EVALUATION OF BIODETERIORATION
IN NEMRUT MOUNT MONUMENT AND TEMPLE OF AUGUSTUS
BY USING VARIOUS TECHNIQUES

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**EVALUATION OF BIODETERIORATION
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ABSTRACT

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Different techniques were studied to evaluate the presence of different microorganisms that played important roles in decay processes of historic stones. In that scope, limestones and sandstones from Nemrut Mount Monument, and marbles and andesites from Temple of Augustus were studied. For measurement of enzymatic activity, fluorescein diacetate (FDA) hydrolysis method previously applied to assess soil microbial activity was carried out. Total microflora method based on countings of colony number was conducted for determination of the level of bacterial and fungal activity of stones. ATP bioluminescence method, developed for the field of hygiene monitoring, was

carried out in order to detect global metabolic activity degree in historic stones. Most probable number (MPN) method was carried out to detect the number of microbial cells, namely nitrifying and sulphur oxidising bacteria which could take part in the decay processes. Moreover, fungi identification was done for determining occurance of detrimental species.

Presence of lichenic and algal zones existed on stones of Nemrut Mount Monument and the presence of black discolorations on stones of Temple of Augustus was common. Results have shown that the bacterial and fungal activity was low, however considerable quantity of FDA hydrolyses has shown the importance of algal population in the stones of two studied historical sites. This study has proved that FDA hydrolyses, total microflora and MPN method were efficient for the evaluation of biodeterioration in historic stones.

Keywords: Total microflora, FDA hydrolyses, most probable number, biodeterioration, historic stones.

ÖZ

NEMRUT DAĞI ANITI'NDAKİ ve AUGUSTUS TAPINAĞI'NDAKİ BİYOLOJİK BOZULMALARIN FARKLI TEKNİKLERLE DEĞERLENDİRİLMESİ

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Bu çalışmada, tarihi taşların bozulma sürecine mikroorganizmaların katkısı farklı teknikler uygulanarak değerlendirilmiştir. Bu kapsamda Nemrut Dağı Anıtı'ndaki kireçtaşları ve kumtaşlarıyla, Augustus Tapınağı'ndaki mermer ve andezitler incelenmiştir. Daha önceleri topraktaki mikrobiyal aktiviteyi ölçmek için kullanılan FDA hidroliz metodu, bu çalışmada enzimatik aktivitenin belirlenebilmesi amacıyla tarihi taşlara uygulanmıştır. Koloni sayım esasına dayanan toplam mikroflora metodu ile taşlardaki bakteri ve fungus aktiviteleri belirlenmiştir. Esas olarak hijyen denetimlerinde kullanılan ATP metodu ile de tarihi taşlardaki toplam metabolik aktivite ölçülmüştür. En muhtemel sayı yöntemi ile taşların bozulma süreçlerinde rol alan, nitrifikasyon ve sülfür

oksidasyonu yapan bakterilerin sayısı belirlenmiştir. Ayrıca, taş yüzeyinde bulunabilecek zararlı fungus türlerinin tanımlanması yapılmıştır.

Nemrut Dağı Anıtı'ndaki taşlarda liken ve alglerin varlığı, Augustus Tapınağı'ndaki mermerlerde ise gri siyah kabuk oluşumunun yaygın olduğu görülmüştür. belirlenmiştir. Sonuçlar, çalışılan tarihi anıtların ikisinde de taşlarda bakteri ve fungus aktivitesinin çok düşük olduğunu göstermiştir. Ancak FDA hidroliz değerlerinin yüksek olması, taşlarda alg aktivitesinin önemli olduğunu göstermiştir. Bu çalışmada kullanılan FDA hidroliz metodu, toplam mikroflora metodu ve en muhtemel sayı metodu, tarihi taşlardaki biyolojik bozulmanın değerlendirilmesi açısından başarılı bulunmuştur.

Anahtar kelimeler: Toplam mikroflora, FDA hidrolizi, en muhtemel sayı, biyolojik bozulma, tarihi taşlar.

To all alone and Beşiktaş,

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TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

LIST OF ABBREVIATIONS

CFU   :   Colony forming unit

NB   :   Nutrient broth

EPS   :   Extracellular polymeric substances

SAB   :   Subaerial biofilms

FDA   :   Fluorescein diacetate

ATP   :   Adenosine triphosphate

MPN   :   Most probable number

CHAPTER 1

INTRODUCTION

In this section; general principles of ecology, mechanisms and phenomology of biodeterioration, biodeterioration of inorganic materials, methods for investigation of biodeterioration and analytical protocol will be explained systematically and sequentially.

1.1.   General Principles of Ecology

Concerning the trophic chains that explain nutritional relationships between organisms in an ecosystem, organisms living in and on historical monuments can be both producers and destroyers. The producers as autotrophic bacteria, algae, lichens, lower and higher plants break substrate both by their metabolism products and through mechanical penetration in an indirect way. The destroyers damage the constituents of substrate in the course of using organic substances for their metabolic requirements. Insects, fungi and most of bacteria are destroyers. The consumers can be considered as the least dangerous group in this field because, they fed on living matter of other micro and macro organisms. On the other hand they have a crucial role in controlling the growth of other

biodeteriogens as snails that eat lichens and the grazing animals (Caneva *et al.*, 1991).
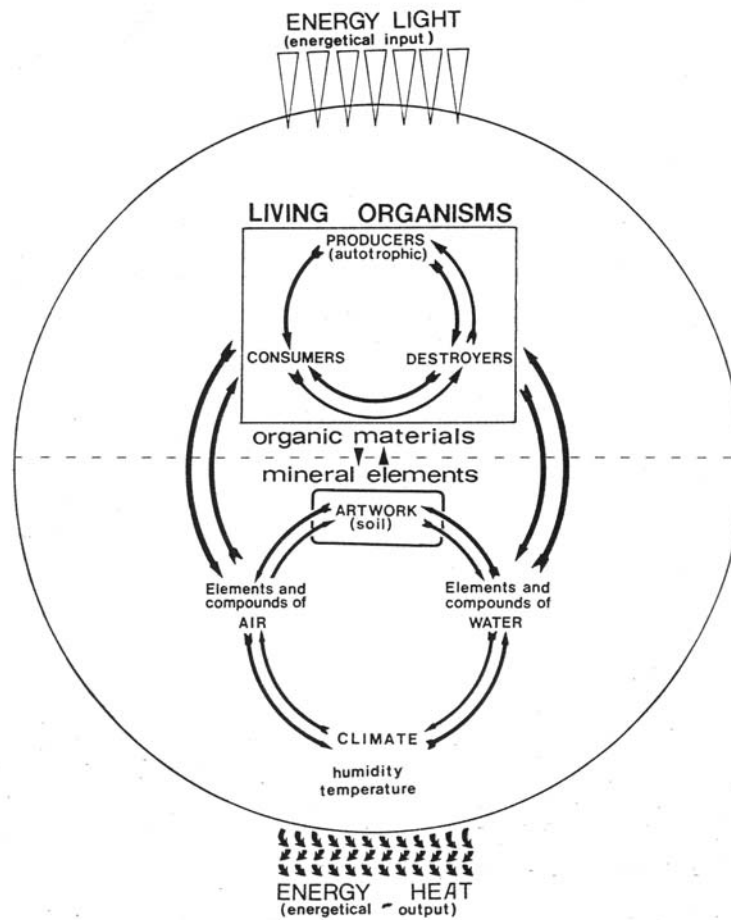
Figure 1. 1 A work of art as an ecosystem (Caneva *et al.*, 1991)

1.1.1. The limiting factors

Generally temperature, light, humidity and salinity can be accepted as limiting factor if values are close to the limits of tolerance of the species. Liebig's law and Shelford's law define the association between biological populations and limiting factors (Caneva *et al.*, 1991).

Liebig's law says that "under conditions of stationary equilibrium, the essential substances become limiting factors if their quantity is close to the minimum". That is, any essential factor presenting in limited quantities is vital for the growth of an organism. For instance, for an algal species, temperature, content of salts and humidity are mostly in optimum quantities in a subterranean environment, and, hence mentioned parameters are not limiting factors. On the contrary, in cases when the light is close to or lower than the minimum limit is of survival, it is considered as a limiting factor (Caneva *et al.*, 1991).

Shelford's law is related with the previous law and says that " organisms have not only an ecological minimum but also a maximum which determines an interval representing the limit is of tolerance" (Caneva *et al.*, 1991).

1.2. Mechanisms and Phenomenology of Biodeterioration

As it's known, life between the lithosphere and atmosphere is an ancient terrestrial niche and nowadays microbial communities rapidly settle and colonize on rock surfaces. In situations as facing with physical stresses, microbial population will continue to prevail throughout the biomass but in the opposite

case macroscopic vegetation will continue to dominate the biomass (Chertov *et al.,* 2004).

Disintegration and decomposition are the processes that play important roles in biodeterioration of materials and these mechanisms eventuate at the same time due to the kind of substrate and biodeteriorating agent. Due to having various chemical constituents, different biodeterioration types eventuate in materials having structure of organic or inorganic substances by the activity of heterotrophic and autotrophic microorganisms, respectively (Caneva *et al.*, 1991). Solar radiation, humidity and temperature are the environmental factors that affect habitation, growth and expansion of microorganisms on rock surfaces (Gorbushina, 2007). For instance, some pH values can condition the growing of acidophilous and basophilous microflora. In the matter of water content and relative humidity (RH), organic materials display more hygroscopic character than inorganic ones (Caneva *et al.*, 1991).

Generally microorganisms that prefer to grow on rock surfaces have tendency to form communities rather than growing as colonies including a single species. As a result of exposure of solid surfaces to the atmosphere, functionally and structurally complex organisms colonize on surfaces ranging from sediments, plant surfaces and soils (Costerton *et al.*, 1987; Guezennec *et al.*, 1998; Ortega-Morales *et al.*, 2001; Lindow and Brandl, 2003). Essentially, rock biofilms form heterogeneous matrices of microorganisms held together and tightly bound to underlying surfaces by extracellular polymeric substances (EPS) that develop when nutrients from the surrounding environment are available (Rosenberg, 1989).

In more detail, rocks that are inhabited by microorganisms remind an appearance like microscopic landscape which is consisted of fissures, pores, cementing materials and grains. Moreover, these landscapes are colonized by

biofilm organisms in various forms depending on their characteristics. For instance; bacteria, algae and microcolonies of yeast-like fungi have tendency to make clusters in the course of their settling on rocks, whereas fungal hyphae move forward by penetration. Depending on their nature as having tendency to generate clusters, microbial organisms settle on surface and depths of rocks via constituting extracellular matrices. However in fungi, hyphae is formed around and between separate cells and microcolonies. EPS is secreted by subaerial biofilm settlers in order to envelop the microcolonies that spread onto the substrate and keep the microbes together to provide adhesion onto and penetration into the substrate (Fig. 1.2) (Gorbushina, 2007).
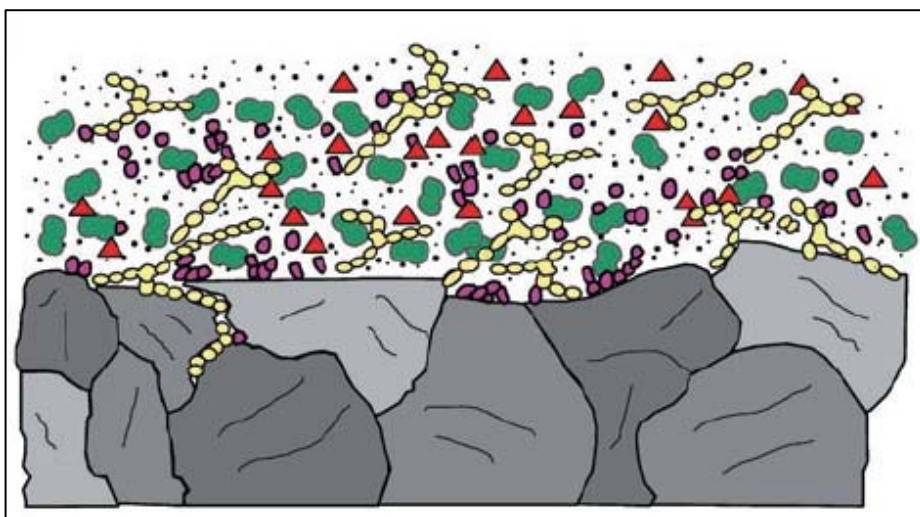
Figure 1. 2 Microorganisms are embedded in EPS and form a miniature microbial ecosystem including both heterotrophic and phototrophic microorganisms (Gorbushina, 2007)

EPS in subaerial biofilms have role in protecting from diffusion and spare them from enormous water stresses in three ways as retaining water for long periods, maintaining the viability of the cells and facilitating access to water vapour in the atmosphere (Gorbushina, 2007). SAB settlers on compact rocks held together by EPS in order to reduce energy consumption in the course of their transportation to other environments. In other words, SAB settlers prefer to be carried out as wind-blown cell aggregates rather than developing as a result of reproduction (Stoodley *et al.*, 2005). Extracellular matrices in other words polymeric substances, are common in all rock communities (Wimpenny, 2000; de los Rios *et al.*, 2002;Kemmling *et al.*, 2004; Omelon *et al.*, 2006). Microorganisms that settle and spread on rocks interface with the substrate, atmosphere and with each other (Fig. 1.3). Thin layers of organic matter that covers the bare rocks comprise organism interactions inside the biofilm, affects on structural components and influenced by climatic and atmospheric factors (Gorbushina, 2007).
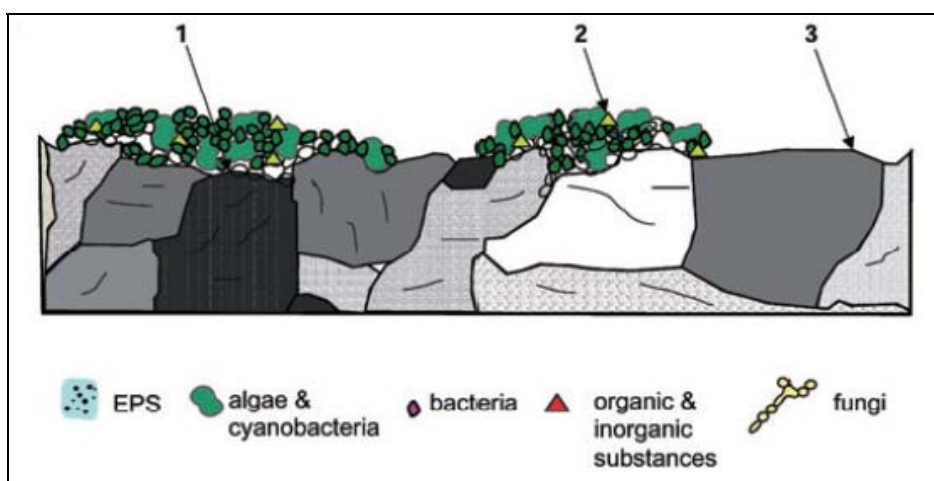
Figure 1. 3 Subaerial biofilms are coupling agents between the lithosphere and atmosphere. Effects seen at the interface include: 1, inter-organims interactions; 2, biofilm-atmosphere interactions; 3, atmosphere-substrate interactions (Gorbushina, 2007).

Depending on breaking up of rocks into it is constituents, SABs and rock substrates function together via penetrating and spreading widely (Ferris and Lowson, 1997). Biofilm formation on rock surfaces consists of several stages as settlement, colonization, penetration and spreading into deeper zones. Biofilms that penetrate these zones preserve their niche from external factors by aid of protective layers of stone. Even if these biofilms are damaged by reason of unsustainable temperatures and extreme solar radiation, they may continue to live as 'isolated inhabited 'islands'' (Nienow *et al.*, 1988; Wierzchos *et al.*, 2006).

Chemo-organotrophic, phototrophic and chemolithotrophic microorganisms are the major constituents of SAB and organisms as algae, fungi, bacteria and protoza may exist in this microorganism aggregation (Gorbushina and Petersen, 2000).

1.2.1. Physical processes

Depending on the mechanical activity of organisms like growth and movements, physical processes lead to mechanical fracturing, abrasion and disruption of the substrate. In general, the deterioration that is brought about by the microorganism activity are less serious than that caused by organisms. Furthermore, substance subjected to decaying process, represents tiny fragments on their surface which then offers greater surface for deterioration. The key point for occurrence of physical processes is the adhesion potential of organisms and microorganisms to the substrate. As a result of characteristics of living organisms, the adherence can be observed in several ways. For instance, fungi, prokaryotes and lichens attach substrate with their various attachment organs (Caneva *et al.*, 1991).

1.2.2. Chemical Processes

Alteration and decomposition of the substrate by the chemical activity of organisms are termed as chemical processes. Owing to general structure of algae, bacteria and fungi as having high surface-volume ratio compared to animals and plants, the diffusion of metabolic products between cells and their environment proceed rapidly. The decomposition of subsrate progress in diffent ways, namely the production of the following: organic and/or inorganic acids, chelating substances, alkalis, enzymes and pigments (Berthelin, 1983; Eckhardt, 1985; Allsopp and Seal, 1986).

1.3.   Biodeterioration of Inorganic Materials

Generally, autotrophic organisms prefer to colonize on inorganic substrates. The existence of organic materials on the surfaces of inorganic substrates is a natural occasion, especially in artifacts subjected to open air conditions. On the other hand, heterotropic microflora develop due to some factors as atmospheric pollution, old treatments, excretion of birds and agricultural pesticides (Caneva *et al.*, 1991).

1.3.1. Stone and Related Materials

The aggregation of one or often more minerals are known as stone or rocks and they can be classified as:

a) Magmatic or igneous rocks,

b) Metamorphic rocks,

c) Sedimentary rocks,

It is redundant to mention about plasters, mortars and ceramic products separately because living deterioration factors are the same as those live on stone. However, some characteristics of these materials as the chemical composition and pigmentation can favor the formation of microflora (Caneva *et al.*, 1991).

1.3.1.1. *Bacteria and actinomycete*

Even though their contribution on weathering of rocks is well known, the exact penetration mechanism of bacteria and actinomycete has not entirely described yet. The bacteria that grow on rocks are ranging in autotrophic, facultative chemolithotrophic and heterotrophic and they cause decays on rocks by their chemical actions (Krumbein, 1972).

Figure 1. 4 Chemoorganotroph bacteria growing on the surface of the primates of Arles, Cloister of Elne, photographed by Eng. Genevieve Orial in 1987.

Even though fungi and heterotrophic bacteria were determined on deteriorated rocks in previous researches (Bachmann, 1916; Paine *et al.*, 1933; Gromov, 1957), the majority have agreed on the fact that biological colonization is formed by the chemolitotrophic processes of nitric and sulfiric acid producing bacteria (Müntz, 1890; Isacenko, 1936) or by the photosynthesis reaction of alg/lichen (Sollas, 1880; Bachmann, 1890). At the same time, chemoorganotrophs are accepted as the first settlers of rocks that utilize minerals and organic substances of air (Viles and Gorbushina, 2003) (Fig 1.4). Moreover, acid production mechanisms of bacteria on rocks are verified by detection of acids in cultural mediums (Caneva *et al.*, 1991). The key issue that has to be considered is to go further analyse to determine whether microbial activity leads to weathering or

their existence is negligible (Caneva *et al*., 1991). The activity of bacteria cause decay zone formations on stones by the action of chemical reactions (Fig 1.4) and they are generally detected in high quantities on weathered stones rather than sound ones (Caneva *et al*., 1991).
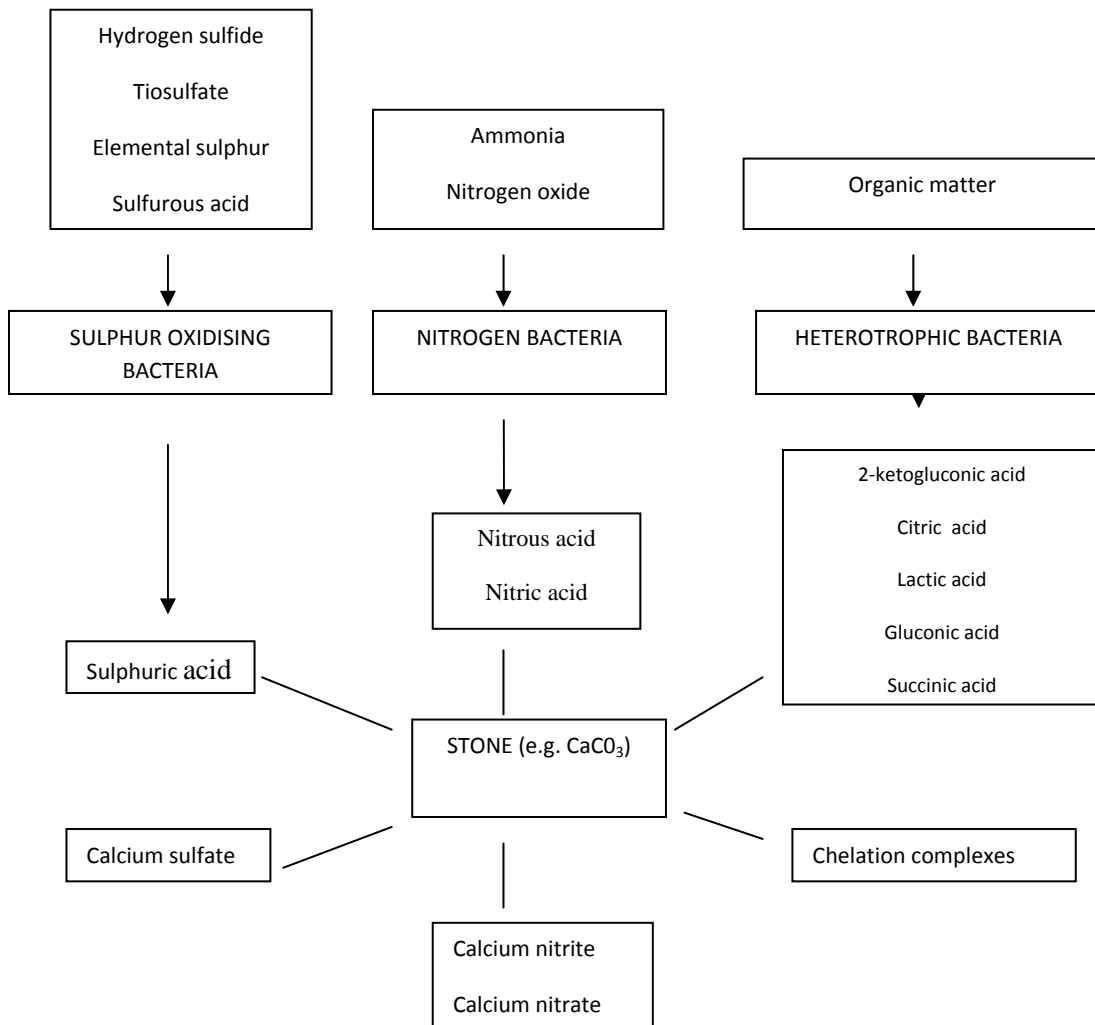
Figure 1. 5 Bacterial activity on stone(Caneva *et al*., 1991).
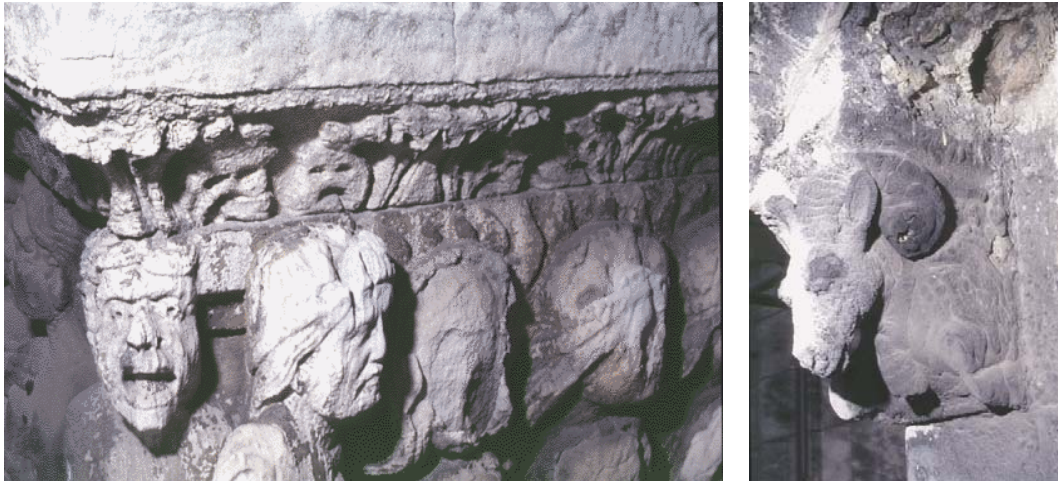
Figure 1. 6 Sulphur oxidising bacteria causing alterations on stones with sulfate formations, 1990s, Saint Trophime Church of ARLES, Bouches du Rhone, photographed by Eng. Genevieve Orial in 1990s.

Gypsum formations, depending on the activity of living organisms, are generally found as weathering products in limestones and sulphur-oxidising bacteria are one of them which take part in utilization of reduced sulphur compunds to produce sulfate ions (SO_4^{2-}) which then reacts with the (Ca^{++}) ions of stone to form ($CaSO_4.2H_2O$) (Pochon and Jaton, 1968) (Fig 1.6). Sulphur compounds circulation in other words sulphur cycle is illustrated in Fig. 1.5.
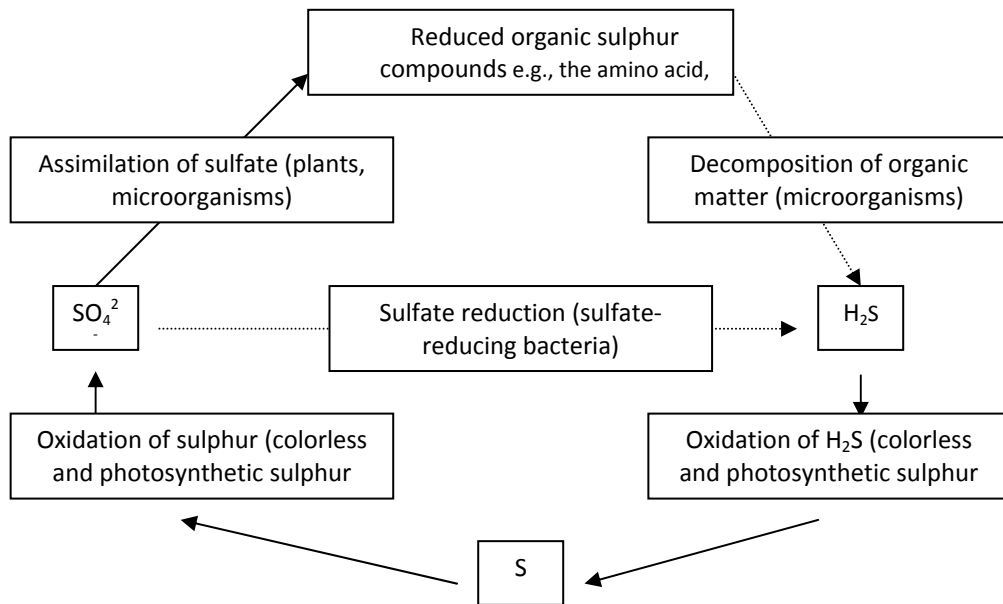
Figure 1. 7 The sulphur cycle (Caneva *et al.*, 1991).

According to the early researches conducted by Pochon and Coppier (1950) sulphur reducing bacteria in soil near the foundations of buildings, turn sulfates to sulfides. Then these sulfides dissolved in solution are penetrated and rised in stone through capillarity and come to the wall surface through surface evaporation. There sulfides are turned into sulfates by Thiobacillus sp. (Fig. 1.6). Nevertheless, this point of view could only be regarded as possible in circumstances as buildings having deterioration in their lower levels by rising damp. But in the cases of deterioration at higher levels, it is essential to pay attention to sulfate accumulation by pollutants or other colonizations just because their determination and isolation in high numbers from French and Italian monuments (Jaton, 1972; Barcellona *et al.*, 1973; Tiano *et al.*, 1975).
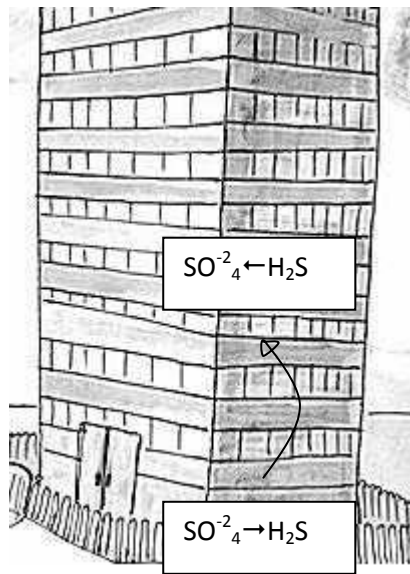
Figure 1. 8 Sulphur cycle at the base of a building according to Pochon and Coppier (1950).

The act of nitrifying bacteria in the deterioration processes of stone monuments are first mentioned in Kauffman's research in 1960. These bacteria are divided into two groups as the ammonia oxidizers (genus *Nitrosomonas*), which turns ammonia to nitrous acid, and the nitrite oxidizers (genus *Nitrobacter*), which turns nitrous acid to nitric acid. Alteration products consisting of calcium nitrates and gypsum are formed as a result of these acid interaction with limestone and then transformation of it is surface into powder. Although nitrifying bacteria are detected in low numbers on weathered marbles and monuments in Italy, their existence is assumed to be the most important factor on German sandstone monuments (Bock *et al*.,1988; Meincke *et al*.,1988; Wolters *et al*., 1988).
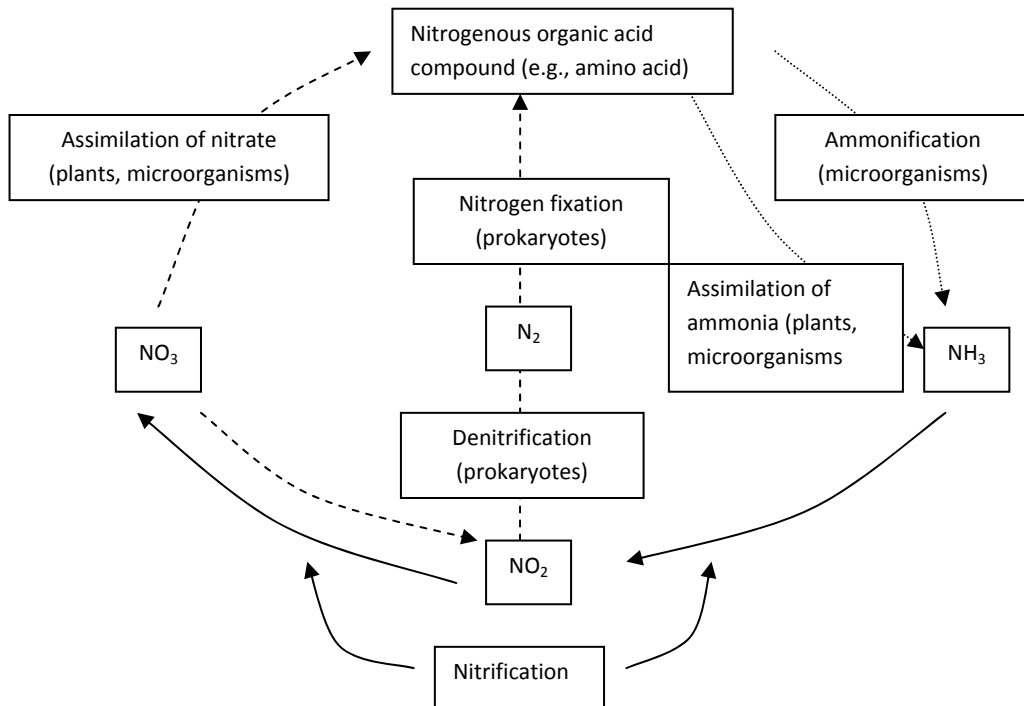
Figure 1. 9 The nitrogen cycle (Caneva *et al*., 1991).

Oxidation process of ferrous ions to ferric ions provide energy to iron bacteria and these bacteria also have ability to oxidize iron containing minerals (Caneva *et al*., 1991).

Hetetrophic bacteria existence in high quantities ranging between 10^6-10^7 CFU/g, (CFU = colony forming unit) indicates their important role in weathering of stone. Generally they deteriorate stone by their products as alkalis, organic and inorganic acids and chelating agents (Duff *et al*., 1963).

Some bacteria act on stone by their ability of mobilizing silica and silicates (Webley *et al*., 1963). By the same strategy in terms of chemical action, insoluble forms of phosphorus (Ca, Al, and Fe phosphates) can be converted into soluble

forms. Moreover, frequently observed Bacillus genus act on minerals as goethite, haematite, limonite, etc. (Ehrlich, 1981).

1.3.1.2. Fungi

Generally fungi species are aerobic and so they prefer to inhabit in the upper layer of the lithosphere . Fungi predominate over in all temperature, polar soils (Kjoller and Struwe, 1982) and warm environments (Mouchacca, 1995). Although they are involved in heterotrophic organisms group, fungi have often been detected on weathered stones (Fig. 1.10), particularly in tropical areas. Natural constituents of stone does not serve an advantageous substrate for their growth whereas several organic residues always exist on stone and favor the growth of fungi (Caneva *et al.*, 1991). Symbiotic and free-living fungi are essential inhabitants on all rock surfaces (Gromov, 1957; Krumbein, 1966; Braams, 1992; May *et al.*, 1993; Diakumaku *et al.*, 1995; Kurakov *et al.*, 1999; Sterflinger, 2000; Warscheid and Braams, 2000). Fungi as common settlers on rocks can be divided into two groups (i) hyphomycetes of soil and epiphytic origin (de Leo *et al.*, 1996) and (ii) black (melanized) MCF (Ascomycetes, orders Chaetothyriales, Dothideales, Capnodiales) that form typical, compact microcolonies (Staley *et al.*, 1982; Gorbushina *et al.*, 1993; Diakumaku *et al.*, 1995; Wollenzien *et al.*, 1995; Sterflinger *et al.*, 1999a).
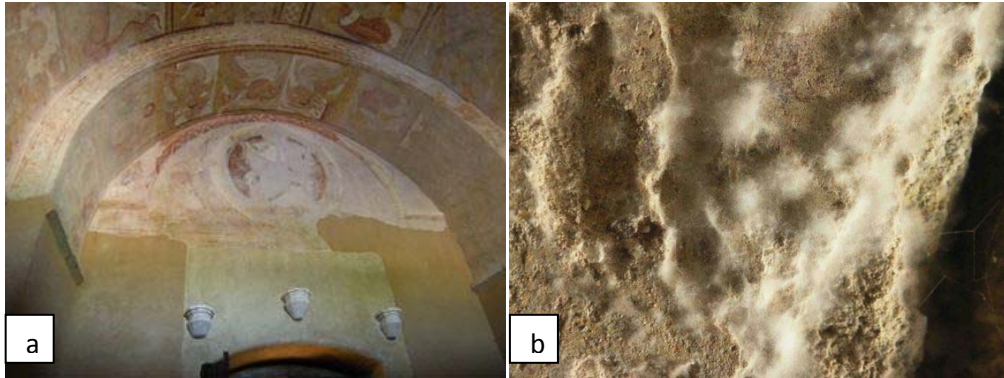
Figure 1. 10 Fungi colonization on paintings of porch (a) and on mortar (b), church of Saint Savine, Vienne (France), photographed by Eng. Genevieve Orial in 2007.

In rock-inhabiting fungi melanin pigmentation provides extra-mechanical power to the hyphae that facilitates fungi movement through cracks of substrates (Dornieden *et al*., 1997; Sterflinger and Krumbein, 1997). Further penetration of fungal hyphae into deeper layers of rock contribute to protection of cells from the UV radiation (Friedmann and Ocampo-Friedmann, 1984; Cockell and Knowland, 1999) and this protection is actually carried out with the synthesis of several pigments comprising carotenoids (torularhodin, phtoene, z-carotene, b-carotene and torulene) (A.A. Gorbushina, E.R. Kotlova and O.A. Sherstnjova, in preparation), mycosporines (Volkmann *et al*., 2003) and melanins. For instance, fungi types that are observed as dark spots causing staining on and within stone generally belong to the Dematiaceae family (Ionita, 1971: Hyvert, 1966b,c) and their disjunction is very difficult most likely due to melanins contained in the mycelium (Leznicka, *et al*., 1988). On the other side, non-dematiaceous fungi also cause stains but shows more unstable character (Caneva *et al*., 1991).

Fungal hyphae penetrate through the calcite crystals in several planes hence creating an etched appearance when observed under SEM (Koestler *et al*., 1985). Furthermore, endolithic fungi species have capability of producing pit is (Danin,

17

1986a) and depending on their filamentous structure penetration through the substrate eventuate easier (Caneva *et al.*, 1991).

As a matter of fact, fungi deteriorate stones mainly by it is chemical actions like producing acids and most familiar ones are nitric, carbonic, sulfiric and many other organic acids. Actually the latter have ability to form chelation complexes with metal cations of the substrate leading to dissolution of limestones, magnesium and iron bearing minerals, silicate minerals and different phosphates (Williams and Rudolph, 1974). In sandstones both of the cementing material and aluminium silicates are deteriorated due to fungal activity. In more detail, big amounts of oxalic and citric acids are produced by certain species as *Aspergillus niger, Spicaria* sp. and *Penicillium* sp. The act of oxalic acid secretion includes an extensive corrosion of the primary minerals and complete decomposition of ferruginous clay minerals. The capability of fungi to form calcium oxalates as a deposition on the outside of hyphae has been displayed and proved empirically on marbles and calcite (Mentler *et al.*, 1986; Chiari *et al.*, 1989).

Some fungi, as *Phoma* spp., *Penicillium* spp. and *Exophiala jeanselmi* could be related in exfoliation and crust formation of stone. On the other hand, it is proven that these fungi species can oxidize manganese that induce extracellular encrustations of hyphae (Kuroczkin *et al.*, 1988; Petersen *et al.*, 1988).

1.3.1.3. *Cyanobacteria and algae*

As it is well known, cyanobacteria have essential roles as photosynthesis and atmospheric nitrogen fixation. Furthermore, they improved their resistance to solar radiation (Garcia-Pichel and Castenholz, 1993; Büdel *et al.*, 1997) and desiccation (Potts, 1999) in a perfect manner. Even though having capability to

resist extreme conditions it is not enough to protect themselves entirely. Cyanobacteria advanced an adaptation method like burrowing into the rocks in order to form endolithic environment when faced with such extreme conditions. (Friedmann, 1968; Friedmann and Ocampo-Friedmann, 1984; Wessels and Büdel, 1995; Büdel, 1999; Büdel *et al*., 2004; Wierzchos *et al*., 2006; de los Rios *et al*., 2007).

Algae not only exist in environments like seepages but also prefer to inhabit in conditions such as mild winter (Rindi and Guiry, 2003). SAB mainly comprise green algae, e.g. *Chlorella*, *Desmococcus*, *Phycopeltis*, *Printzina*, *Trebouxia*, *Trentepohlia* and *Stichococcus* (Hoffmann, 1989; Ortega-Morales *et al*., 2000; Gorbushina *et al*., 2002b; Zurita *et al*., 2005; Gaylarde *et al*., 2006). On the other hand diatoms sometimes prefer to exist in hypogean or chasmolithic environments (Anagnostidis *et al*., 1983; Wohler *et al*., 1998; Rindi and Guiry, 2003).

Generally, we can observe two different algae groups, such as Cyanobacteria (blue green algae) and Chlorophytes (green algae) (Fig. 1.11) on or within stones. But other kinds of algae like diatoms can be present on stones (Caneva *et al*., 1991). For instance, big amounts of diatoms have been detected on sandstone and several bulding surfaces in tropic regions (Wee and Lee, 1980).

Figure 1. 11 Alg formations, Church of Thouars (France), photographed by Genevieve Orial in the 2000s.

Due to their involvement with the substrate, algae can be divided into two groups such as epilithic and endolithic. Epilithic and crypto-endolithic (Golubic *et al.*, 1981) cyanobacterial biofilms, as well as crusts of cyano-lichens can be found in hot and cold deserts, temperate regions, semideserts, savannas, rain forests and even polar regions (Frit isch, 1907; Diels, 1914; Jaag, 1945; Fjerdingstad, 1965; Golubic, 1967; Broady, 1981; Wessels and Büdel, 1995; Büdel, 1999; Büdel *et al.*, 2004). While epilithic ones have tendency to grow on exposed surfaces, the latter one prefer to colonize through the interior parts of semitranslucent and translucent rocks. In more detail, endolithic algae comprise chasmoendolithic, cryptoendolithic and euendolithic forms. The first form prefer to live inside the fissures and cavities exposed to the air and the second form have a tendency to colonize the structural cavities of porous rocks and the last one actively penetrate into the substrate (Golubic *et al.*, 1981; Danin, 1986a; Hoffman, 1989).

The most significant factors that contribute to the formation of algae populations are humidity, temperature, light intensity and pH. Green algae and especially cyanobacteria are generally determined as first colonizers of stone depending on their slight requirements in terms of light, water, inorganic compounds and alkaline substrate (pH 7-8) to continue their lives. Limestone is more susceptible to be colonized than other types of stone. Cyanobacteria have a structure as gelatinous sheath that enables absorbing and retaining of water for a long time. Indeed, this make them to survive in harsh environmental conditions, as in the event of freezing. Actually, algae can switch their metabolism on and off as a response to changing environmental conditions (Lefevre, 1974; Pietrini *et al.*, 1986; Albertano and Grilli Caiola, 1988). The species of Cyanobacteria such as *Chroococcus, Nostoc, Oscillatoria, Lynbya, Gloeocapsa, Scytonema* and *Myxosarcina* are frequently determined on mural paintings and stone of historical monuments. Besides, the species of green algae as *Chlorella, Chlorococcum, Haematococcus, Scenedesmus, Ulothrix, Stichococcus* are commonly isolated from monuments (Caneva *et al.*, 1991).

Epilithic algae type can be frequently determined on stones of monuments as patinas ranging in different thickness and color by simple observation. They display thick and gelatinous character in various colours such as yellow, orange, green in damp places whereas in dry places their structure is thin and though and sometimes green, very frequently grey or black**.** Different colors of patina existence is thought to be occurred due to biocoenosis types, growth phase and development stage of the algae (Ricci *et al.*, 1985). Furthermore, soil, dust, spores and other substances attach to these patinas and let them to deteriorate and meanwhile act like a rich substrate with organic constituents, hold up the establishment of organisms such as lichens, ferns, mosses and heterotrophic microflora. Chasmoendolithic algae generate green areas parallel to the surface,

commonly under slightly detached scales, therefore, it is just possible to observe them in the cases of detachment of scales (Caneva *et al.*, 1991). Cryptoendolithic algae settle on light-coloured sandstone and limestone, generating a colored zone parallel to the surface at a depth of a few milimeters. Eventhough their occurrence is described mainly in extreme conditions, recent studies pointed out their ability to survive at varying ecological valences (Sainz- Jimenez et al., 1990). Euendolithic algae (only blue-green algae) acts as three different ways, such as, dissolution of carbonates, penetration into the substrate, and the formation of microcavities, depending on various species. Last and most important to be mentioned briefly is that algae lead to stone decay by respiration processes, by retaining water which then bring about problems in freeze-thaw cycles or by releasing acids and chelation compounds (Degelius, 1962).

1.3.1.4. Lichens

Lichens have the capability to act and maintain their lives at changing water content and so called poikilohydric organisms (Hawksworth and Hill,1984). Combination with cyanobacteria, they perform as pioneer organisms in the colonization of rocks and can establish on man made substrate in a very short period of time.

Lichens damage their substrate by exerting physical force in the course of contraction and expansion act under dry or wet conditions. Due to this occasion, mineral fragments are detached from the substrate and combined into the thallus (Fry, 1924). The most crucial damage that is eventuated by chemical reactions of lichen, can be explained in three ways, such as the "formation of

carbonic acid, the excretion of oxalic acid and the production of lichen compounds with chelating abilities" (Jones and Wilson, 1985).

Oxalic acid is excreted by the mycobiont(fungus) partner of lichen. In most cases the production of oxalic acid increases due to age of lichens and it is much more observed in calcicolous rather than in silicicolous species (Ascaso *et al.*, 1982). Nonetheless, this case isn't comply with all lichens, because some species also from the same genus do not produce oxalic acid and sometimes same species produce low amounts due to the composition of substrate they are holding on (Salvadori and Lazzarini, 1988). Oxalic acid is accepted as more active than other organic acids and it leads the formation of pit is under the thalli via surface etching and causes accumulation of oxalates as crystallized forms in the thallus. There is a strong relationship between the type of insoluble oxalates that deposited in the thallus and the mineral composition and the hydration state of stone (Caneva *et al.*, 1991). Well-known calcium oxalates weddellite and whewellite are usually generated on sandstone, limestone and other kinds of stone with a calcitic matrix; they can also arised from deteriorated calcium containing minerals (Caneva *et al.*, 1991). Actually, oxalates can be detected in certain regions of the thallus, such as on the external surface, in the central part of the thallus or at the lichen –rock interface (Salvadori and Lazzarini, 1989).

1.3.1.5. *Lower and higher plants*

Anytime the substrates and environmental conditions are suitable, liverworths, mosses and vascular plants tend to establish on buildings and archaelogical sites. In more detail, water content, lighting and porosity of subsrate are deterministic

parameters for the act of mechanical penetration of roots and rhizines (Fischer, 1972: Allsopp and Drayton, 1975).

Root growth commonly detected in regions with lowest resistance like in mortar or plaster between stones and bricks (Caneva,1985). In addition, plant growth can be observed even in compact zones due to the attenuation of materials depending on the effect of physico-chemical factors (e.g rain, frost) (Caneva *et al.*, 1991). In some cases plants can lead to generation of coloration of stone owing to excretion of organic compounds into the pores of the substrate and when it is white or light colored observation becomes easier (Lewin and Charola, 1981).

The existence of trees in archaeological areas bring about critical occasions in terms of their development of root systems both in depth and parallel (Caneva, 1988).

1.3.1.6. *Animals*

Historical monuments, located in open field, are exposed to outdoor environment conditions constantly. For this reason, being damaged by several bird species is a natural state that can not be disregarded. Their feces include certain acids which then reacts with stone and cause corrosion (Caneva *et al.*, 1991). On the other hand, the indirect damage of birds are eventuated by the organic substances of excrement as a nutritive substrate for hetetrophic microflora consisting of actinomycetes, bacteria and fungi (Bassi and Chiatante, 1976). In subterrestrial regions birds, such as bats deteriorate environment by

the accumulation of their excretement that is generally composed of urea (Caneva *et al.*, 1991).

Bovine animals, such as goats, ships and cows can also cause damage to the monuments located in archaeological sites (Caneva *et al.*, 1991).

Insects, molluscs, spiders and snails, exist on stone monuments without visible deteroration impact. They carry on their lives by feeding on algae and lichens, displaying a micrograzing action (Danin, 1986b).

1.4.   Methods for Investigation of Biodeterioration

Biodeterioration of stones of Nemrut Mount Monument and Temple of Augustus are exposed to atmospheric conditions, the degree of biodeterioration was investigated in terms of microorganims to determine their role in the decay processes of stones. For this purpose various techniques as fluorescence, luminescence and microbial counting were used in order to determine the level of bacteria and fungi existing on the surface of stones.

Here some of those techniques were explained below:

Fluorescence Techniques

A variety of studies have used the fluorescein diacetate to measure microbial activity in soils (Schnurer and Rosswall, 1982; Adam and Duncan, 2001; Green *et al.,* 2006) and stone surfaces (Prieto *et al.,* 2004). Moreover, fungal activity in

fungal batch cultures (Ingham and Klein, 1982) and algal activity in lichens (Berglund and Eversman, 1987) were determined with the product of enzymatic convertion of FDA, fluorescein, either by measuring in spectrophotometer or visualising under a microscope. All these mentioned studies are based to the direct relationship between the enzymatic activity and microbial population.

The method proposed in this study for measuring enzymatic activity in other words FDA hydrolysis on stones, was taken from the thesis of Ustunkaya (2008), which was slightly adapted from the studies of Adam and Duncan (2001) and Green et al (2006). According to the assay of Adam and Duncon (2001), 0.7 μg fluorescein/g of soil is regarded as low fluorescein concentration, while 1.1 μg fluorescein/g of soil is regarded as intermediate. In addition, above 2 μg fluorescein/g of soil is assumed as having high fluorescein concentration. Based on the study of Diack and Stott (2001), the microbial activity value was in the range of 83-96 μg fluorescein/g of soil for good agricultural soil. According to the studies of Green *et al.* (2006), the average microbial activity of soils containing high clay contents, gave values in the range of 66-226 μg fluorescein/g of soil.

Luminescence Technique

The ATP bioluminescence method has been used commonly in the field of hygiene monitoring to assess the degree of contamination (Sigler *et al*., 2002; Kaskova *et al*., 2005; Green *et al*, 1999; Vilar *et al*., 2008). Moreover, in recent years, this technique was used to measure the global metabolic activity with Adenosine Triphosphate (ATP) of historical stone surfaces (Orial *et al*., 2009).

The ATP bioluminescence method was adapted from the studies of Orial (2009), in order to detect global microbial activity degree of Nemrut Mount Monuments

and Temple of Augustus stones. According to the studies of Orial *et al.,*(2009), 2-3000 RLU (Relative Light Unit is) was considered as very low for microbial activity to start taking part in stone decay processes.

Total Microflora Technique

The method of total microflora have been widely used in the past researches of scientists working at counting of aerobic heterotrophic bacteria and fungi (May and Lewis, 1988; Eckhardt, 1988; Wilimzig *et al.,* 1992; Wilimzig and Bock, 1992; Gorbushina *et al.*, 2001). Actually, total microflora method have been studied since 1933 for enumaration of the bacterial population of sound and decayed stones (Paine *et al.*, 1933).

The method proposed in this study for determining the distribution of bacteria and fungi on stone, was taken from the studies of Jaton (1971) . Acccording to the studies of Jaton (1971) cell number of 10^6 CFU/g of stone is considered as normal microflora for bacteria living on stones. Based on the studies of Wilimzig *et al.*(1992), cell number of 10^3 CFU/g of stone is low for bacteria and fungi to play part in decaying process. Furthermore, in weathered stones cell numbers were generally existed in the ranges of 10^6-10^7 CFU/g as stated in the literature of Caneva *et al.*, (1991).

As applied in the studies of Hueck van der Plas (1968) and Krumbein (1972), the first step of Koch's postulates which is adapted from his approach based on establishing link between 'the presence of a microorganism and disease symptoms' (May and Lewis, 1960) was applied on the the method of total microflora.

MPN (Most Probable Number) Method together with Biochemical Tests

Together with biochemical tests, most probable number method has been widely used in the past researches both to asses microbial biomass of soils (Molina and Rovira,1964; Belser and Mays, 1982; Stout *et al.,* 1984, Papen and Von berg, 1996) and to understand if the microbial activity is responsible for the formations of salt deposit is of/on stones (Leite Magalhaes and Sequeira Braga, 2000; Wilimzig *et al*, 1991, 1992; Gorbushina *et al*., 2001; Manch and Bock, 1998).

Following the comprehensive study on detecting the number of microbial cells, namely nitrifying and sulphur oxidising bacteria, protocols of nitrification and sulphur oxidation were adapted from the studies of Pochon and Tardieux (1962) in which quantification of microbial cells was obtained by the most probable number method as microorganism / gram of stone. According to the studies of Leite Magalhaes and Sequeira Braga (2000), concerning nitrifying and sulphur oxidising bacteria, cell number of 1×10^3 cells /g of stone was regarded as low. At the same time, based on the studies of Gorbushina *et al*., (2001), the amount of bacterial activity of several monuments were in the range of $1\text{-}10^4$ cells/g of stone expressing low microbial activity as well. On the other hand, as stated in the report of Wilimzig *et al.,* (1991), average cell number of nitrite oxidising bacteria was found to be 1×10^5 cells/g of stone which made them conclude about bacteria related stone decay .

1.5. Analytical protocol

Before starting anywork regarding biodeterioration, a comprehensive plan having six main topics are required to accomplish the study successfully. So these topics can be explained as follows;

1) The existing circumstances are evaluated by gathering information about environmental conditions such as temperature, relative humidity, condensation and dew point, leaks, defect of ventilation, etc.; life of the artwork and the products as resins, glues, binders that were used in the past restoration studies.

2) Different substrates as paintings, wall paintings, stone, wood, tapestries, carpets, etc. are specified to consider their contamination.

3) Biological contamination kind, abundance and first distinction are assessed separately. For instance deteriorated stones having visible coverings draw attention to fungi, algae and lichens,on the other hand in damaged stones haven't got visible coverings, possibility of bacterial deterioration point out complementary microbiological analysis.

4) Sampling method is selected according to the appearance of deterioration and microorganism type.
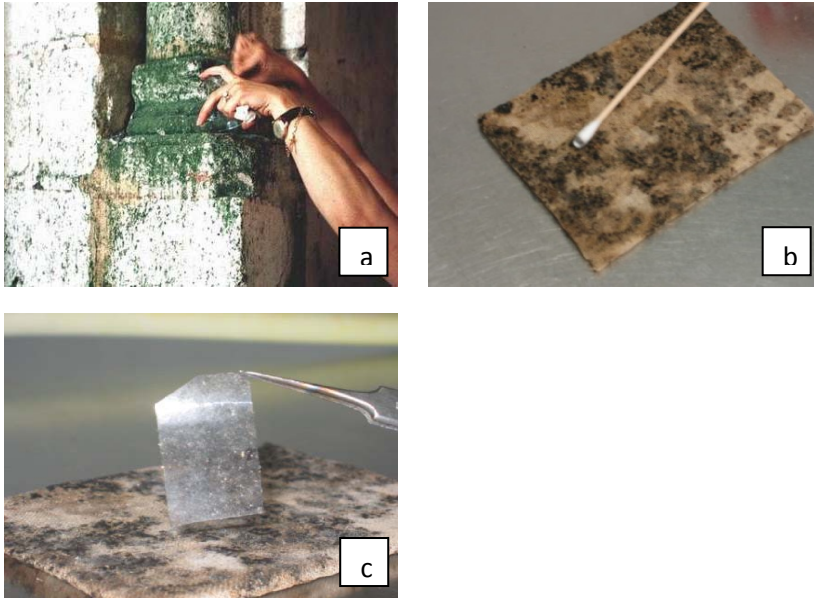
Figure 1. 12 Matter sampling (a), cotton swab(b), adhesive piece (scotch tape)(c) (Photographs were taken from the archieve of LRMH)

| Body of the device which looks like a vacuum cleaner | Petri dish containing specific medium | Air flow including micro organisms (germs, spores, algal cells) |
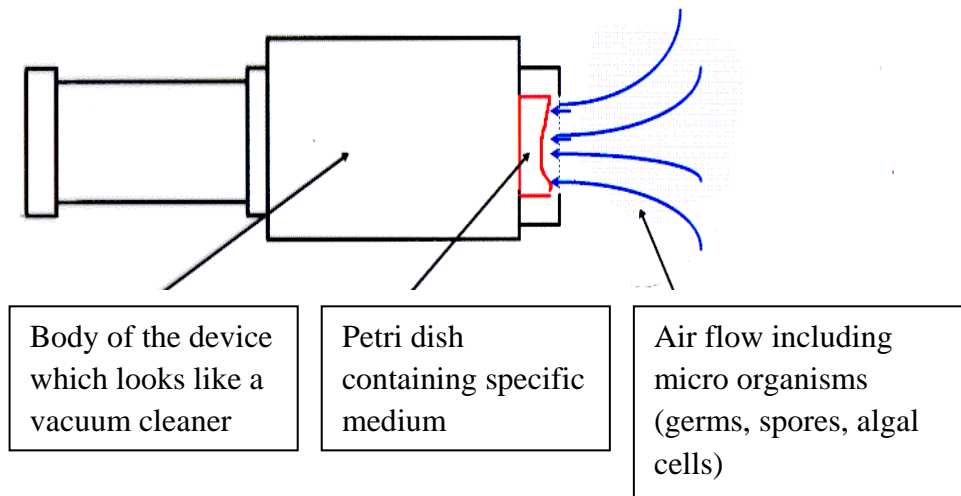
Figure 1. 13 Ambient air sampling

After incubation period each spore existing in the air give a visible fungal colony which can be counted. Eventually it it possible to know the pollution level accroding to the number of the colonies per cubic meter of air.
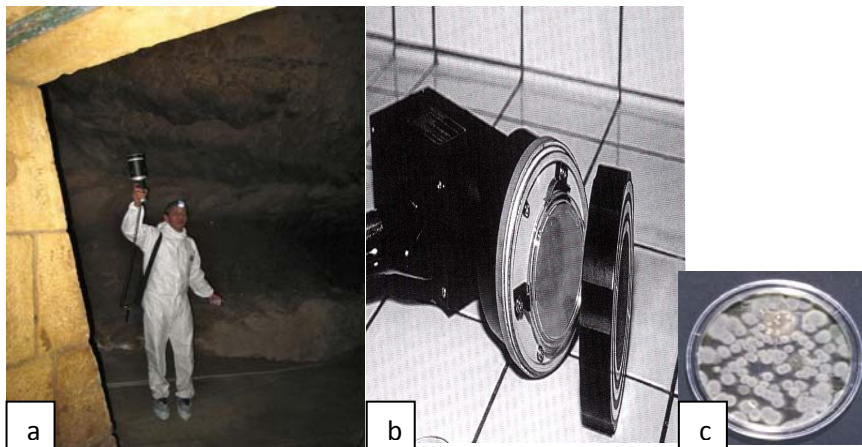
Figure 1. 14 During sampling process (a), sampling device (b), fungal growth after incubation period (c) (Photographs were taken from the archieve of LRMH)

5) After the protocol that will be applied is determined and described briefly, the steps of the protocol are mentioned in a detailed manner. The protocol can be conducted from a list of experiment as given below;

- Chemical analysis (sulphates, nitrates)

- Microbiological analysis;

    ✓ Global metabolic activity

    ✓ Assessment of micro-organisms viability by fluorescence

✓ Counting and species identification

   ✓ Antibiogram application

6) Synthesis of results are performed in order to examine the whole results of the experiment to conclude if there is or not biological deterioration.

7) Characterization of the appropriate treatment and it is implementation are carried out in situations where it is needed. The subjects in the following list correspond to this mentioned situation;

   - Cleaning of the substrate

   - Removal of the biological coverings

   - Spraying or applying pads impregnated with effective biocides

   - Atmosphere treatment

8) The assessment of the external effects is carried out in order to eliminate damage during conservation of artworks. In the following, there is an example of management list relevant to that occasion;

   - Management of the environmental conditions

   - Investigation of the climate and water

   - Control and maintenance of artworks

   - Observation of artworks regularly in an attentive manner, moreover detailed studies in environments having high risks.

   - Implementation of the ventilation.

   - Improvement of the drying

- Water drainage maintenance of buildings, (gutters, drainpipes cleaning, etc.) (Orial,2002).

1.6. Aim of the study

Stones suffer from biodeterioration since they are exposed to cyclic weathering conditions, especially in the changes of humidity and temperature. There is a necessity to better understand the weathering mechanisms of biodeterioration on stones in order to develop protective measures for the control of biodeterioration. Some investigation methods are used for the assessment of biodeterioration on stone in terms of types of microganisms causing biodeterioration, their distribution, and the degree of biodeterioration, such as measurements of Fluorescence, Luminescence, Total Microflora, Most Probable Number and ATP.

Some of those methods are used to analyse the microbial activity in soil and in hygienic environments such as FDA hydrolyses and ATP, respectively. Here, some of those methods were adapted for the biological activity analyses on stone with an emphasis on improved sampling from the stones and evaluation of experimental results. Due to the difference in evaluation of data in recent years, there is a necessity of using more than one investigation method, for measuring the quantity of microorganisms causing deterioration on stone surfaces. The joint interpretation of the data obtained from various analyses, therefore, gained importance for the diagnostic and monitoring studies of stone biodeterioration. In this study, several investigation methods were tried to be used together for the analyses of stone biodeterioration in order to improve the accuracy of the

scientific study on the determination of biological activity playing role on the deterioration of stones.

Two cases were selected to be studied: Nemrut Dag Monument (Adiyaman, Turkey) and Temple of Augustus (Ankara, Turkey). Both historical monuments had particular importance since they were listed as World Cultural Heritage and Cultural Heritage in Danger, respectively. The Nemrut Dag Monuments suffer from prevailing harsh climatic conditions of Nemrut Dag at an altitude of 2200m while stones of Temple of Augutus suffer from the dense air pollution of the city together with the effect of continental climate. The types and levels of biological activity on Nemrut Dağ limestones and sandstones, Temple of Augustus marbles and andesites and the role of that biological activity on deterioration of those stones were the key concerns of the study.

The objective of this study was compare and develop standardized procedures for measuring metabolic activity and counting microorganisms growing on stones of historical monuments in order to determine if these microorganisms played important roles in the decay processes. For this purpose, FDA hydrolysis, Total Microflora Method and Most Probable Number Method together with Biochemical tests were conducted on the limestones and sandstones of Nemrut Mount Monument, and marbles and andesites belonging to the Temple of Augustus. Moreover, fungi identification were done for determining the occurance of detrimental species on those historic stones.

CHAPTER 2

MATERIALS AND METHODS

In this study, representative samples from deteriorated stones having visible and non visible coverings, taken from sandstones and limestones of Nemrut Mount Monument were analysed in three seasons with different techniques to measure the degree of biological deterioration. At the same time, marbles belonging to the Temple of Augustus having deteriorated regions as soiling, salt crystals and colour changes, were analysed in order to detect if the biological organisms play important roles in the aforementioned decay forms. For that purpose, FDA hydrolyses, Total Microflora method, ATP-bioluminescence method and MPN method together with biochemical tests were conducted in the samples taken from Nemrut Mount Monument and Temple of Augustus.

2.1. Nomenclature of the Samples

Collected samples were coded in order to simplify their identification during different types of analyses. The nomenclatures of the samples are demonstrated below in a detailed manner.

```
11  M  N  Q  L  1
```

- → Sample number
- → The first letter of stone type, Limestone
- → The first letter of location, Quarry
- → The first letter of monument, Nemrut Mount Monument
- → The first letter of month, May
- → The last two digit of the year, 2011
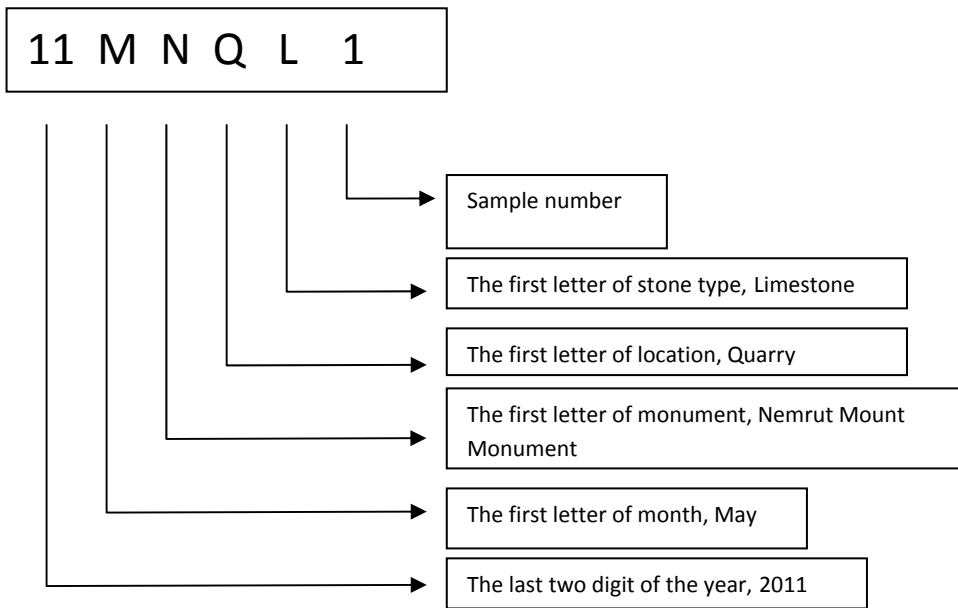
Figure 2. 1 An example of nomenclature made for samples taken from Nemrut Mount Monument.

```
11  M  N  Q  L  BA1
```

- → The first letter of the part of the stone where ATP is conducted. Bottom
- → The first letter of stone type, Limestone
- → The first letter of location, Quarry
- → The first letter of monument, Nemrut Mount Monument
- → The first letter of month, May
- → The last two digit of the year, 2011
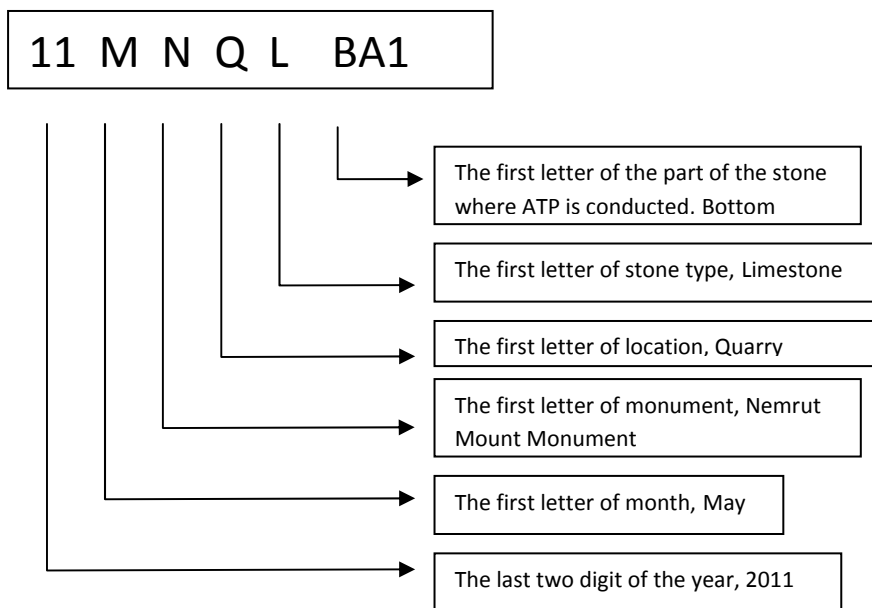
Figure 2. 2 An example of nomenclature made for samples taken from Nemrut Mount Monument.

The first letter of stone type, Andesite

The first letter of side of the wall, Interior

The first letter of location, East

The first letter of monument, Temple of Augustus

The first letter of month, July

The last two digit of the year. 2011
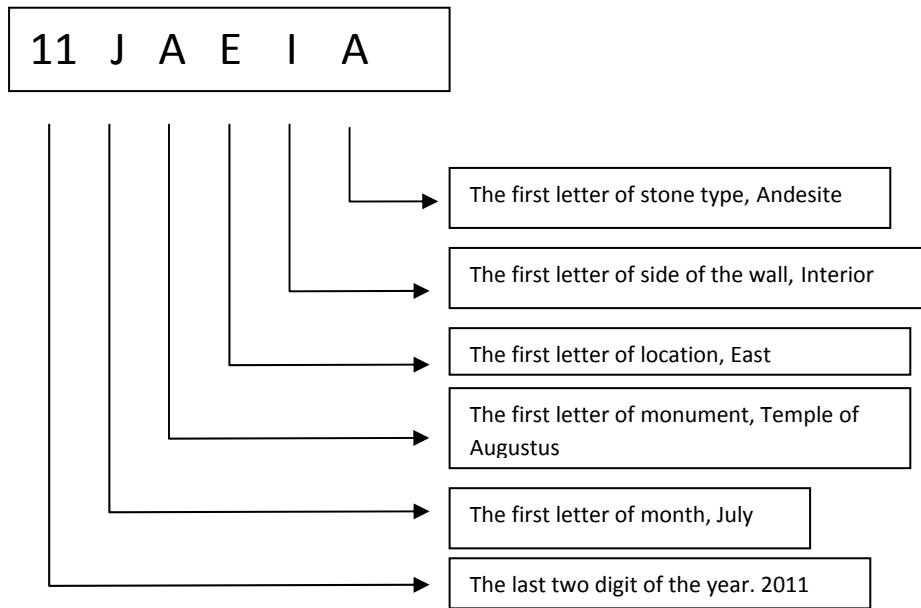
Figure 2. 3 An example of nomenclature made for samples taken from Temple of Augustus.

The first letter of stone type, Andesite and test of ATP

The first letter of side of the wall, Interior

The first letter of location, East

The first letter of monument, Temple of Augustus

The first letter of month, July

The last two digit of the year, 2011
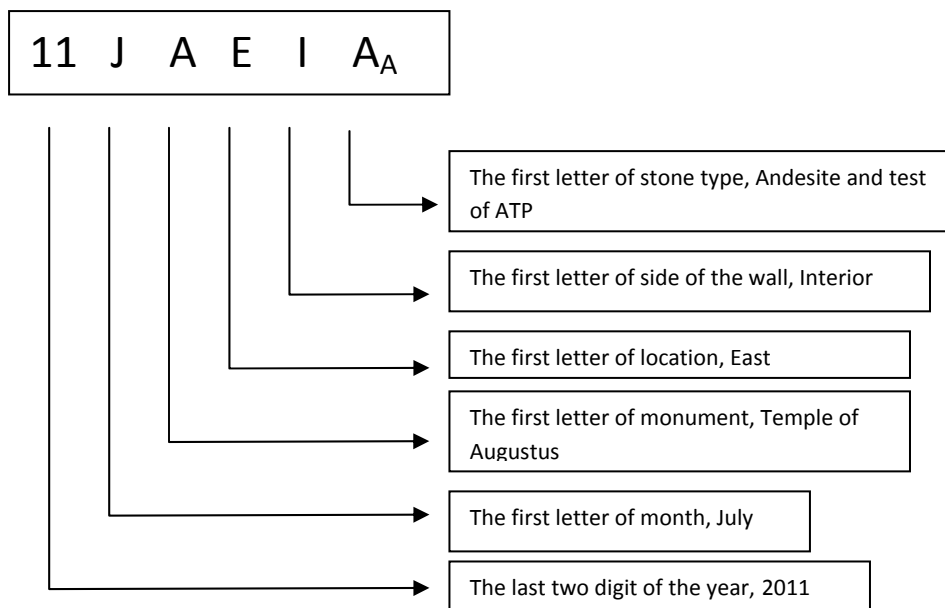
Figure 2. 4 An example of nomenclature made for samples taken from Temple of Augustus.

37

2.2. Historic Buildings and the Description of Samples

In this section, the samples, location of the monuments, the sampling places and the planned analyses were described briefly.

2.2.1. Nemrut Mount Monument

Nemrut Mount Monument is located in the town of Kahta, Adiyaman. The monument was constructed during the reign of King Antiochus I (163 (?) or 80 (?) BC – 72 AD) (Şahin Güçhan, 2011).
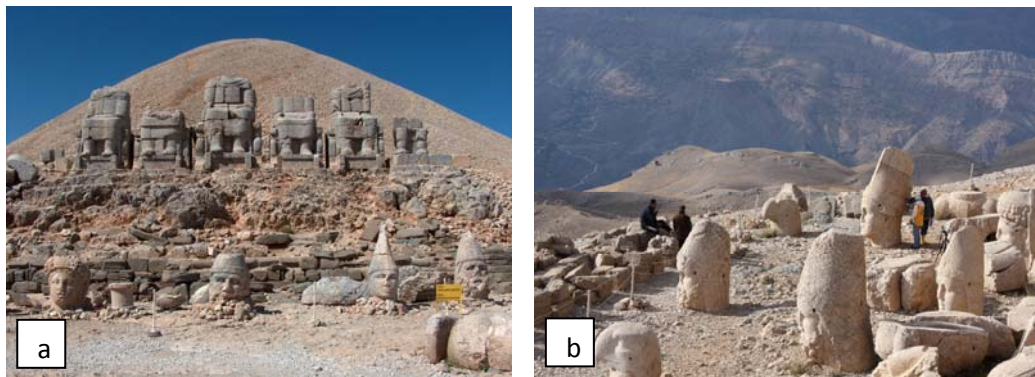
Figure 2. 5 East(a) and West Terrace (b) of Nemrut Mount Monument

Spread over an area of 2.6 hectars (26.000 m^2), the Hierothesion is comprised of a conical tumulus at the center, and three terraces surrounding on the west, east and north sides, together with processional ways leading to the tumulus from the northeast and the southwest. (Şahin Güçhan, 2011)

In the east and west terraces, there are five limestone god statues with their backs turned to the tumulus, flanked by guardian animals, lions and eagles, on each side. In each of these terraces, the gods situated between the guardian animals are, from left to right, Antiochus, Commagene, Zeus, Apollon and Herakles. (Şahin Güçhan, 2011)

Sandstone and limestone samples of Nemrut Mount Monument and the analyses applied on these samples are given in Table 2.3, 2.4, 2.5.

Table 2. 1 Sandstone and Limestone samples of Nemrut Mount Monument. (June, 2010)

SAMPLE NAME	DATE	LOCATION	PHOTOGRAPH	TYPE OF ANALYSIS
10JNWS1	23.06.10	WEST TERRACE SANDSTONE		FDA, TOTAL MICROFLORA (BACTERIA, FUNGI)

Table 2.1 Continued

10JNWS2	23.06.10	WEST TERRACE SANDSTONE		FDA, TOTAL MICROFLORA (BACTERIA, FUNGI)
10JNWS3	23.06.10	WEST TERRACE SANDSTONE		FDA, TOTAL MICROFLORA (BACTERIA, FUNGI)
10JNES1	23.06.10	EAST TERRACE SANDSTONE		FDA, TOTAL MICROFLORA (BACTERIA, FUNGI)
10JNES2	23.06.10	EAST TERRACE SANDSTONE		FDA, TOTAL MICROFLORA (BACTERIA, FUNGI)

Table 2.1 Continued

10JNES3	23.06.10	EAST TERRACE SANDSTONE		FDA, TOTAL MICROFLORA (BACTERIA, FUNGI)
10JNNS1	23.06.10	NORTH TERRACE SANDSTONE		FDA, TOTAL MICROFLORA (BACTERIA, FUNGI)
10JNNS2	23.06.10	NORTH TERRACE SANDSTONE		FDA, TOTAL MICROFLORA (BACTERIA, FUNGI)

Table 2. 2 Sandstone and Limestone samples of Nemrut Mount Monument. (October, 2010 and May, 2011).

SAMPLE NAME	DATE	LOCATION	PHOTOGRAPH	TYPE OF ANALYSES
10ONWS1 11MNWS1 10ONWS2 11MNWS2	08.10.10 27.05.11	WEST TERRACE SANDSTONE		FDA, TOTAL MICROFLORA (BACTERIA, FUNGI), QUALITATIVE ANALYSES OF SOLUBLE SALTS

Table 2.2 Continued

10ONWL1 11MNWL1	08.10.10 27.05.11	WEST TERRACE LIMESTONE		FDA, TOTAL MICROFLORA (BACTERIA, FUNGI), QUALITATIVE ANALYSES OF SOLUBLE SALTS
10ONWL2 11MNWL2	08.10.10 27.05.11	WEST TERRACE LIMESTONE		FDA, TOTAL MICROFLORA (BACTERIA, FUNGI), QUALITATIVE ANALYSES OF SOLUBLE SALTS
10ONES1 11MNES1	07.10.10 27.05.11	EAST TERRACE SANDSTONE		FDA, TOTAL MICROFLORA (BACTERIA, FUNGI), QUALITATIVE ANALYSES OF SOLUBLE SALTS
10ONES2 11MNES2	07.10.10 27.05.11	EAST TERRACE SANDSTONE		FDA, TOTAL MICROFLORA (BACTERIA, FUNGI), QUALITATIVE ANALYSES OF SOLUBLE SALTS

Table 2.2 Continued

10ONEL1 11MNEL1	07.10.10 27.05.11	EAST TERRACE LIMESTONE		FDA, TOTAL MICROFLORA (BACTERIA, FUNGI), QUALITATIVE ANALYSES OF SOLUBLE SALTS
10ONEL2 11MNEL2	07.10.10 27.05.11	EAST TERRACE LIMESTONE		FDA, TOTAL MICROFLORA (BACTERIA, FUNGI), QUALITATIVE ANALYSES OF SOLUBLE SALTS
10ONEL3 11MNEL3	07.10.10 27.05.11	EAST TERRACE LIMESTONE		FDA, TOTAL MICROFLORA (BACTERIA, FUNGI), QUALITATIVE ANALYSES OF SOLUBLE SALTS
10ONNS1 11MNNS1	08.10.10 27.05.11	NORTH TERRACE SANDSTONE		FDA, TOTAL MICROFLORA (BACTERIA, FUNGI), QUALITATIVE ANALYSES OF SOLUBLE SALTS

Table 2.2 Continued

10ONNS2 11MNNS2	08.10.10 27.05.11	NORTH TERRACE SANDSTONE		FDA, TOTAL MICROFLORA (BACTERIA, FUNGI), QUALITATIVE ANALYSES OF SOLUBLE SALTS

Table 2. 3 Sampling from sandstone and limestone for ATP analyses May, 2011).

SAMPLE NAME	DATE	LOCATION	PHOTOGRAPH	TYPE OF ANALYSES
11MNQL$_{BA1}$	26.05.11	QUARRY LIMESTONE		ATP ANALYSES
11MNQL$_{TA1}$	26.05.11	QUARRY LIMESTONE		ATP ANALYSES

Table 2.3 Continued

11MNQL_{BA2}	26.05.11	QUARRY LIMESTONE		ATP ANALYSES
11MNQL_{TA2}	26.05.11	QUARRY LIMESTONE		ATP ANALYSES
11MNES_{TA}	26.05.11	EAST TERRACE SANDSTONE		ATP ANALYSES
11MNES_{BA}	26.05.11	EAST TERRACE SANDSTONE		ATP ANALYSES
11MNEL_{TA2}	26.05.11	EAST TERRACE LIMESTONE		ATP ANALYSES
11MNEL_A	26.05.11	EAST TERRACE LIMESTONE		ATP ANALYSES
11MNWS_A	26.05.11	WEST TERRACE SANDSTONE		ATP ANALYSES

2.2.2. Temple of Augustus

Temple of Augustus ,also known as "Momentum Ancyranum", has great significance in terms of architecture and archaeology. It is located next to the Hacı Bayram Mosque in Ulus which is the center of Ankara in older times. As well as the architectural and archaeological value of it, the Augustus Temple is regarded as a written document due to the inscriptions on it (Caner-Saltık, 2005). The text *'Resgestae Divi Augusti'* praising the Emperor Augustus (30 BC - AD 14) is probably embroidered on the walls of the monument with Greek and Latin letters after his death. Latin inscriptions are located on the inside walls of pronaous while the greek ones are on the outside of the south-east facing wall of naos. The subscriptions are the only written documentation of the world from the time of Emperor Augustus (Caner-Saltık, 2005).

Temple of Augustus, located next to the Hacı Bayram Mosque, is about 12 m. high and 32.5 m wide. Pronaos, side walls of the portion "cella" and additional walls of the Byzantine church are the remnants of the monument today. The remains of the monument are currently below the road level. Columns surrounding the environment of the monument are still visible (Caner-Saltık, 2005)**.**

Marble and andesite samples of Temple of Augustus and the analyses applied on these samples are given in Table 2.6, 2.7.

Table 2. 4 Marble and Andesite samples of Temple of Augustus (July, 2011).

SAMPLE NAME	DATE	LOCATION	PHOTOGRAPH	TYPE OF ANALYSES
11JAEEM$_i$	27.07.11	EXTERIOR OF EAST WALL MARBLE		FDA, TOTAL MICROFLORA (BACTERIA, FUNGI), QUALITATIVE ANALYSES OF SOLUBLE SALTS BIOCHEMICAL TESTS
11JAEIA$_e$	27.07.11	INTERIOR OF EAST WALL BOTTOM PART ANDESITE		FDA, TOTAL MICROFLORA (BACTERIA, FUNGI), QUALITATIVE ANALYSES OF SOLUBLE SALTS BIOCHEMICAL TESTS
11JAEEM$_{SSc}$	27.07.11	EXTERIOR OF EAST WALL MARBLE		FDA, TOTAL MICROFLORA (BACTERIA, FUNGI), QUALITATIVE ANALYSES OF SOLUBLE SALTS BIOCHEMICAL TESTS

Table 2.4 Continued

				FDA, TOTAL MICROFLORA (BACTERIA, FUNGI), QUALITATIVE ANALYSES OF SOLUBLE SALTS, BIOCHEMICAL TESTS
11JAWIM1 d	27.07.11	INTERIOR OF WEST WALL MARBLE		FDA, TOTAL MICROFLORA (BACTERIA, FUNGI), QUALITATIVE ANALYSES OF SOLUBLE SALTS, BIOCHEMICAL TESTS
11JAWIM2 b	27.07.11	INTERIOR OF WEST WALL MARBLE		FDA, TOTAL MICROFLORA (BACTERIA, FUNGI), QUALITATIVE ANALYSES OF SOLUBLE SALTS BIOCHEMICAL TESTS
11JAWIM3 k	27.07.11	INTERIOR OF WEST WALL MARBLE		FDA, TOTAL MICROFLORA (BACTERIA, FUNGI), QUALITATIVE ANALYSES OF SOLUBLE SALTS BIOCHEMICAL TESTS

Table 2. 5 Sampling from Andesite and Marbles for ATP analyses (July, 2011)

SAMPLE NAME	DATE	LOCATION	PHOTOGRAPH	TYPE OF ANALYSES
11JAEEM$_{A1}$	27.07.11	EXTERIOR OF EAST WALL MARBLE		ATP ANALYSES
11JAEIA$_A$	27.07.11	INTERIOR OF EAST WALL ANDESITE		ATP ANALYSES
11JAEEM$_{A2}$	27.07.11	EXTERIOR OF EAST WALL MARBLE		ATP ANALYSES

Deterioration zones on the marble surfaces of Temple of Augustus are shown in Table 2.2, 2.3, 2.4, 2.5, 2.6, 2.7.
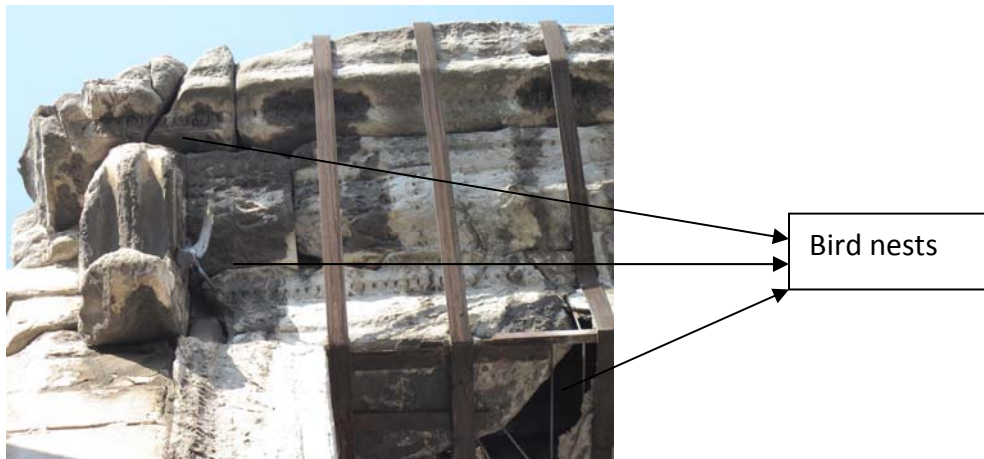
Figure 2. 6 Deterioration zones of marble (Temple of Augustus, 2011).
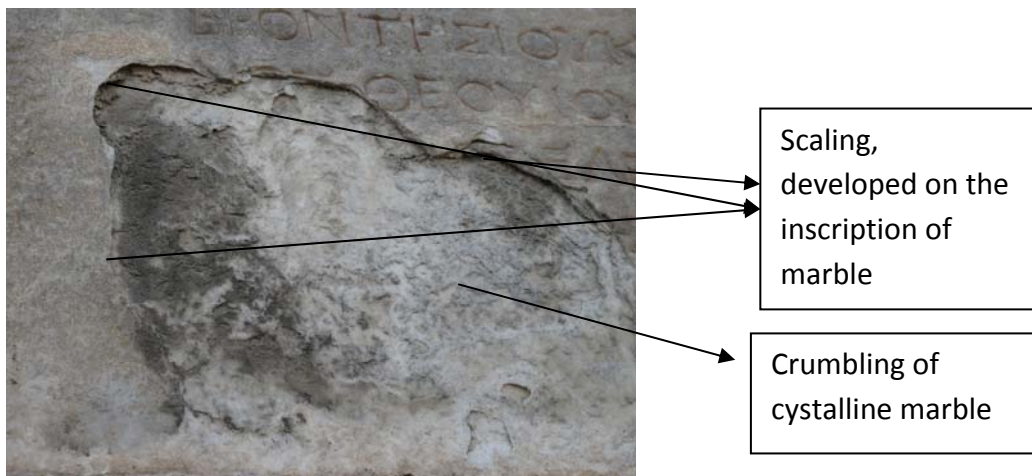
Figure 2. 7 Deterioration zones of marble (Temple of Augustus, 2011).

Blistering of
marble surface
caused by
expansion of
the weathered
surface.
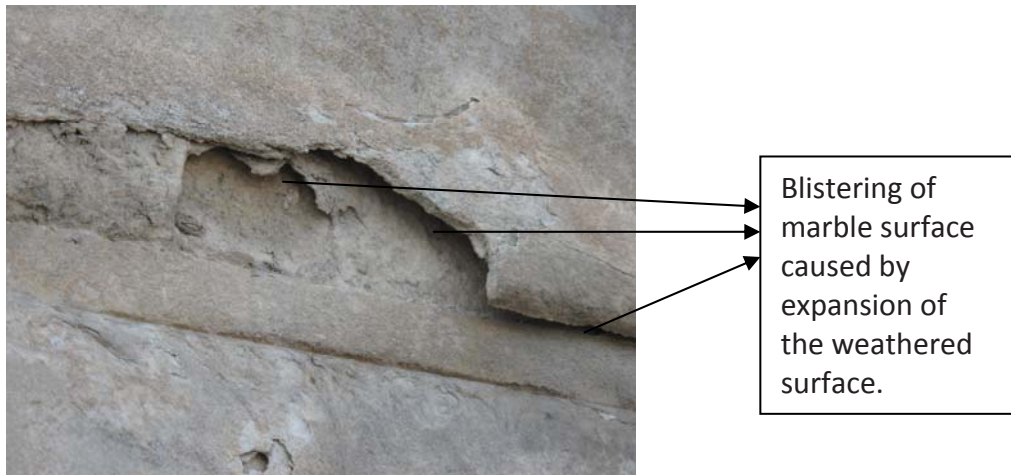
Figure 2. 8 Deterioration zones of marble (Temple of Augustus, 2011).

Crumbling of
cystalline
marble
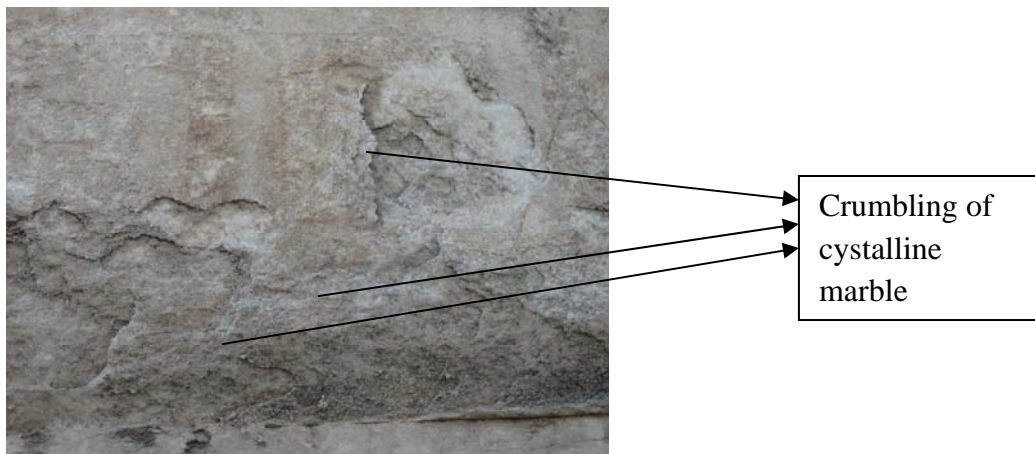
Figure 2. 9 Deterioration zones of marble (Temple of Augustus, 2011).

Contour scaling developed as thin detachments on the wall of the temple
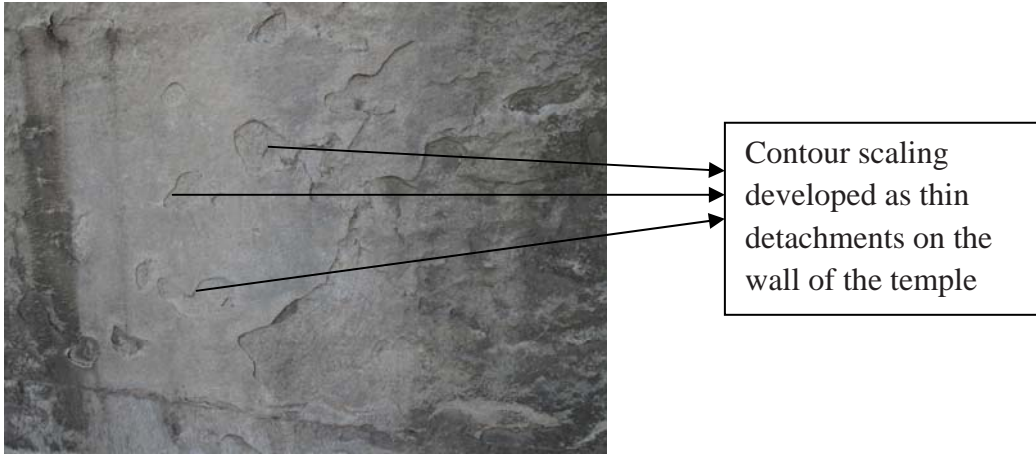
Figure 2. 10 Deterioration zones of marble (Temple of Augustus, 2011).

Pigeon hole in the cavities of marble
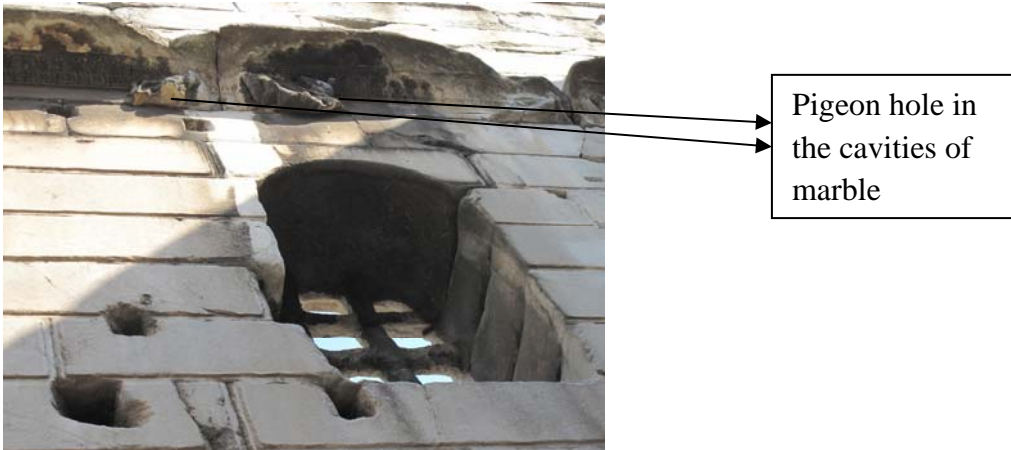
Figure 2. 11 Deterioration zones of marble (Temple of Augustus, 2011).

2.3.   Determination of Biological Activity

Biological activity samples were examined by using FDA, MFT and ATP analyses.

Sampling Technique

Samples up to one cm size were collected without damaging the monuments by using disposable gloves; equipments such as chisel, spatula and pens were washed with %70 alcohol.

Stone samples were crushed to a fine powder in a sterile agate mortar and stored in sterile caps at +5°C until the day of the experiment.

2.3.1.   Measurement of Total Microbial activity using fluorescein diacetate (FDA) in powdered stone samples

Quantitative analyses of biological activity were done by using fluorescence techniques in powdered samples of Nemrut Mount monuments and Temple of Augustus. The FDA analysis in spectrophotometric measurements relies on the direct proportion of biological activity and enzymatic activity with the fluorescence formed:
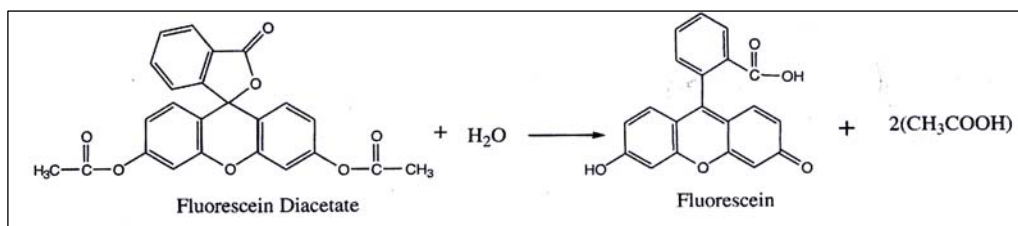
Figure 2. 12 The convertion of fluorescein diacetate to fluorescein (Green *et al.*, 2005)

The solutions used were buffer solution, FDA stock solution and fluorescein stock solution (see Appendix A). The FDA method applied to the samples were adapted from the studies of Adam and Duncon (2001).

2.3.1.1. *Chemicals*

Table 2. 6 Chemicals used in the FDA analyses

Chemical	Producer
Fluorescein Diacetate	Sigma-Aldrich, Germany
Fluorescein Sodium Salt	Sigma-Aldrich, Germany
K_2HPO_4	Merck, Germany
KH_2PO_4	Merck, Germany
Acetone	Sigma-Aldrich, Germany

2.3.1.2. Scientific Equipment

Table 2. 7 Instruments used in the FDA analyses

Instrument	Model	Producer
UV/VIS Spectrophotometer	SP-3000 plus	OPTIMA INC., Japan
Oven (30 °C)	OV-18SC	Blue M Electric Company, USA
Filter paper	No:1440 125	Whatman ^{TM}, UK
Centrifuge	Rotofix 32A	Hettich Zentrifugen, Germany
Quartz cells		Optima, Japan
pH –indicator strips	1.09535.0001	Merck, Germany

2.3.1.3. The FDA method application to powdered stone samples

FDA method was done in order to determine the total microbial activity of bacteria and fungi in powdered limestone (Nemrut Mount Monument) and marble samples (Temple of Augustus).

Samples prepared for 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 throughly and placed in an oven at 30°C for 30 minutes. After removing the samples from the oven, the

powdered samples were centrifuged at 4000 rpm for 3 minutes. The supernatant was filtered with Whatman No 40 filter paper and the the filtrate was measured at 490 nm in a spectrophotometer (Optima SP-3000 Plus UV/VIS spectrophotometer).

2.3.2. Total Microflora Method

In this study, nutrient agar medium, czapek dox agar medium and malt extract agar medium were used for determining bacterial and fungal numbers. In the course of this experiment, serial dilutions were made by sterile distilled water and inoculated to triplicate plates from each dilution until the bacteria reached optimum concentration in order to count accurately. After all, final plates in the series of medium should have been between 30 and 300 colonies. Less than 30 colonies are not acceptable for statistical reasons, and more than 300 colonies on a plate are likely to produce colonies too close to each other to be distinguished as distinct colony-forming unit is (CFUs).

2.3.2.1. Scientific Equipment

Table 2. 8 Instruments used in the Total Microflora Method

Instrument	Model	Producer
Autoclave	CL-32L	ALP Co.Ltd, Japan

Table 2.8 Continued

Microbiological Safety Cabinet	KS9	Thermo SCIENTIFIC, Germany
Incubator	BK800	Thermo SCIENTIFIC, Germany
Pipette tips	Yellow,Blue	LP ITALIANA SPA, Italy
Mikro Pipette	SL 200, SL 1000, SL 5000	RAININ, USA
Glass bottles	50 ml, 500 ml	SIMAX, UK
Magnetic stirrer	MR Hei-Standard	Heidolph Instruments, Germany
Vortex	Reax top	Heidolph Instruments, Germany.
Weighing scale	GP3202	Sartorius, Germany

2.3.2.2.   Procedure

Experiment was started with preparing the 10^{-1} dilution by adding a certain gram (usually 1 g) of powdered stone to a 9 ml sterile distilled water and continued by making dilution series in the order of 10^{-2} to 10^{-6} through transferring 100 μl solution to 900 μl sterile distilled water. Afterwards, triplicate mediums were inoculated with each dilution in an amount of 100 μl and this process was applied for each type of medium named as nutrient agar (Bacteria), malt extract agar (all fungi types) and czapek dox agar (soil fungi) (Fig. 2.10). The preparation of media used in this method was explained in Appendix B.
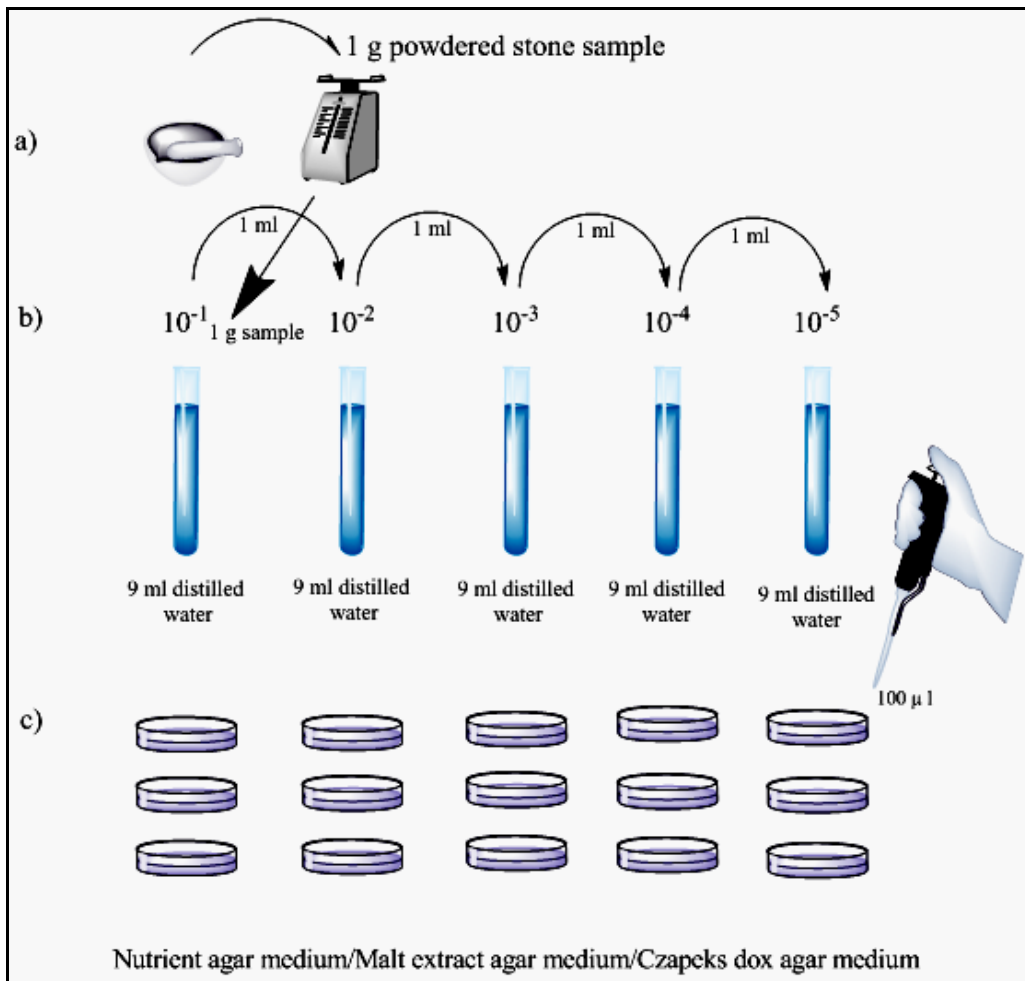
Figure 2. 13 Total microflora schematic demonstration

Nutrient agar medium and czapek dox agar medium were incubated at 30 °C for 1 week and malt extract agar medium was incubated at 24 °C for 4 days. After the incubation period, petri dishes having colonies between 30 and 300 were counted and the colony forming unit (CFU) in 1 g of the original sample calculated as follows:

N = (C/3) × D × V

N: CFU per gram or ml

C: Total colony count of three petri dishes

D: Dilution Factor

V: Transfered volume (mL)

2.3.3. Measurement of the Global Microbial Activity with Adenosin Triphosphate (ATP)

ATP (adenosine 5'-triphosphate) is a compund existing in all living cells as a viability marker and their determination in stones gives an idea about global microbial activity. ATP is tracked specifically by reaction with a Luciferin/Luciferase mixture in buffered solution.
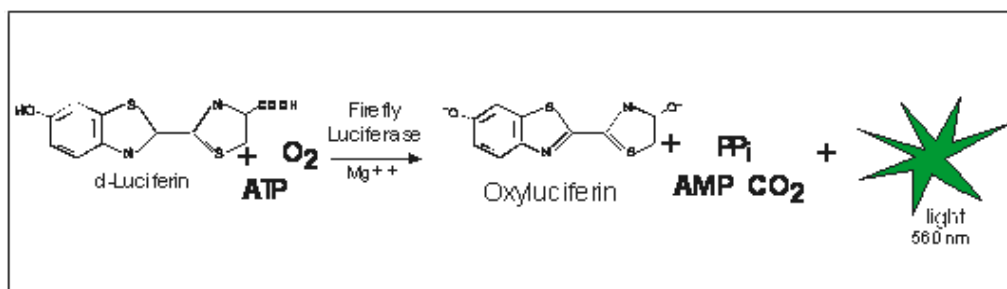
Figure 2. 14 ATP molecule luminescence reaction by firefly luciferase enzyme (Held, 2004).

The ATP proportion is based on the evaluation of the quantity of emitted light and the intensity of emitted light is proportional to the concentration of ATP because any ATP present reacts with the enzyme and produces light. In this method, the amount of light emitted was measured by the HY-LiTE® 2 luminometer where the values are between 0-99,999 as RLU (Relative Light Unit is) (Lundin *et al*., 1986)

2.3.3.1. Scientific Equipment

Table 2. 9 Instruments used in the Bioluminescence Method

Instrument	Model	Producer
HY-Lite	Plus 2 luminometer	Merck, Germany
Sterile cotton swab	0051	Merck, Germany

Table 2.9 Continued

Kit (sampling pen)	M130101	Merck, Germany
Mini cooler		Rubbermaid, USA

2.3.3.2.   *Procedure*

Surface samples were taken with cotton swabs from certain areas of the historical monument by making small movements paralel to each other. Following this step, cotton swabs were submerged into the buffer solution part of the kit, then the cap of the bottom part was removed and the long and thin pen like part was pushed through the solution containing luciferin/luciferase enzyme. Finally, pen was put inside the device and measured in a short time.

Figure 2. 15 Hy-Lite Device (a), enzyme kit (b)

2.4. Qualitative analyses of soluble salts by spot tests

Water soluble salts as nitrites, nitrates and sulfates may be present in materials of buildings and monuments due to the activity of some organisms such as nitrosomonas, nitrobacter and thiobacillus. Therefore spot tests were made to detect their existence as a first step ,and then based on positive test results biochemical tests were conducted in order to detect the origin of the salt formations

2.4.1. Chemicals

Table 2. 10 Chemicals used in the Qualitative Analyses of Soluble Salts by Spot Tests

Chemical	Producer
Barium Chloride solution ((BaCl$_2$ 10%)	Riedel, Germany
Hydrochloric acid solution (HC1 2N)	Sigma-Aldrich, Germany
Acetic acid	Sigma-Aldrich, Germany
Sulfanilic acid	Sigma-Aldrich, Germany
α-naphthylamine solution	Sigma-Aldrich, Germany
Zinc dust	

2.4.2. Scientific Equipment

Table 2. 11 Instruments used in the Qualitative Analyses of Soluble Salts by Spot Tests

Instrument	Model	Producer
Glass tubes		
Porcelain spot plate		
Transferring pippettes		
Weighing scale	GP3202	Sartorius, Germany

2.4.3. Spot test for sulphate SO_4^{-2}

In the beginning of this test few drops of test solution were put in a tube. Then 1-2 drops dilute hydrochloric acid solution (HCl 2N) and 1-2 drops of barium chloride solution (BaCl$_2$ 10%) were added respectively. Finally, white precipitate, indicator of sulphate ions, were investigated in those samples

$$SO_4^{-2} + BaCl_2 \implies BaSO_4 + 2Cl^-$$
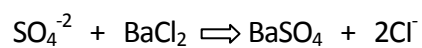
2.4.4. Spot test for nitrite NO_2^-

For detection of nitrite ions, few drops of test solution were put on a spot plate and 1 drop of sulfanilic acid solution and α naphthylamine solution were added respectively. If nitrite ions are present, _solution turns to pink._

2.4.5. Spot test for nitrate NO_3^-

In this test few drops of test solution were put on a spot plate as first stage. Then one drop of acetic acid solution (2N), sulfanilic acid, α naphthylamine solution and a few mg of zinc dust were added in turn. If nitrate ions are present, _solution turns to pink._

2.5. Most Probable Number (MPN) Method and Biochemical Tests

In this section, the detection of nitrifying and sulphur oxidising bacteria were enabled by the Most probable number method together with biochemical tests

2.5.1. Nitrification

The activity of nitrous and nitric fermenters were investigated separately in two different type of medium.

For identifying nitrous fermenters, nitrogen source was provided as ammonium sulphate and after incubation period, nitrit and nitrat existence were examined by the chemical compound of diphenylamine in sulphuric acid. On the other hand the investigation for the formation of nitrate were done with the same reactive substance after the elimination of nitrites by urea with using sodium nitrite in medium as a source of nitrogen.

2.5.1.1.   Chemicals

Table 2. 12 Chemicals used in the nitrification section

Chemical	Producer
Ammonium sulfate ($(NH_4)_2SO_4$)	Merck, Germany
Calcium carbonate ($CaCO_3$)	Pro analysi, Germany
Sodium nitrite ($NaNO_2$)	BDH, UK
Diphenylamine ($C_{12}H_{11}N$)	Riedel, Germany
Sulfiric Acid ($2H_3O^+ + SO_4^{2-}$)	Sigma-Aldrich, Germany
Urea	Merck, Germany
Dipotassium phosphate (K_2HPO_4)	Merck, Germany
Magnesium sulfate ($MgSO_4$)	Pro analysi, Germany
NaCl	Saray, Turkey
Ferric (III) sulfate ($Fe_2(SO_4)_3$)	Merck, Germany
$MnSO_4$	Pro analysi, Germany

2.5.1.2. Scientific Equipment

Table 2. 13 Instruments used in the nitrification section

Instrument	Model	Producer
Autoclave	CL-32L	ALP Co.Ltd, Japan
Microbiological Safety Cabinet	KS9	Thermo SCIENTIFIC, Germany
Incubator	BK800	Thermo SCIENTIFIC, Germany
Pipettus		Hirschmann Laborgerate, Germany
Vortex	Reax top	Heidolph, Germany

2.5.1.3. Inoculation

Same soil suspension-dilutions prepared in total microflora count experiment were used for the inoculation of media in an amount of 0,5 ml for each tube from the dilution series of 10^{-1} to 10^{-6} as triplicates and then placed in an incubator at 28 °C for 20 days.

The preparation of media used for both sets of nitrification was explained in Appendix C.

2.5.1.4.   Control of the results

A) For nitrous fermenters;

The presence of nitrite and nitrate formations were investigated by using diphenylamine ($C_{12}H_{11}N$) sulphuric acid solution prepared as follows;

Diphenylamine (10g) + Sulphuric acid (1000ml) + Distilled water (200ml)

After the incubation period, 10 drops of sulphuric acid and the reactive were added to each tube. Depending on the presence of nitrites and nitrates, blue colour formation were observed in some tubes and for these tubes results were accepted as positive.

B) For nitric fermenters;

The existence of nitrate formations were identified after approximately 50 mg urea addition to each tube just after incubation period to deplete nitrite formations. Then 10 drops of sulphuric acid and diphenylamine reactive were added in turn. The tubes that have blue color formations due to nitrate presence were considered as positive result.

2.5.1.5.   Interpretation

The positive tube results in each dilution of nitrous and nitric series were counted and then the number of bacteria/g of powdered stone were calculated by using Mc Grady table

Table 2. 14 Mc Grady table (Pochon and Tardieux, 1962).

3 tubes per dilution					
Characteristic number	Number of cells	Characteristic number	Number of cells	Characteristic number	Number of cells
0 0 0	0,0	2 0 1	1,4	3 0 2	6,5
0 0 1	0,3	2 0 2	2,0	3 1 0	4,5
0 1 0	0,3	2 1 0	1,5	3 1 1	7,5
0 1 1	0,6	2 1 1	2,0	3 1 2	11,5
0 2 0	0,6	2 1 2	3,0	3 1 3	16,0
1 0 0	0,4	2 2 0	2,0	3 2 0	9,5
1 0 1	0,7	2 2 1	3,0	3 2 1	15,0
1 0 2	1,1	2 2 2	3,5	3 2 2	20,0
1 1 0	0,7	2 2 3	4,0	3 2 3	30,0
1 1 1	1,1	2 3 0	3,0	3 3 0	25,0
1 2 0	1,1	2 3 1	3,5	3 3 1	45,0
1 2 1	1,5	2 3 2	4,0	3 3 2	110,0
1 3 0	1,6	3 0 0	2,5	3 3 3	110,0
2 0 0	0,9	3 0 1	4,0		

2.5.2. The oxidation of sulphur

The liquid mineral media that is inoculated with soil suspension dilutions have hidrojen sulphur rich atmosphere in which sulphur only exists as element form.

2.5.2.1. Chemicals

Table 2. 15 Chemicals used in the sulphur oxydation section

Chemical	Producer
Dipotassium phosphate (K_2HPO_4)	Merck, Germany
Magnesium chloride ($MgCl_2$)	BDH, UK
Sodium chloride (NaCl)	Saray, Turkey
Ammonium nitrate (NH_4NO_3)	Merck, Germany
Sodium sulfide (Na_2S)	Merck, Germany
Hydrochloric acid (HCl^-)	Sigma-Aldrich, Germany
Baryum chloride ($BaCl_2$)	Riedel, Germany
Sublimed sulphur	Merck, Germany
Magnesium sulfate (MgSO4)	Pro analysi, Germany
NaCl	Saray, Turkey
Ferric (III) sulfate (Fe2(SO4)3)	Merck, Germany
MnSO4	Merck, Germany

2.5.2.2. Inoculation

Same soil suspension-dilutions prepared in total microflora count experiment were used for the inoculation of media in an amount of 1 ml for each tube from the dilution series of 10^{-1} to 10^{-4} as triplicates and 50 mg sublimed sulphur was added to each tube aseptically.Then all these tubes were placed in an incubator at 28 °C for 20 days.

The preparation of media used for oxidation of suphur was explained in Appendix C.

2.3.2.4. Control of the results

After 3 week of incubation period, 1 or 2 ml of sample were taken from the top brilliant part of the solution without shaking and transferred to empty tube and this process was applied to all tubes. Following this step, 2 drops of hydrochloric acid (HCl^-) solution and 5 drops of aqueous barium chloride solution were added in turn. The presence of sulfates were determined by observing white precipitate formations in comparison with controls (not inoculated medium).

2.5.2.3. Interpretation

For each dilution, positive tube results were counted and then the number of sulphur oxidising bacteria/g of powdered stone were calculated by using Mc Grady table (Table 2.8).

2.6. Identification of Fungi Species

Identifications were done in order to have a thorough knowledge of distribution and type fungi species. Moreover, identification is crucial to determine and list detrimental species that live in and on stone.

2.6.1. Chemicals

Table 2. 16 Chemicals used in the identification of fungi

Chemical	Producer
Lactophenol blue	Merck, Germany

2.6.2. Identification of fungi

A drop of lactophenol blue were instilled on slide, then the sample of isolated fungi colony were taken by scotch tape. Following this step, scotch tape was put on to the lactophenol drop and covered with slip, then observed under optical microscope, generally at 400X and 600X magnification.

CHAPTER 3

RESULTS

Nemrut limestones and sandstones, Temple of Augustus marbles and andesites were studied for determining their biological activity degree in order to better understand their contribution to decay process of stones by using different methods such as; FDA hydrolysis, Total Microflora, ATP (Adenosine triphosphate) bioluminescence and MPN (Most Probable Number) method. Bacteria and fungi identifications were also done for determining occurence of detrimental species. At the same time, qualitative analyses of soluble salts were carried out by spot tests in order to determine the necessity of applying MPN method.

3.1. Determination of Biological Activity

3.1.1 FDA Results in Spectrophotometric Measurements

Spectrophotometric measurements were carried out on sandstones and limestones of Nemrut Mount Monuments and marbles of Temple of Augustus.

Biological activity, in other words, enzymatic activity of the samples was stated as μg of fluorescein per gram of powdered sample after calculating the concentration in 1 ml by using standard curve and also taking into account weight of the samples.

Samples taken in June of 2010 showed that sandstones of east and west terrace, and limestones of west terrace, had almost the same and low enzymatic activity. The limestone sample taken from east terrace had the highest biological activity (15.9 μg fluorescein/g) in comparison to other stone types. Moreover sandstone without visible biological covering showed lowest enzymatic activity(2.52 μg fluorescein/g) (Fig. 3.1).

	10JNWS	10JNNS1	10JNES1	10JNEL	10JNWL
■ Absorbance (490 nm)	0.175	0.061	0.208	0.385	0.149
■ Concentration (ppm)	0.88	0.31	1.04	1.93	0.75
□ Enzymatic activity (μg fluorescein/g of stone)	7.24	2.52	8.60	15.92	6.16
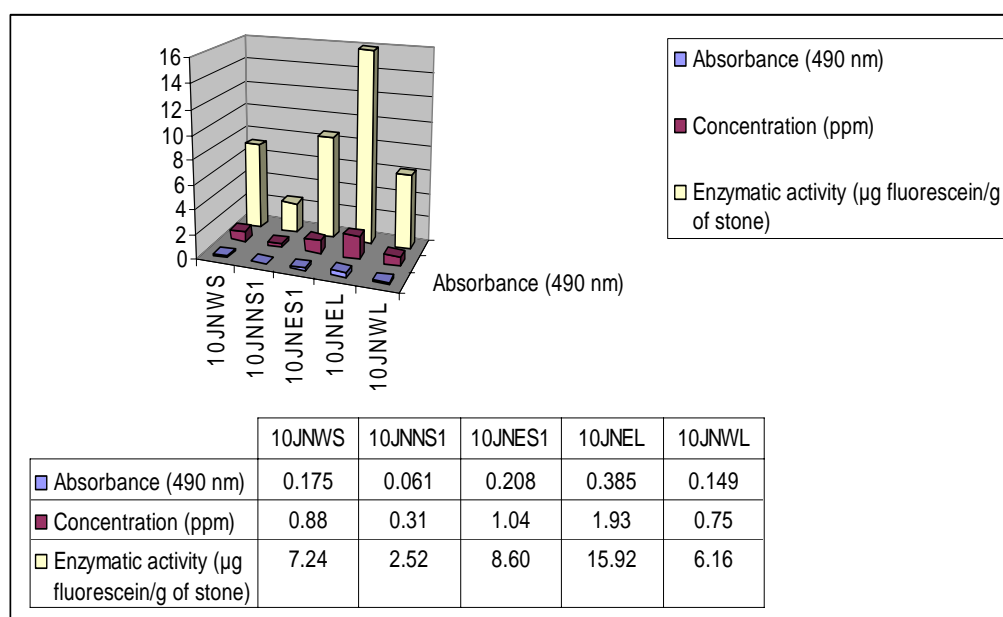
Figure 3. 1 Diagram of the FDA analyses experiment results, applied on stones of Nemrut Mount Monument in June of 2010.

Limestones and sandstones taken from east terrace in October of 2010, had highest biological activity (20.55, 18.05 µg fluorescein/g) among other stones of west and north terraces. On the other hand, sandstones of west and north terrace, and limestones of west terrace showed slightly same and low enzymatic activity (6.49 µg fluorescein/g) (Fig. 3.2).

	10ONES	10ONWS	10ONNS	10ONEL	10ONWL
■ Absorbance (490 nm)	0.4365	0.107	0.1605	0.497	0.157
■ Concentration µg/ml	2.19	0.54	0.80	2.49	0.79
☐ Enzymatic activity (µg fluorescein/g of stone)	18.051	6.27	6.64	20.55	6.49
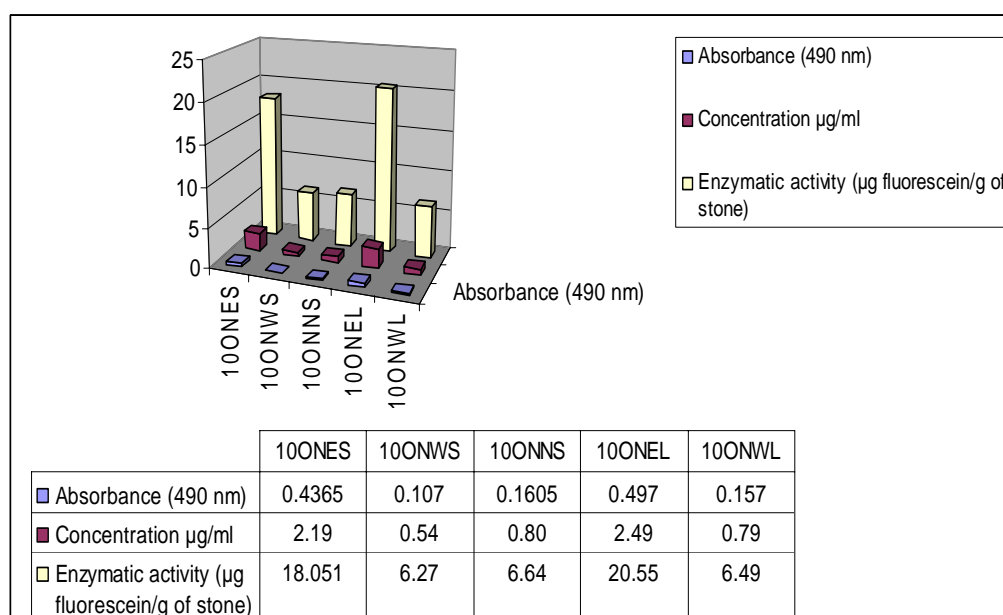
Figure 3. 2 Diagram of the FDA analyses experiment results, applied on stones of Nemrut Mount Monument in October of 2010.

Samples collected during field studies in May of 2011 showed that east and north terrace sandstones had high biological activity level (50.02, 11.35 µg fluorescein/g). Although limestones of east terrace had higher biological activity (9.32 µg fluorescein/g) than sandstones (6.06 µg fluorescein/g) and limestones (4.34 µg fluorescein/g) of west terrace, these aforementioned stones of west terrace almost had the same enzymatic activity as well (Fig. 3.3).

	11MNES	11MNNS	11MNEL	11MNWS	11MNWL
☐ Absorbance (490 nm)	1.210	0.275	0.214	0.147	0.105
☐ Concentration µg/ml	6.06	1.38	1.07	0.73	0.53
☐ Enzymatic activity (µg fluorescein/g of stone)	50.02	11.35	9.32	6.06	4.34
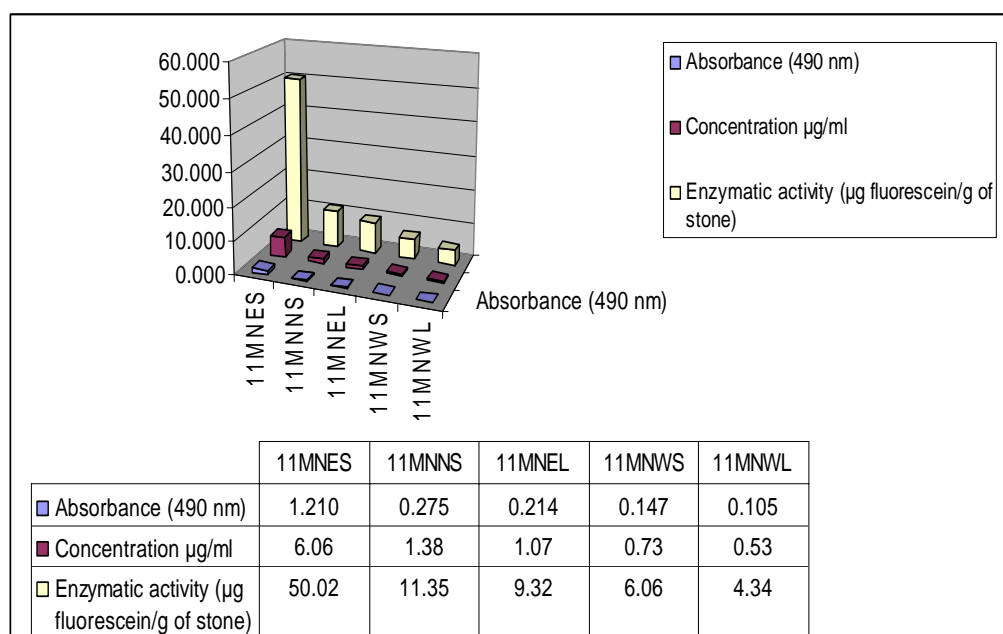
Figure 3. 3 Diagram of the FDA analyses experiment results, applied on stones of Nemrut Mount Monument in May of 2011.

When sandstones and limestones of west terrace were compared among themselves regarding enzymatic activity value, the concentration of fluorescein detected were, nearly in the same amounts in all samples (Fig 3.4).

	10JNWS	10JNWL	10ONWS	10ONWL	11MNWS	11MNWL
■ Enzymatic activity (µg fluorescein/g of stone)	7.24	6.16	6.27	6.49	6.06	4.34
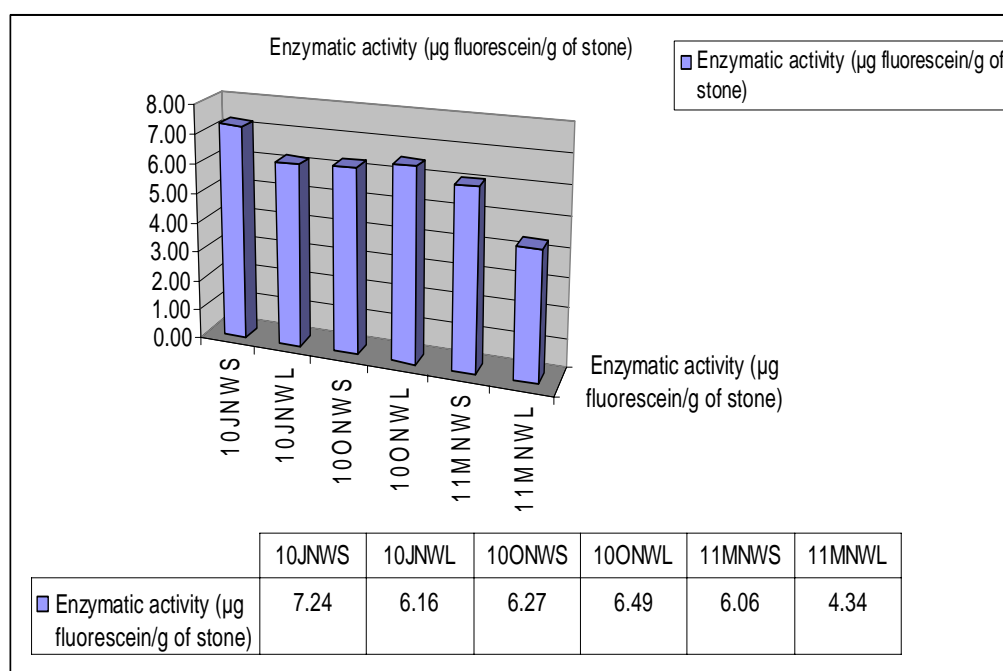
Figure 3. 4 Comparison of the FDA test results applied on stones of Nemrut Mount Monument in three seasons, such as June, October and May.

For samples belonging to Temple of Augustus, the hydrolysis of FDA was found low (1.19 µg fluorescein/g) in the sample, collected from the interior part of the wall facing towards the west side in comparison with discolored marble, taken from the exterior part of the wall facing towards the east side . Moreover, andesite sample, taken from the bottom part of the interior of east wall, had the highest enzymatic activity (23.35 µg fluorescein/g) (Fig. 3.5).

	11JAWIM1	11JAEIA	11JAEEMSS
▦ Absorbance (490 nm)	0.029	0.564	0.175
▦ Concentration (ppm)	0.15	2.83	0.88
▢ Enzymatic activity µg fluorescein/g of stone	1.19	23.35	7.24
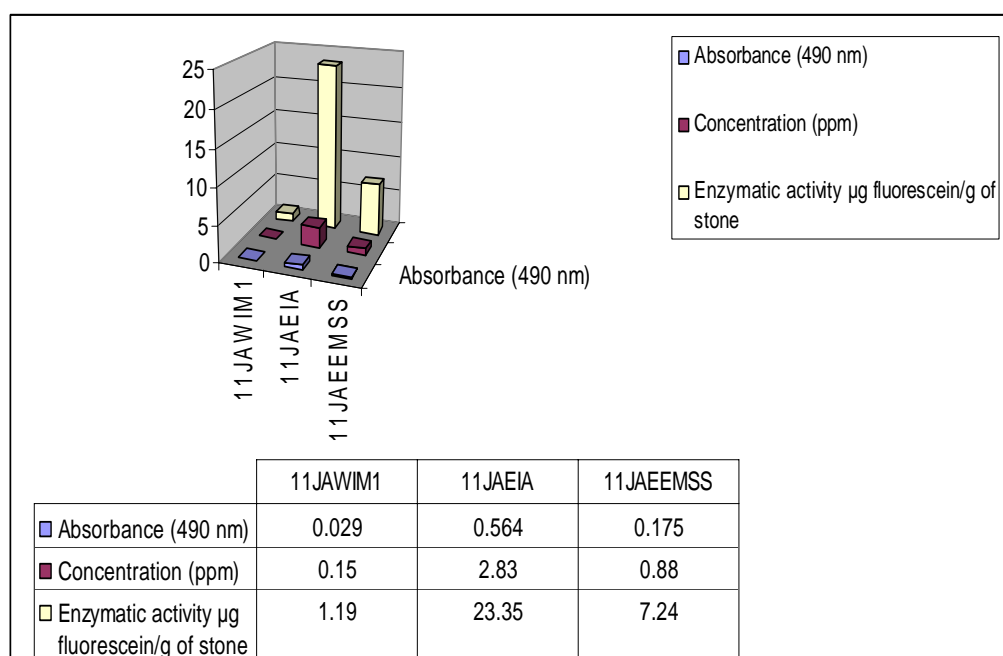
Figure 3. 5 Diagram of the FDA analyses experiment results, applied on andesite and marbles of Temple of Augustus in July of 2011.

3.1.2   Results of Total Microflora Method

For samples taken in June of 2010, the sandstones of the west terrace had the highest number of bacteria. The powder sample taken from the crack of limestone of the west terrace, and sandstones of north and east terrace almost had the same and low bacterial activity. On the other hand, for all of the stone samples, mold spores showed very low and almost the same values with each other(Table 3.1).

Table 3. 1 The determination of aerobic heterotrophic bacteria and fungi as colony forming unit per g of stone by total microflora method. (Samples of Nemrut Mount Monuments, June, 2010)

Sample code	Nutrient Agar Medium (CFU/g of stone)	Czapek Dox Agar Medium (CFU/g of stone)
**10JNES	1.6×10^5	7.6×10^3
**10JNWS	2.6×10^6	3×10^2
*10JNNS	4.9×10^5	5×10^2
10JNWLc	4.4×10^2	1×10^2
*The surfaces of the samples have slightly visible biological covering.		
**The surfaces of the samples have visible biological covering.		

Following the steps of inoculation and incubation period, of samples taken in October of 2010, the counting of microorganisms were carried out precisely. After all steps of the experiment were completed, stone samples showed very low number of bacteria and mold spores (Table 3.2).

Table 3. 2 The determination of aerobic heterotrophic bacteria and fungi as colony forming unit per g of stone by total microflora method. (Samples of Nemrut Mount Monuments, October, 2010)

Sample code	Nutrient Agar Medium (CFU/g of stone)	Malt Extract Agar Medium (CFU/g of stone)
*10ONES	3.5×10^4	2×10^2
*10ONWS	2.5×10^4	0
*10ONNS	4.1×10^4	0
*10ONEL	4.8×10^4	3×10^1
*10ONWL	0.8×10^4	9×10^2
*The surfaces of the samples have slightly visible biological covering.		

For the samples collected during field studies in May of 2011, sandstones of west and north terrace gave the highest numbers in bacterial counts, whereas limestones of east and west terrace , and sanstones of east of terrace gave the lowest. The number of viable spores was found low being in the range of 0.45×10^4 - 0.15×10^4 CFU/g of Stone (Table 3.3)

Table 3. 3 The determination of aerobic heterotrophic bacteria and fungi as colony forming unit per g of stone by total microflora method. (Samples of Nemrut Mount Monuments, May, 2011)

Sample code	Nutrient Agar Medium (CFU/g of stone)	Malt Extract Agar Medium (CFU/g of Stone)
**11MNNS	7.6×10^5	4.5×10^3
**11MNWS	1.03×10^6	1.8×10^3
*11MNES	1.1×10^5	2.3×10^3
*11MNEL	8.02×10^4	1.5×10^3
*11MNWL	6.9×10^4	1.5×10^3
*The surfaces of the samples have slightly visible biological covering. **The surfaces of the samples have visible biological covering.		

For the samples collected from the Temple of Augustus in July, CFU counting showed that bacterial biomass was found in the ranges of 10^3-10^7 CFU/g, whereas fungal biomass was detected in the ranges of 10^3-10^5 CFU/g. The highest cell numbers of bacteria and fungi were observed in the samples of andesite. In addition, marble taken from the exterior of east wall also showed high bacterial and fungal activity. Marble of east wall, having black discolorations on it is surface, exhibited intermediate level of microbial activity in comparison to marble of west wall (Table 3.4).

Table 3. 4 The determination of aerobic heterotrophic bacteria and fungi as colony forming unit per g of stone by total microflora method. (Samples of Temple of Augustus, May, 2011)

Sample code	Nutrient Agar Medium (CFU/g of stone)	Malt Extract Agar Medium (CFU/g of Stone)
11JAEEM	1.3×10^7	4.8×10^4
11JAEIA	3.2×10^7	4.5×10^5
11JAEEM$_{SS}$	2.1×10^6	1.83×10^4
11JAWIM1	9.3×10^3	6.3×10^3
11JAWIM2	3.9×10^3	2.3×10^3
11JAWIM3	7.4×10^4	9.5×10^3

3.1.3 Results of ATP analyses

After sampling from upper and bottom part of lichens, growing on sandstones and limestones, with cotton swabs, global metabolic activity level was found very low being in the ranges of 5-26 RLU (Table 3.6).

Table 3. 5 The determination of global metabolic activity as relative light unit per cm^2 of stone surface by ATP bioluminescence method. (Nemrut Mount Monuments, July, 2011)

Sample code	Surface area (cm^2)	ATP content (RLU)	ATP activity (RLU/cm^2)
11MNQL$_{BA1}$	3.0×0.5	26	17.3
11MNQL$_{TA1}$	2.5×0.5	7	5.6
11MNQL$_{BA2}$	2.0×0.5	6	6
11MNQL$_{TA2}$	2.0×0.5	16	16
11MNES$_{TA1}$	2.5×0.5	19	15.2
11MNES$_{TA2}$	2.5×0.5	9	7.2
11MNES$_{BA}$	2.5×0.5	5	4
11MNEL$_A$	1×1	25	25
11MNWS$_A$	1×1	19	19

According to the ATP measurements conducted on the surfaces of marble and andesite, global metabolic activity level was found very low being in the range of 18-25 RLU in Temple of Augustus (Table 3.5).

Table 3. 6 The determination of global metabolic activity as relative light unit per cm^2 of stone surface by ATP bioluminescence method. (Temple of Augustus, July, 2011)

Sample code	Surface area (cm$^{2)}$	ATP content (RLU)	ATP activity (RLU/cm^2)
11JAEEM$_{A1}$	1×1	21	21
11JAEEM$_{A2}$	1×1	18	18
11JAEIA$_A$	1×1	25	25

3.2   Results of Qualitative Analyses of Soluble Salts by Spot Tests

All stone samples of Nemrut Mount Monuments were salt free as seen in Table 3.7.

Table 3. 7 Soluble salt results of spot test analysis (Samples of Nemrut Mount Monuments, October, 2010)

Sample code	SO_4^{2-}	NO_2^-	NO_3^-
10ONES	(-)	(-)	(-)
10ONWS	(-)	(-)	(-)
10ONNS	(-)	(-)	(-)
10ONEL	(-)	(-)	(-)
10ONWL	(-)	(-)	(-)

All marble and andesite samples had sulphate and nitrate ion as the anion, whereas nitrite was detected only in one sample, belonging to marble of west wall given as in Table 3.8.

Table 3. 8 Soluble salt results of spot test analysis (Samples of Temple of Augustus, October, 2010)

Sample code	SO_4^{2-}	NO_2^-	NO_3^-
11JAEEM$_{SS}$	(+) (+)	(-)	(+) (+)
11JAEEM	(+) (+)	(-)	(+)
11JAEIA	(+)	(-)	(+)
11JAWIM1	(+) (+)	(-)	(+) (+) (+)
11JAWIM2	(+) (+)	(+) (+)	(+) (+) (+) (+)
11JAWIM3	(+) (+)	(-)	(+) (+)

3.3   Results of Biochemical Tests

Among the marble and andesite samples collected from the Temple of Augustus during the field studies in July of 2011, marble taken from the interior of west wall gave the highest numbers in nitrosifying bacterial counts, while marbles taken from the exterior of east wall, and interior of west wall gave the lower and slightly the same nitrosifying bacterial numbers. In addition, marble collected from the interior of west wall showed lowest values in comparison with other parts (Table 3.9).

Table 3. 9 Most probable number of nitrosifying bacteria per g of Stone for the marble and andesite samples collected from the Temple of Augustus.

Sample code	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Characteristic number	Number of microorganisms/ g of stone
11JAWIM2	+++	+++	+ ++	- - -	- - -	- - -	330	250
11JAEEM$_{ss}$	+++	+++	- - -	- - -	- - -	- - -	330	250
11JAWIM1	+++	+ - +	- - -	- - -	- - -	- - -	320	95
11JAEIA	+++	- - -	- - -	- - -	- - -	- - -	300	25
11JAEEM	+++	+++	- - -	- - -	- - -	- - -	330	250
11JAWIM3	+++	+++	+- -	- - -	- - -	- - -	331	450

From samples taken in July of 2011, marble and andesite samples of Temple of Augustus showed slightly the same and low nitrifying bacterial counts as being in the range of 95-250 microorganisms/g of stone (Table 3.10)

Table 3. 10 Most probable number of nitrifiying bacteria per g of stone for the marble and andesite samples collected from the Temple of Augustus.

Sample code	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Characteristic number	Number of microorganisms/g of stone
11JAWIM2	+++	+++	- - -	- - -	- - -	- - -	330	250
11JAEEM$_{ss}$	+++	+++	- - -	- - -	- - -	- - -	330	250
11JAWIM1	+++	+++	- - -	- - -	- - -	- - -	330	250
11JAEIA	+++	+++	- - -	- - -	- - -	- - -	330	250
11JAEEM	+++	+ - +	- - -	- - -	- - -	- - -	320	95
11JAWIM3	+++	+++	+++	- - -	- - -	- - -	330	250

The samples taken in July of 2011, marble samples of Temple of Augustus showed low sulphur oxidising bacteria being in the range of 10^{-2}-10^{-3} number of microorganims/g of stone, whereas andesite and, marble of west wall interior and east wall exterior gave numbers in the range of 10^{-3}-10^{-4} number of microorganims/g of stone.

Table 3. 11 Most probable number of sulphur oxidising bacteria per g of stone for the marble and andesite samples collected from the Temple of Augustus.

Sample code	10^{-1}	10^{-2}	10^{-3}	10^{-4}	Characteristic number	Number of microorganisms/g of stone
11JAWIM2	+++	+++	- - -	- - -	330	250
11JAEEM$_{ss}$	+++	+++	- - -	- - -	330	250
11JAWIM1	+++	+++	- - -	- - -	330	250
11JAEIA	+++	+++	+++	+ - -	331	4500
11JAEEM	+++	+++	+++	- - -	330	2500
11JAWIM3	+++	+++	+++	- - -	333	1100

3.4   Results of Identification of Fungi

Aureobasidium sp., *Mucor sp.* and different *Penicillium sp*. were isolated and identified in the samples taken from limestones and sandstones of Nemrut Mount Monument and marbles of Temple of Augustus.
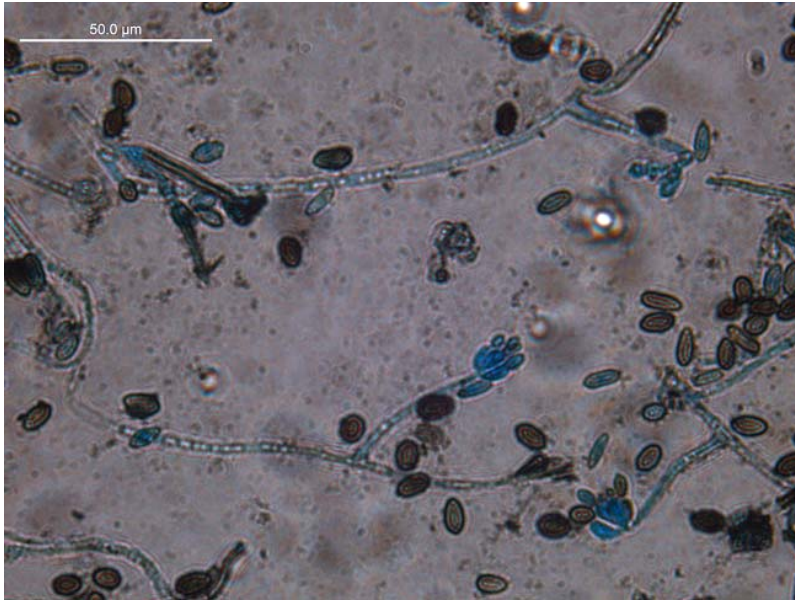
Figure 3. 6 *Penicillium* sp. under optical microscopy (X600, bar=50μm)(11MNNS1)
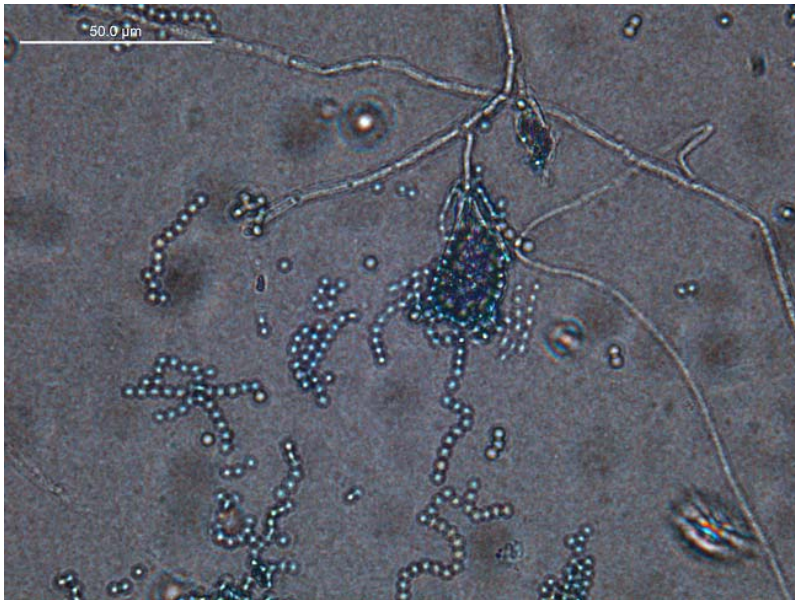
Figure 3. 7 *Penicillium* sp. under optical microscopy (X600, bar=50μm)(11MNNS2)
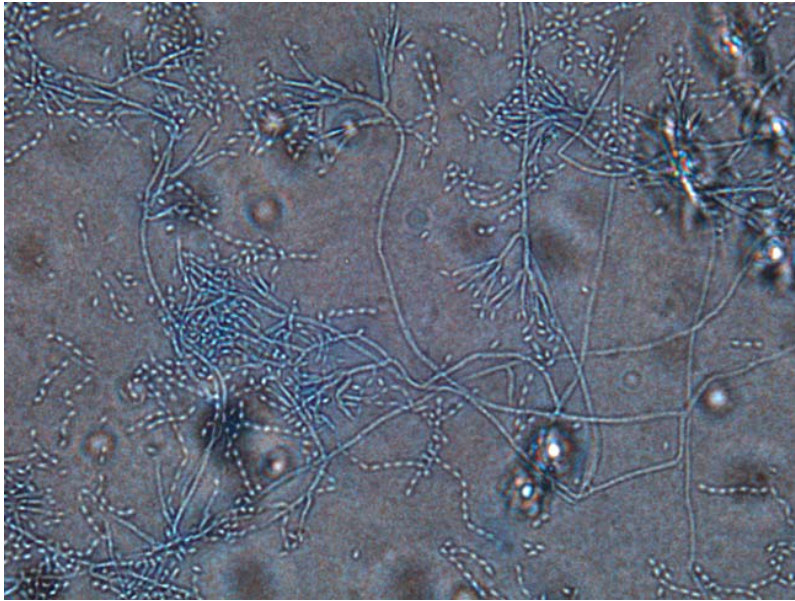
Figure 3. 8 *Penicillium* sp. under optical microscopy (X600, bar=50μm) (11MNNS2)

Figure 3. 9 *Penicillium* sp. under optical microscopy (X600, bar=50μm) (11JAEEM)
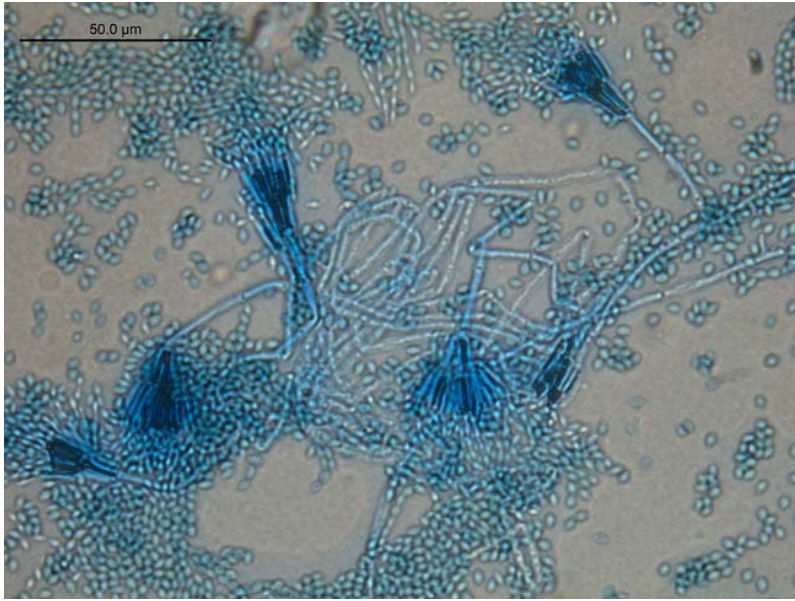
Figure 3. 10 *Penicillium* sp. under optical microscopy (X600, bar=50µm) (11JAWIM1)
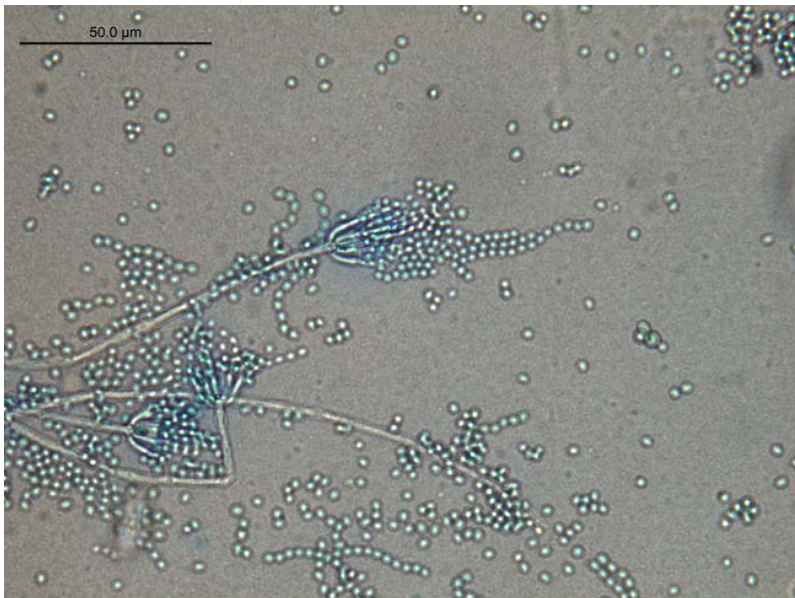
Figure 3. 11 *Penicillium* sp. under optical microscopy (X600, bar=50µm) (11JAWIM1)
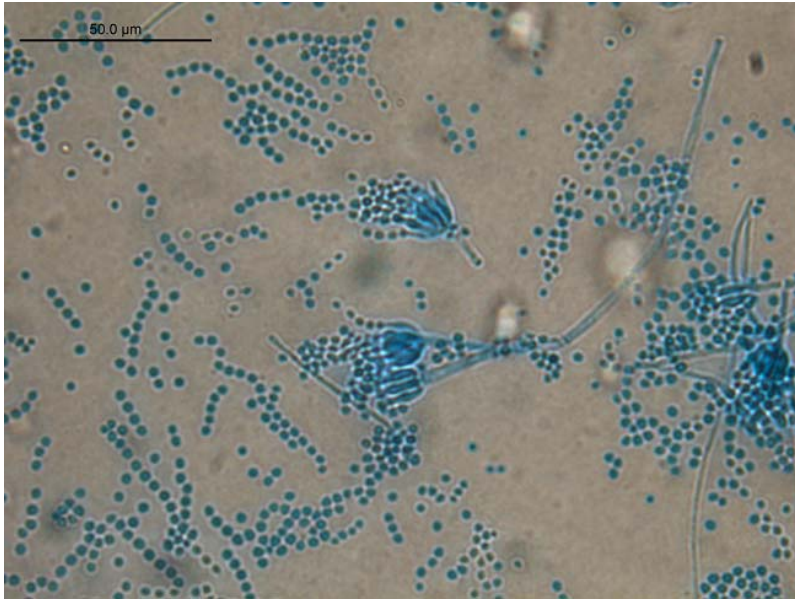
Figure 3. 12 *Penicillium* sp. under optical microscopy (X600, bar=50μm) (11JAWIM2)
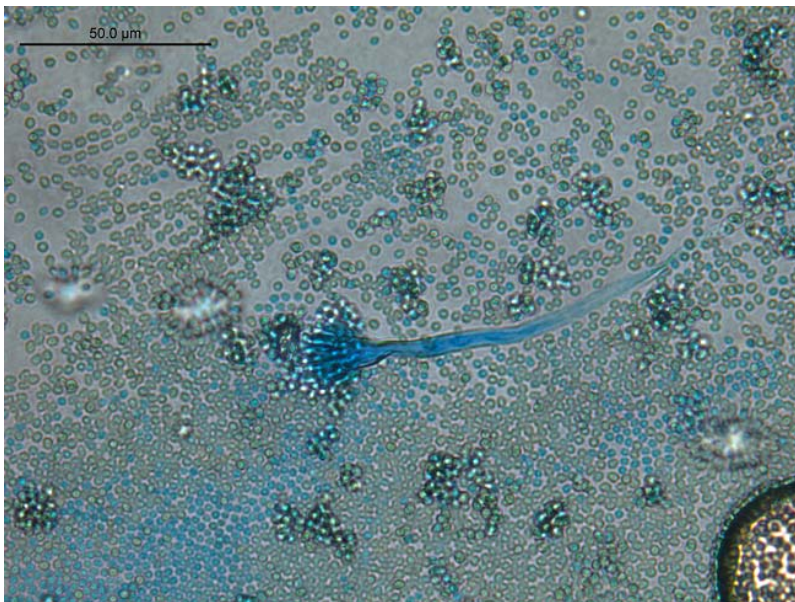
Figure 3. 13 *Penicillium* sp. under optical microscopy (X600, bar=50μm) (11JAWIM2)
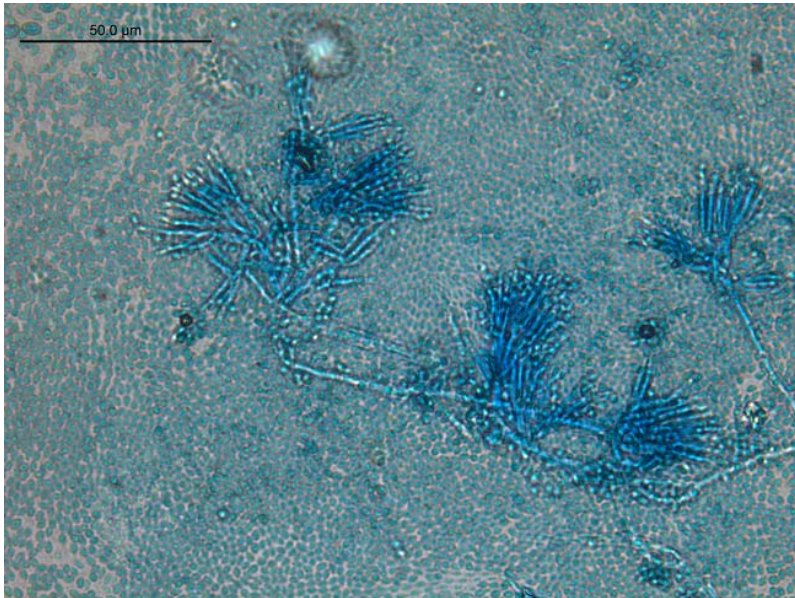
Figure 3. 14 *Penicillium* sp. under optical microscopy (X600, bar=50μm) (11JAWIM2)
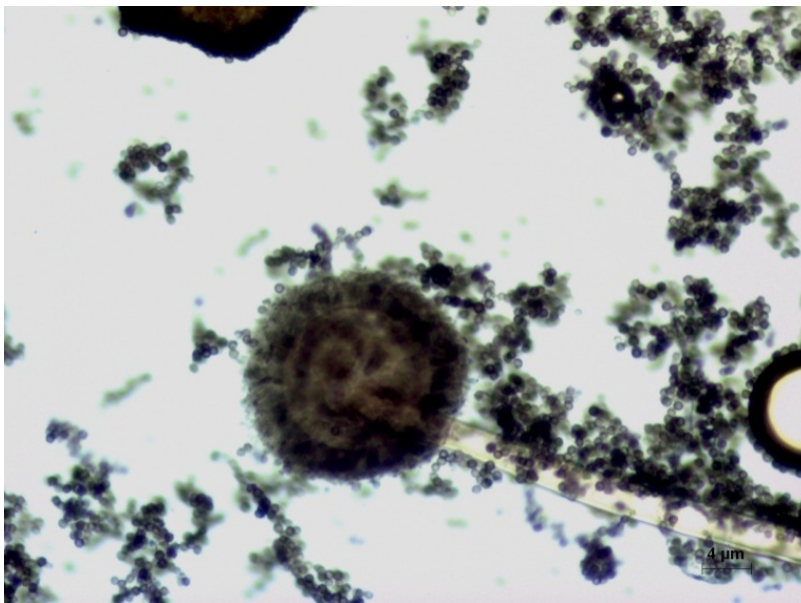
Figure 3. 15 *Mucor* sp. under optical microscopy (X400, bar=4μm) (10ONEL1)

Figure 3. 16 Aureobasidium sp. under optical microscopy (X400, bar=4µm) (10ONES1)

CHAPTER 4

DISCUSSION

In this chapter, the experimental results of biological activity that were obtained by the examination of representative samples from Temple of Augustus and Nemrut Mount Monuments were discussed. Results of biological activity measurements were evaluated under the titles of 'Evaluation of FDA results', 'Evaluation of Total Microflora Countings', 'Evaluation of ATP Bioluminescence Method', 'Evaluation of Most Probable Number Method together with Biochemical Tests' and 'Identification of fungi and bacteria species'. Results of those biological activity measurements were discussed for the importance and contribution of biological activity on stone deterioration problems of those monuments.

4.1   Evaluation of Biological Activity Measurements

In this section, the results of FDA analyses, total microflora method and ATP analyses were considered individually on the subject of different cellular functions.

4.1.1   Evaluation of FDA in Results Spectrophotometric Measurements

In the present study, quantitave method of FDA hydrolysis were applied on powdered stone samples of Nemrut Mount Monument and Temple of Augustus estimate the microbial biomass of the stone surfaces having visible coverings, slightly visible coverings, no visible covering and discoloration. As the first step, the results of spectrophotometric measurements were evaluated by comparing the stones having different surface textures and mineral components. After that, stone samples, especially having similar surface textures were considered in terms of change in microbial activity levels corresponding to different seasons, such as June (2010), October (2010) and May (2011).

The results of FDA hydrolysis for limestones and sandstones of Nemrut Mount Monument were as follows; the stones, having slightly visible biological coverings showed values in the range of 4.34-9.32 μg fluorescein/g of stone, whereas the stones having visible biological coverings, gave values in the range of 11.35-50.02 μg fluorescein/g of stone. The sample which had no visible biological covering on its surface, had the lowest biological activity as 2.52 μg fluorescein/g of stone (Figure 3.1, 3.2, 3.3, 3.5). Obtaining high values from lichenic zones of stones were regarded as normal (Swisher and Carroll, 1980)

because lichens showed considerable enzymatic activity owing to their symbiotic partners as alga and fungus (Berglund and Eversman, 1987).

FDA hydrolysis, measured at Temple of Augustus marbles and andesites , gave the highest value at andesite of east exterior wall and lowest value at marble of west interior wall. The marble of east exterior wall, having black discoloration on its surface, had 7.24 µg fluorescein/g of stone. Obtaining high values of fluorescein on andesite and marble having discolorations may be related with algae existence on the surfaces of those stones.

When all obtained results of FDA hydrolysis were compared with the soil fluorescein concentrations found in other studies, some conclusions could be made about the level of biological activity in those samples. Most stones of Nemrut Mount Monuments and Temple of Augustus were found to have high biological activity when compared with the studies of Adam and Duncan (2001), since values above 2 µg fluorescein/g were considered to indicate high biological activity in soil. FDA hydrolysis values 4.34-9.32 µg fluorescein/g indicated high biological activity for limestones and sandstones of Nemrut Mount Monument. However, according to Diack and Stott (2001), good agricultural soil should have 83-96 µg fluorescein/g of soil. Those were well above the values found on stone surfaces and in soil of high biological activity determined by Adam and Duncan (2001).

In fact, the results of FDA hydrolyses were evaluated among themselves, because soil ecology had rich biological population, unlike stone, even if some microorganisms had the ability to grow both on stone and in soil. On the other hand, after applying this technique to several types of stones, exposed to different climatic conditions, a database about µg fluorescein/g of stone could be constituted together with data of other experiments, such as total microflora

and biochemical tests, in order to minimise the risk of errors and increase reliability of evaluations.

It might be considered that stones of Nemrut Mount Monument and Temple of Augustus, represented low values in fluorescein concentrations in comparison to the results of total microflora method. The reason of evaluating those results in comparison with total microflora method was that the bacteria and fungi species, showing enzymatic activites, also had the ability to grow on nutrient agar medium and malt extract agar medium, respectively. However, Total Microflora method did not reflect the algal population.

Moreover, it was observed that lichens growing on stone surfaces and algae growing especially in cracks of stones were responsible from the great majority of biodeterioration problem in Nemrut Mount Monuments and Temple of Augustus. In fact, biominerals of calcium oxalates as weddelite and whewellite detected on surfaces of those stones have indicated the presence of considerable biological activity (Caner and Boke, 1989; Üstünkaya, 2008). Therefore, those detected fluorescein concentrations might indicate considerable biological activity due to the algal population in the stones

4.1.2 Evaluating the Countings of Total Microflora

The total microflora method used in this study was found to be useful to reveal the degree of microbial, namely, bacterial and fungal activity on stone surfaces, and enabled the isolation of fungal species as seen in section 2.6.

The samples for analysis were collected from marble and andesite monuments situated in the Temple of Augustus (Ankara), and limestones and sandstones

situated in the Nemrut Mount Monument in Adıyaman. The quantitave method of Total Microflora was applied on those powdered stone samples in order to determine the microbial biomass of the stone surfaces having visible coverings, slightly visible coverings, no visible covering and discolorations.

Regarding the results of total microflora of sandstones and limestones belonging to the Nemrut Mount Monument, bacterial counts were found in the ranges of 10^2-10^6 CFU/g in June of 2010 and 10^4-10^6 CFU/g in May of 2011. Moreover, the samples, taken in October of 2010, showed intermediate bacterial activity (10^4 CFU/g). Besides, the numbers of fungi, counted on Czapeks dox (June and October, 2010) and Malt Extract Agar (July, 2011), were slightly in the same values being in the ranges of 10^2-10^3.

On the other part, marbles and andesites of Temple of Augustus (July, 2011) gave colony counts in the range of 10^3-10^7 for bacteria and 10^3-10^5 for fungi.

When all obtained results of total microflora were compared with the colony counts detected in the studies of Caneva *et al.*, (1991), Wilimzig *et al.*(1992) and Jaton (1971), various conclusions could made. To begin with the studies of Jaton (1971), the stones of Nemrut Mount Monuments showed low bacterial and low fungal activity which could be estimated as being a normal microflora on stones. But, even those lower numbers, when activity of stone surfaces were compared with each other in changing seasons, bacterial and fungal activity gave the highest numbers in the seasons of summer and spring in comparison to Autumn. According to Caneva *et al.*,(1991), the level of bacterial activity in the stones of Temple of Augustus, was found closer to the limit of risk than Nemrut Mount Monument in terms of its contribution to stone decay process. Nevertheless, based on the studies of Wilimzig *et al.*(1992), considering the mean value of the

colony counts, the activity of fungi and bacteria were found at intermediate levels since being slightly higher than 10^3 CFU/g.

Concerning the relationship of biological coverings (lichens) on stones and with the microbial activity, it was determined that there were no significant correlation between them.

Eventually, the method of total microflora showed that stones of Nemrut Mount Monuments and Temple of Augustus presented low bacterial and fungal activity except some of the samples taken in warm periods, most probably due to harsh weather conditions (Turer *et al.*, 2010). In general, detecting microbial activity in low amounts seemed to be related with the climatic conditions being not sufficient for the survival of microorganisms (Wilimzig and Bock, 1992).

In the aspect of biodeterioration, there are no significant numbers of aerobic heterotrophic microflora and fungi on stones of mentioned historical monuments. In the case of Nemrut Mount Monument, considering biodeterioration, lichens, distributed on the surface of limestones and sandstones, played the greatest part in the decay process either by physical or chemical actions In addition, according to the recent studies the main factor, causing and/or accelerating decay processes of stones, are wetting-drying cycles and thermal fluctuations (Akoğlu, 2011; Caner, 2011). On the other hand, stones belonging to Temple of Augustus revealed higher microbial activity in comparison to stones of Nemrut Mount Monument. Biominerals weddelite and whewellite were found on marble surfaces of Temple of Augustus (Caner and Boke, 1989) as well as Nemrut Mount Monument (Ustunkaya, 2008), which should be related with biological activity. Moreover, air pollution**,** seemed to be the main factor (Caner *et al.,* 1989) causing deterioration of marbles in Temple of Augustus.

4.1.3 Evaluation of ATP Bioluminescence Method

In the present study, sampling was conducted by sterile cotton swabs in order to determine the microbial biomass of the Nemrut Mount Monument and Temple of Augustus on the stone surfaces having visible biological coverings, slightly visible coverings, no visible covering, having $Ca(OH)_2$ treatments and discolorations. When all obtained results were compared with the studies of Sigler *et al.,*(2002); Kaskova *et al.,* (2005); Green *et al,* (1999); Vilar *et al.,* (2008) and also Orial *et al.,*(2009), luminometer gave very low values ranging in 4-25 RLU/cm^2 of stone.

Considering ATP bioluminescence method, since the stones of those two historical sites had both low porosity and low moisture content on their surfaces, it was not possible to take proper samples of microorganism with cotton swabs to make measurements with Hy-Lite device. Although ATP bioluminescence method had advantages in terms of being practical and giving results in a short time, sampling method and protocol were not efficient to measure global microbial activity of stones belonging to Temple of Augustus and Nemrut Mount Monument.

4.1.4 Evaluating the Results of Biochemical Tests together with Soluble Salt tests

In the study, in July 2011, stone samples of Temple of Augustus were tested in order to understand if the salt deposits were originated from the activites of bacteria. Concerning sulphur oxidising bacteria, the microbial cells existed with

an average of 1×10^2 cells/g of stone. Moreover, regarding nitrifying bacteria, the nitric and nitrous, microbial cells were detected in small amounts of 1×10^2 cells/g of stone, 10^2-10^3 cells/g of stone, respectively.

When all obtained results were compared with the the cell numbers detected in the studies of Gorbushina *et al*., (2001), Wilimzig *et al.,* (1991), stone samples, including marble and andesite, of Temple of Augustus represented low autotrophic bacteria activity on their surfaces.

Concerning the relationship of severely decayed zones of stones and microbial activity, it was determined that the values of bacterial counts were generally the same in terms of microbial cell numbers/g of stone except one sample, coded as 11JAEIA, taken from the cavity of marble as soil deposit**.** The number of nitrosifying bacteria were detected 10 times higher in the mentioned sample than the others (See Table 3.11)

Existence of ammonia and nitrite oxidizers in low cell numbers could be related with the inverse relationship between these bacteria and ammonia.

Even sulphur oxidising bacteria existed in low cell numbers in the stones of Temple of Augustus, the results of qualitative soluble salt test showed the presence of sulfate. That could be explained by potential salt sources being air pollution (Caner and Boke, 1989; Mansch and Bock, 1998).

At the time of field survey of Temple of Augustus, only the samples at the surface of marbles and andesites were taken. However , according to the findings of Bock and Mansch (1987), nitrifying bacteria commonly colonize in deeper layers of stones. Therefore sampling of the deeper decay zones would prevent underestimations of bacterial colonization. On the other hand, detailed knowledge about environmental conditions such as humidity and pH would be

helpful while evaluating the bacterial biomass of stones (Bock and Mansch, 1987).

Consequently, even the MPN method underestimates the cell numbers of nitrifying bacteria, (Besler and Mays, 1982; Cooper, 1983), applied method seems to be appropriate in terms of determining the cell numbers of ammonia, nitrite and sulphur oxidising bacteria. In other words, liquid media enables bacteria to grow efficiently in the absence of heterotrophs.

4.2   Evaluation of Identification of Fungi

In this study, which focused on fungi, the most frequently occuring fungal genera was *Penicillium* in the samples of limestones of Nemrut Mount Monument and marbles of Temple of Augustus., but *Mucor sp. and Aureobasidium sp* were also identified in the stones of Nemrut Mount Monument. All those samples taken from the two historical sites showing evidence of biological colonization. Since the counting results of fungi showed their existence as normal microflora, those identified fungi species were not found to be harmful to the stones of the two studied historical sites.

CHAPTER 5

CONCLUSION

In this study, historic marbles, sandstones and limestones, exposed to atmospheric conditions, were analysed by means of different techniques such as FDA hydrolysis, Total Microflora and Most Probable Number Method in order to show the level of biological activity based on different types of microorganisms Furthermore , the identification of microorganisms growing on solid media,was done particulary for fungal species.

The stones of Temple of Augustus showed higher microbial activity ,compared to Nemrut Mount Monuments, in the experiments of FDA hydrolyses and Total microflora most likely due to colonized by different type of species such as ammonia, nitrit and sulphur oxidising bacteria, whereas sandstones and limestones of Nemrut Mount Monuments revealed that only stones having visible biological coverings (lichens) gave values closer to intermediate level. In that manner, together with the methods of Total Microflora and FDA hydrolysis, it can be concluded that there was no biodeterioration due to aerobic hetetrophic bacteria and fungi on the stone surfaces of two studied historical sites. On the other hand, considerable fluorescein levels detected by FDA hydrolyses in this study and the presence of biominerals as calcium oxalates

determined by other studies in those monuments, indicated the importance of algal activity in biodeterioration of those stones.

Regarding different types of species colonized on stones of Temple of Augustus, nitrifying and sulphur oxidising bacteria were detected as normal microflora on the surfaces of stones. Therefore, it was concluded that the origin of gypsum formation on the surfaces of marbles were not originated from biological activity. However, more studies were needed to investigate the possibility of their existence in lower layers of marble surfaces.

Application of ATP bioluminescence method on the stone surfaces of two studied historical sites, resulted with very low RLU/cm^2 on stone surfaces having variable states of deterioration.

This study has shown that FDA hydrolyses, Total Microflora and Most Probable Number method was efficient for the evaluation of biodeterioration in historic stones.

Further analyses should be conducted with more samples to enhance the accuracy of the examination and to have a rich database. Moreover, conducting experiments regarding organic acid production, would be useful for detecting the existence of bacteria and fungi having ability to solubilise minerals

Moreover, the presence of algae and its critical concentrations at the surface layers of stone and in the surface cracks of the stone has to be studied by measurable parameters. e.g amount of Chlorophyll a / g of stone

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APPENDIX A

PREPARATION OF SOLUTIONS USED IN FDA ANALYSES

1. Buffer solution

The buffer solution was prepared in order to make fluorescein standard solution from certain amount of K_2HPO_4 and (Riedel – de Haen, Sigma Aldrich Co. Ltd, Analar) and KH_2PO_4 (Merck, BDH Analar). According to the studies of Adam and Duncon (2001) the formation of fluorescein compound from FDA eventuates optimum between pH 7.0 and 8.0.

In this experiment, a buffer solution with pH 7.5 was prepared as follows;

8.7 grams of K2HPO4 and 1.3 grams of KH2PO4 were dissolved in 800 ml distilled water and the volume was completed to one litre with the addition of distilled water. The pH of buffer solution was measured with pH papers before each usage (Adam and Duncon, 2001).

2. FDA stock solution

0.1 grams 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 stored in refrigerator below 0 °C except applications. The solution was prepared over again for each experiment (Adam and Duncon, 2001).

3. Fluorescein stock solution

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

4. Preparation of 20 µg fluorescein ml^{-1} standard solution

1 ml of stock solution (2 mg fluorescein) 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 Duncon, 2001). Then a standard curve was drawn with above mentioned 5 standard solutions.
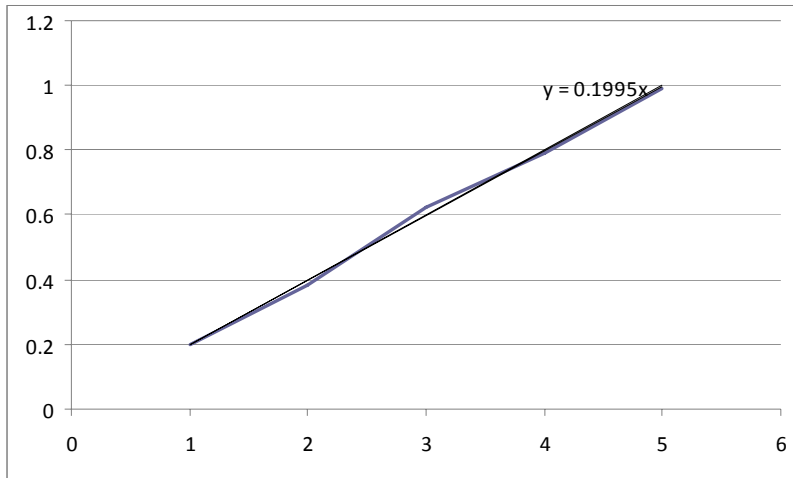
Figure A.1 Standard curve prepared by the standard solution of fluorescein. (x axis: concentration, (μg/ml); y axis: absorbance (490nm))

APPENDIX B

COMPONENTS OF MEDIA USED IN MICROFLORA TOTAL METHOD

1. Nutrient Agar

Nutrient Broth: Peptone from meat 5.0; Meat extract 3.0 (g/litre)

Agar Agar

8 g nutrient broth and 7.5 g agar agar was solubilized in 1 liter distilled water and autoclaved 15 min at 121 °C. After sterilization process, medium was poured into petri plates aseptically.

2. Czapek Dox Agar

Sucrose 30.0, $NaNO_3$ 3.0, $MgSO_4$ 0.5, KCl 0.5, Iron(III)sulfate 0.01, K_2HPO_4 1.0, Agar Agar 13.0 (g/litre)

Chemical compounds, disaccharide and agar agar was dispersed one liter distilled water and autoclaved 15 min at 121 °C. After sterilization process, medium was poured into petri plates aseptically

3. Malt Extract Agar

Malt extract 30.0, Mycological peptone 5.0, Agar No.2 15.0 (g/litre)

50 grams of powder was dispersed in 1 liter distilled water and autoclaved 10 min at 115 °C. After sterilization process, medium was poured into petri plates aseptically.

APPENDIX C

COMPONENTS OF MEDIA USED IN MPN METHOD

1. For nitrous ferments;

Standard saline solution → 50 ml

Ammonium sulfate → 0,5 g

Calcium carbonate → 1 g

Distilled water → 950 ml

2. For nitric ferments;

Standard saline solution → 50 ml

Sodium nitrite → 1 g

Calcium carbonate → 1 g

Distilled water → 950 ml

All components were dissolved in distilled water to have a final volume of 1L. 1 ml media were transferred to the tubes for both sets of nitrification and after plugging the ends of the tubes with cotton sterilization process was performed at 110 °C for 20 minutes.

3. For oxidation of sulphur

Dipotassium phosphate → 0,25 g

Magnesium chloride → 0,10 g

Sodium chloride → 0,10 g

Ammonium nitrate → 2 g

Calcium carbonate → 5 g

Distilled water → 1000 ml

Minerale solution → 1ml

5 ml media were transferred to the tubes and after plugging the ends of the tubes with cotton, sterilization process was performed at 110 °C for 20 minutes