15.39 |
| * The data given corresponds to the average of at least two measurements. | | | |

**Table 3. 2 Biological activity of stone samples calculated from standard curve and converted to microgram of fluorescein per gram of sample.**

| Sample | Absorbance* | Concentration (ppm) | µg fluorescein/g of Stone |
|---|---|---|---|
| NEL1U** | 0.083 | 0.47 | 3.52 |
| NEL5L | 0.136 | 0.77 | 5.77 |
| NEL5F | 0.086 | 0.49 | 3.65 |
| PM1U | 0.519 | 2.95 | 22.06 |
| PM2U | 0.245 | 1.39 | 10.24 |
| PM3U | 0.241 | 1.37 | 10.24 |
| PM4L** | 0.035 | 0.19 | 1.46 |
| PM4F** | 0.029 | 0.16 | 1.20 |
| NES1U | 0.068 | 0.39 | 2.89 |

* The data given corresponds to the average of at least two measurements.

U: Untreated sample, L: Lyophilized bacteria treated sample, F: Fresh bacteria treated sample.

**The surfaces of the samples did not have visible biological patina.

### 3.3. Colorimetric Measurements Before and After Treatment with Bacteria

Colorimetric measurements of the samples were done on the samples before and after the application of bacteria solution to the surfaces. The results of measurements were given in Table 3.3. It was seen that the SCI and SCE data expressed as L*a*b* values did not show significant changes in colour due to bacterial treatment. L* values of limestones changed by 3 points (from 57 to 60) before and after surface treatment with bacteria. L* values of Pessinous marbles changed at most by 2 points (from 79 to 81) before and after surface treatment with bacteria.

**Table 3. 3 Colourimetric results of Nemrut limestone and Pessinous marble samples before and after bacteria treatment (fresh and lyophilized).**

| Type of Stone | Sample | SCI Data | | | SCE Data | | |
|---|---|---|---|---|---|---|---|
| | | L*(D65) | a*(D65) | b*(D65) | L*(D65) | a*(D65) | b*(D65) |
| Nemrut Limestone | Untreated | 56.86 | 6.99 | 19.47 | 57.02 | 6.97 | 19.37 |
| | Fresh | 59.46 | 6.85 | 19.07 | 59.50 | 6.85 | 19.08 |
| | Lyophilised | 58.40 | 5.43 | 16.47 | 58.40 | 5.43 | 16.46 |
| Pessinous Marble | Untreated | 80.16 | 0.53 | 5.61 | 80.28 | 0.56 | 5.61 |
| | Fresh | 80.58 | -0.31 | 7.56 | 80.49 | -0.28 | 7.56 |
| | Lyophilised | 78.73 | -0.42 | 8.93 | 78.69 | -0.39 | 8.91 |

# CHAPTER 4

# DISCUSSION OF EXPERIMENTAL RESULTS

In this chapter, the experimental results that were obtained by the examination of representative samples from Nemrut Mount Monument and Pessinous Archaeological Site were discussed in terms of changes in mineralogical and petrographical characteristics of stone by biodeterioration, FDA analyses adapted as a method to study biodeterioration in historic stones, evaluation of biodeterioration determined by FDA treatment and evaluation of biomineralisation treatment with *B.cereus* in lyophilized and fresh forms.

## 4.1. Changes in Mineralogical and Petrographical Characteristics of Stone by Biodeterioration

The petrographic analyses carried out on thin sections, XRD analyses of powdered samples and SEM-EDX analyses revealed that Nemrut limestones were composed of micritic calcite with fossil remains (Fig. 3.1, 3.13, 3.20). Biological patina formation on the surface was discontinuous and its thickness was about 2 mm (Fig. 3.14). Biodeterioration zone detected on limestones had a thickness up to 4 mm starting from the surface spreading to the interior parts (Fig. 3.15, 3.20a, 3.20b). Compact structure of limestone composed of micritic calcite was deteriorated as a result of biological activity at the sites where biological patina was attached with the stone surface (Fig. 3.16, 3.20a, 3.20b). Biomineral formations were seen at the XRD patterns and SEM-EDX analyses of patinas (Fig. 3.2-3.7, 3.21a, 3.22). XRD patterns of patinas showed the peaks of biominerals such as calcium oxalates: wedellite and whewellite, and minor

minerals being quartz, dolomite, clay minerals, and amorphous clay minerals (Fig. 3.2-3.7). The organic structural features of biological patina and biominerals were visible in SEM images (Fig. 3.20, 3.22). The EDX analyses of patina on limestone showed Si and Al elements indicating the presence of clay minerals in the biological patina (Fig. 3.21b). Presence of dolomite in white coloured patina might be resulted from either its in situ recrystallization during biodeterioration or simply a contamination through the deposition of clays from the environment (Fig. 3.2). Clay minerals found in all the biological patinas were thought to be deposited from environment. However, the presence of amorphous silica minerals (Opal A) and amorphous clay minerals in the patina indicate the possibility that they might be the products of biodeterioration (Fig. 3.3, 3.6, 3.7). However, further experimental evidence is needed for its clarification such as the analyses of environmental clay minerals in the nearby soils.

XRD analyses, thin sections examination and SEM-EDX analyses of Pessinous marbles revealed that they were composed of heteroblastic textured calcite grains with twins (Fig. 3.9, 3.17, 3.28). Variable amounts of dolomite existed as minor mineral in their composition (Fig. 3.9). Biological activity was visible especially in intergranular zones and exposed surfaces where wedellite (calcium oxalate mineral) was found as seen in XRD patterns (Fig. 3.9, 3.17-3.19, and 3.28). Intergranular zones around the coarse grains of calcite were exposed to the biodeterioration agents. The penetration of biological activity was visible approximately up to 5 mm from the surface of the marble. Root-like structures of biological growth and colour changes were visible in thin sections (Fig. 3.17, 3.29). In the biological patina of Pessinous marbles the XRD patterns revealed the presence of dolomite, wedellite and quartz along with the main component calcite (Fig. 3.10). The biomineral wedellite was formed by microorganisms acting upon the calcite minerals. Dolomite found at the biodeterioration zone might be either in the composition of the stone or was recrystallized in the biodeterioration zone.

XRD patterns and SEM-EDX analysis revealed that sandstone from Nemrut Mount Monument was composed of quartz, feldspars, calcite and small amount

of clay minerals being mostly kaolinite (Fig. 3.12, 3.30b). Presences of calcite grains along with other mineral phases were visible on SEM images (3.30a). Si, Ca, Fe, Mg, Al, and S were the elements detected on the biodeterioration zone at the surface indicating the main minerals of the stone (Fig. 3.30b). The presence of iron oxides at the surface seemed to be the reason of colour change at the exposed surfaces of sandstones due to the migration of those minerals from interior parts to the surface.

## 4.2. FDA Analyses Adapted as a Method to Study Biodeterioration in Historic Stones

The method used in this study was adapted from literature by following the studies about the development of this method and the research to find the optimum conditions for its application (Adam and Duncan, 2001; Green *et al.*, 2006). The method of FDA hydrolysis was simple, rapid and sensitive for detecting microbial activity in both soil and pure cultures (Adam and Duncan, 2001). In the years 2000 the FDA hydrolysis method used by Shnürer and Rosswall (1980) was optimized with respect to the temperature and time of incubation during hydrolysis with FDA as well as the optimum pH of the buffer solution by Adam and Duncan (2001) and Green *et al.* (2006).

In this study FDA method was adapted as a qualitative method for detection of biological activity through the structure of stone by staining the cross sections of the samples. The solution prepared for the quantitative measurements of fluorescence by spectrophotometry was used as a staining solution to be applied to cross sections of the historic stone samples. Fluorescence developed on the cross section surfaces was examined under light microscope with external light source for fluorescence excitation under blue filter (450-490 nm).

In this study, the method was also applied to powdered stone samples having biological patina for quantification of the microbial biomass through

spectrophotometric measurements of fluorescence as described in Chapter 2, Section 2.4.

Both qualitative and quantitative application of FDA to historic stone samples proved to be useful methods for the examination of biological activity in historic stones as discussed in more detail in the following section.

### 4.3 Evaluation of Biodeterioration Determined by FDA Hydrolysis

FDA hydrolysis was adapted as a qualitative and quantitative method for the analyses of biological activity on historic stone samples.

Evaluation of the qualitative analysis results done on cross sections of stone samples had shown the thickness and the depth of biological activity. The depth of biological activity was up to 5 mm from the surface of Nemrut Mount limestone samples (Fig. 3.32). Biological activity that could not be detected under visible light was efficiently detected by this method. Biological activity was quiet dense around the microcracks and at the interior parts of the stone (Fig. 3.33). Root-like structures of biological deterioration agents were visible with FDA treatment. Those structures penetrated to about 0.2 mm depth into the stone (Fig. 3.31). It was seen that there were heavily populated areas of biological activity on Nemrut limestones (Fig. 3.31-3.33).

FDA staining on the cross sections of Pessinous marble samples supported the thin section images showing the biological activity around intergranular and intragranular zones of calcite grains (Fig. 3.17-3.19, 3.39, 3.42, 3.43). Biological activity reached up to 1 cm thickness from surface spreading through the interior parts. Heavy biological activity was detected in marble samples especially around intragranular zones in areas close to the surface (Fig. 3.40, 3.41).

The results of sandstones stained with FDA revealed that the depth of biological activity in sandstones from Nemrut Mount Monument was up to 1 cm from the exterior surface. The activity was high at the surface and decreased through the interior parts (Fig. 3.46).

The results obtained from the FDA staining of cross sections revealed that in Pessinous marbles the biological activity had spread more and penetrated to deeper parts of the stone up to 1 cm depth in comparison to Nemrut limestones where the penetration of biological activity was restricted to the microcracks and the average depth of biological activity was about 5 mm (Fig. 3.32, 3.40). Although few samples of sandstones from Nemrut Mount were examined, the results have shown that biological activity penetrated to deeper parts of the sandstones in comparison to Nemrut Mount limestones (Fig. 3.32, 3.33, 3.46).

Quantification of biological activity in other words the microbial biomass by using FDA hydrolysis was done on Nemrut limestones, Pessinous marbles and Nemrut sandstones. Evaluation of the results of spectrophotometric measurements was compared with the results based on soil microbial activity. According to Adam and Duncan (2001), 0.7 μg fluorescein/g of soil represented low microbial activity, 1.1 μg fluorescein/g of soil represented intermediate microbial activity and above 2 μg fluorescein/g of soil represented the high microbial activity (Adam and Duncan, 2001). According to Diack and Stott (2001) the microbial activity was in the range of 83-96 μg/g for good agricultural soil where microbial biomass C was in the range of 614-800 mg/kg of soil. In studies of Green *et al.* (2006) the mean value of μg fluorescein/g of soil was in range of 66-226 the values being higher for soils with higher clay content.

The results of biological activity expressed as μg fluorescein/g of sample were given in Table 3.1. Samples represented the biologically active zones of Nemrut limestones, Pessinous marbles and Nemrut sandstones. Most samples were prepared by powdering the cross sections cut from the stone surfaces being slices of 2 mm thickness and 1 cm depth if not indicated otherwise.

In Nemrut limestones, biological activity was 3.52 μg fluorescein/g of stone. Measurements of biological activity in the powder scraped from the visible biological patinas alone were in the range of 10-16 μg fluorescein/g of patina in Nemrut limestones (Table 3.2).

The biological activity measured at Pessinous marbles was quite high being in the range of 10-22 μg fluorescein/g of stone. The Pessinous quarry marble slice

(PM1U) with the value of 22.06 μg fluorescein/g of stone had a thick biological patina formation on the surface that may have contributed to higher value of biological activity.

Nemrut sandstone had a biological activity value of 2.89 μg fluorescein/g of stone on its surface.

Pessinous marbles had the highest biological activity in comparison to Nemrut limestones and sandstones. The higher biodeterioration of the Pessinous marbles was also detected in thin sections and FDA analysis of cross sections (Fig. 3.15, 3.17). Quantitative analysis with FDA had shown that Nemrut limestone samples had higher values of biological activity in comparison to Nemrut sandstones. On the other hand the FDA staining of the samples showed deeper penetration of biological activity in sandstones and fluorescence developed in the cross sections of those samples were more intense than Nemrut limestones. Those results might be due to the following reasons: it was not possible to do the qualitative and quantitative analyses in the same sample and biodeterioration was not homogeneously distributed in the stones. Therefore, more precise quantification of biological activity is needed to be done on quite a number of samples.

When the FDA hydrolysis results of this study were compared with the values of soil microbial activity found in literature, variable comments could be made. If compared with the results of Adam and Duncan (2001) all the stone samples and biological patinas showed high microbial activity. However, the evaluation of the results with respect to the studies of Diack and Stott (2001) and Green *et al.* (2006) showed that the microbial activity on samples were quite low, the maximum amount found on historic stones showing about 1/3 of microbial activity found in high quality agricultural soil. In any case it was seen that surface deterioration of historic stones included high biological activity.

## 4.4. Evaluation of Biomineralisation Treatment with *B.cereus* in Lyophilized and Fresh Forms

The evaluation of the biomineralisation treatment was based on the results derived from XRD, SEM-EDX, FDA and colourimetric analyses.

XRD patterns of Nemrut limestone samples that were treated with lyophilized or fresh bacteria showed only calcite peaks (Fig. 3.8). There were not any biomineral formations after bacterial treatment of Nemrut limestones. SEM images of the lyophilized and fresh bacteria treated samples of Nemrut limestones revealed the bacterial corpses surrounded by calcite particles (Fig. 3.24, 3.26). Qualitative evaluation of FDA hydrolysis of treated limestones from Nemrut Mount showed that the biological activity at the surface was decreased in parts treated with bacteria (Fig. 3.35-3.38). However, the results from quantitative FDA analysis showed that the biological activity increased after biomineralisation treatment (Table 3.1). The values of biological activity went up to 5.77 μg fluorescein/g of stone for lyophilized bacteria and to 3.65 μg fluorescein/g of stone for fresh bacteria. The increased biological activity found in the samples might have resulted from heterogeneous biological patina on the samples.

Regarding the colour change in Nemrut limestones by bacteria treatment, L* values have differed from 57 to 59 in untreated and fresh bacteria applied samples, while the difference was from 57 to 58 in untreated and lyophilized bacteria applied samples (Table 3.3). Those changes in colour values were insignificant.

Pessinous marbles treated with lyophilized and fresh bacteria showed only calcite minerals in XRD patterns showing no biominerals (Fig. 3.11). FDA staining applied on cross sections of Pessinous marbles revealed that the surface treatment decreased the biological activity both at the surface and at the interior parts of the marbles (Fig. 3.44, 3.45). Quantitative FDA hydrolysis of Pessinous marbles supported that the microbial activity of the stone was decreased after biomineralisation application (Table 3.1). The microbial activity values decreased

from 10-22 μg fluorescein/g of stone to 1.46 μg fluorescein/g of stone with lyophilized bacteria, and to 1.20 μg fluorescein/g of stone with fresh bacteria application (Table 3.1).

Regarding the colour changes of Pessinous marbles L* values were almost same (being about 80) with fresh bacteria application, while L* became 79 with lyophilized bacteria application. Colour measurements had shown that no significant change occurred with bacterial treatment of marble samples.

The qualitative results of FDA staining on Nemrut sandstones had shown that after lyophilized bacteria application the microbial activity considerably decreased (Fig.3.46-3.48).

It was concluded that bacteria treatment could control biological activity on the surface of historic stones where biodeterioration was in considerable amounts.

# CHAPTER 5

# CONCLUSION

In this study, historic limestones, marbles and sandstones being in atmospheric conditions since about 2000 years had been investigated for their biological deterioration. There were visually detectable biodeterioration on monumental stones in the forms of colour change, patina formation, detachments, fissures and cracks. Biofilms (patina) formation seemed to be discontinuous on stone surfaces; in fact on some stone surfaces no biofilm was visually detected. The biodeterioration was examined using different analytical techniques such as: XRD, light microscopy, SEM-EDX analyses, FDA hydrolysis and colorimetric measurements. The conclusions obtained from the results are given below.

Nemrut limestones were composed of micritic calcite with fossil remains. Biological patina formation on the surface was discontinuous and its thickness was about 2 mm. On the deteriorated surfaces, biominerals, wedellite, whewellite, and minor minerals being quartz, dolomite, clay minerals, and amorphous clay minerals were found. Biological patina that was visually detected on some samples signified deeper biological deterioration below it. FDA staining had clearly shown that the biological activity has penetrated through the microcracks to the interior parts of the stone. Biological activity resulted in deterioration of stone by physical and chemical mechanisms. Quantitative results by FDA hydrolysis indicated that the level of biological activity in Nemrut limestones were comparable with the soils having intermediate or low microbial activity.

Pessinous marbles were composed of heteroblastic textured calcite grains with twins. Variable amounts of dolomite existed as minor mineral in their

composition. Small amounts of dolomite were found at the deterioration zones together with wedellite and quartz minerals. Biological patina formation on the surface was discontinuous. The thickness of the heavily biodeteriorated zone could be up to 1 cm below the surface. FDA staining had clearly shown that the biological activity was high at intergranular and intragranular zones around calcite minerals. Quantitative results of FDA hydrolysis had showed that the level of biological activity in Pessinous marbles were very high comparable with soils having high microbial activity.

Nemrut sandstones were mainly composed of quartz, feldspars and with small amounts of calcite, and clay mineral kaolinite. Depth of biological activity in sandstones having black biological patina was up to 1 cm from the surface. Quantitative FDA analysis had shown that biodeteriorated layers of sandstone had low or intermediate biological activity comparable with soils of low microbial activity. No biominerals were detected on sandstones.

This study has proved that qualitative and quantitative use of FDA hydrolysis method was an efficient method for the evaluation of the biodeterioration in historic stones.

Finally, it can be concluded that biodeterioration of those historic stones can be taken under control with the help of biomineralisation with *B.cereus*. It caused no colour changes in historic stones. However, its side effects have to be examined. The soluble salts in the nutritive solution need to be cleaned. The efficiency of cleaning has to be verified.

Further studies are needed to verify the quantitative efficiency of FDA hydrolysis method as a tool to detect total microbial activity. Other quantitative methods such as the evaluation of biodeterioration with the determination of chlorophylls, and its comparison with FDA hydrolysis need to be done. It seems that further studies such as the determination of the rate of biological decay on stone monuments with respect to time, space and environmental conditions can be carried out by FDA analyses.

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# APPENDIX A

## NEMRUT MOUNT MONUMENT

**Figure A1. 1 Topographic view of Mount Nemrut**

**Figure A1. 2 Statues of Mount Nemrut**

# APPENDIX B

## PESSINOUS ARCHAEOLOGICAL SITE

**Figure A2. 1 View of Pessinous Archaelogical site**

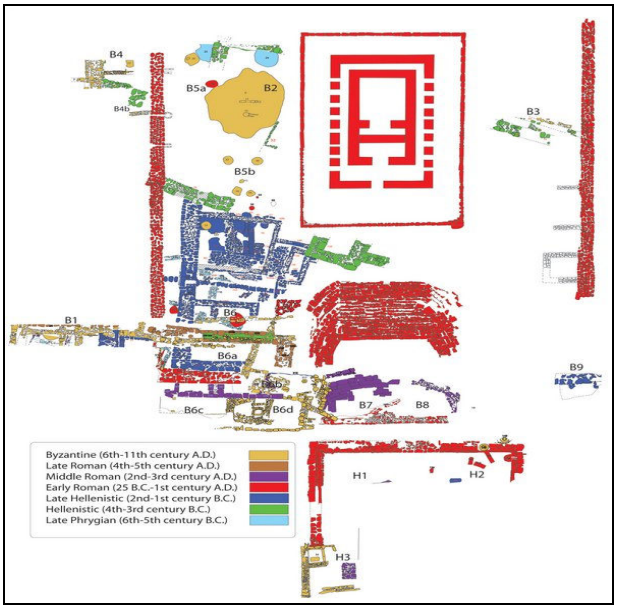


**Figure A2. 2 Scheme of Pessinous Archaelogical site**