University of Évora

ARCHMAT
(ERASMUS MUNDUS MASTER IN ARCHaeological MATerials Science)

Mestrado em Arqueologia e Ambiente (Erasmus Mundus – ARCHMAT)

Investigation of marble limestone biocolonization: The case study of Convento das Maltesas in Estremoz

Sriradha Bhattacharya
m38419

Ana Teresa Caldeira
(Supervisor - Universidade de Évora)

Evora, September 2018

Universidade de Évora

ARCHMAT
(ERASMUS MUNDUS MASTER IN ARCHaeological MATerials Science)

Mestrado em Arqueologia e Ambiente (Erasmus Mundus – ARCHMAT)

Biocolonização de pedra calcária: O caso de estudo do Convento das Maltesas em Estremoz

Sriradha Bhattacharya
m38419

Ana Teresa Caldeira
(Orientador - Universidade de Évora)

Evora, Setembro 2018

JURY MEMBERS:

Nicola Schiavon (President of the Jury)
Maria do Rosário Martins (Arguente)
Mary Anne Tafuri (Vowel)
Ana Teresa Caldeira (Orientador)

This page is intentionally left blank

"Whoever expects to find a stone that will stand from century to century, deriding alike the frigid rains and scorching solar rays, without need of reparation, will indeed search for the 'philosopher's stone'" – C.H. Lewis, 1840

In Memory of my Grandmother

This work was supported by the following projects: ColourStone - Colour of commercial marbles and limestone: causes and changings" (ALT20-03-0145-FEDER-000017) and "MEDUSA-Microorganisms Monitoring and Mitigation– Developing and Unlocking novel Sustainable Approaches" (ALT20-03-0145-FEDER-000015).

TABLE OF CONTENT:

LIST OF FIGURES:

LIST OF TABLE:

LIST OF APPENDIX TABLES:

LIST OF ABBREVIATIONS:

CM – Convent Maltesas

CRB – Cook Rose Bengal

DGGE - Denaturating Gradient Gel Elecrophoresis

DNA - Deoxyribonucleic acid

FISH - Fluorescence *In Situ* Hybridisation

FORS – Fibre Optic Reflectance Spectroscopy

FTIR ATR – Fourier Transform-Infrared Spectroscopy with Attenuated Total Reflectance

MCS – MiSeq Control Software

MEA – Malt Extract Agar

MRD-Maximum Recovery Diluent

N.A. – Nutrient Agar

NA - Non- altered

NGS – Next Generation Sequencing

PCR – Polymerase Chain Reaction

RH – Relative Humidity

RNA - Ribonucleic acid

SEM-EDS – Scanning Electron Microscopy coupled with Energy Dispersive X-Ray Spectroscopy

SERS - Surface Enhanced Raman Spectroscopy

SORS - Spatially Offset Raman Spectroscopy

XRD – X-Ray Diffraction

XRF – X-Ray Fluorescence

ACKNOWLEDGEMENTS

This thesis “Investigation of Marble Limestone Biocolonization: The case study of Convento das Maltezas in Estremoz, Portugal” would not have come through, if it was not for a number of people, who helped and encouraged and believed in me. First and foremost I would like to thank my supervisor Professor Ana Teresa Caldeira for giving me the opportunity to learn about something that was a completely a new venture for me. It would have been extremely difficult to finish this dissertation without her constant support, encouragement and help. I would like to also express my sincerest thanks, gratefulness and indebtedness to Tania Rosado and Luis Dias, who have painstakingly taught me everything, developed and nurtured my interest and introduced me to the fascinating world of microorganisms and has always been available for my several questions. A special thank you to Professor José Mirão for taking time out of his busy schedule to first introduce me to the site and then helping me with the data analysis of XRD and SEM.

I am tremendously grateful to many at HERCULES Laboratory especially Professor António Candeias, Professor José Mirão and Professor Cristina Barrocas Dias and the fantastic professors who have helped us over the past two years. My appreciation to Centro de Ciência Viva in Estremoz, for making the Convent available to us for the material that was used for completing this thesis.

I owe a very big big thank you to my friend, Maitreyee, who actually got me to apply for this course and have been a constant support always, rebuking me when I slaked and encouraged me at every step. My friends, here at HERCULES and back home who have always been very positive about me taking this step even when I was not very confident about myself. A big thank you to Ana for taking time out of her busy schedule to help me with the FTIR-ATR spectra interprettions. Maitreyee, without whose support I wouldnot have enrolled in this course. A special mention to Mara, Isabel, Guncem and Patricia who was calm when I was not and in true Portuguese way was always unruffled, unperturbed and relaxed making me see that I was worrying for nothing. Jota, who has been the biggest stressbuster, punching bag, making sure I was ship-shape and reminding me that it was important to take a break every now and then and also provided me with much needed criticism on my thesis. Lastly but not the least there is no words with which I would like to show my gratitude and indebtedness to my family. Without their support and encouragement and their trust in me I would not be here

ABSTRACT

Over centuries, different lithotypes – either calcitic such as limestones and marbles, and/or silicious such as sandstones and granites have been used in historical monuments, whose deterioration/degradation differs as per the hardness, porosity and chemical composition of each stone. However, over the last decades living microorganisms have been associated with structural and aesthetical damages to building stone in historical monuments. The current research focuses on the investigation of degradation/deterioration in architectural marble monuments, with the aim to contribute to a better understanding the role of microbial agents on the biodegradation/biodeterioration of monuments built in marble.

A multidisciplinary approach was employed to perform material characterisation and alteration products detection involving *in situ* and laboratory analysis in order to arrive at a comprehensive assessment of biocolonisation. Studying the microbial proliferation gave a wider perspective on recognising the role of microorganisms and their ability to degrade cultural heritage materials, which in turn helps understand and chalk out a mitigation process for future.

The micro-analytical techniques distinguished and identified the alterations processes like patina formation, pigmentation and biofilms formation. The biocontamination was characterised by SEM-EDS, culture-dependent methods (CDM) and Next Generation Sequencing (NGS). CDM and NGS confirm the presence of several strains of bacteria, filamentous fungi and yeasts that appears to contribute to the presence of calcium oxalates, carotenoids and biofilms formation.

Thus, it is imperative to study and comprehend the causes for marble degradation/deterioration, and recognise the source for the alteration of these materials, in order to define effective strategies to prevent marble decay and safeguard our cultural heritage.

Keywords: Biodegradation/Biodeterioration, biofilms, marble, Maltesas Convent, stone alteration

.

RESUMO

Biocolonização de pedra calcária: O caso de estudo do Convento das Maltesas em Estremoz

Ao longo dos séculos, diferentes litotipos - calcíticos, como calcários e mármores, e / ou siliciosos como arenitos e granitos - têm sido utilizados em monumentos históricos, cuja degradação/deterioração difere de acordo com a dureza, porosidade e composição química de cada pedra. Nas últimas décadas, a presença de microrganismos tem sido associada a danos estruturais e estéticos de pedra utilizada em monumentos históricos. O presente trabalho tem como objetivo o estudo da degradação/deterioração de mármore aplicado em património arquitetónico, com o objetivo de contribuir para uma melhor compreensão do papel dos agentes microbianos na biodegradação/biodeterioração deste material, em contexto histórico.

Foi utilizada uma abordagem multidisciplinar na caracterização de materiais e na deteção de produtos de alteração, envolvendo análises *in situ* e em laboratório, no sentido de obter uma avaliação abrangente da biocolonização. O estudo da proliferação microbiana permite uma perspetiva mais ampla no reconhecimento do papel dos microrganismos e da sua capacidade para degradar os materiais do património cultural, o que, por sua vez pode vir a ser útil na definição de estratégias de mitigação, para o futuro.

As técnicas microanalíticas permitiram distinguir e identificar alguns processos de alteração, como formação de pátinas, pigmentação e formação de biofilmes. A biocontaminação foi caracterizada por SEM-EDS, através de métodos dependentes de cultura (CDM) e por Sequenciação de Nova Geração (NGS). CDM e NGS confirmam a presença de várias espécies de bactérias, fungos filamentosos e leveduras que parecem contribuir para a presença de oxalatos de cálcio, formação de carotenóides e de biofilmes.

Assim , é imperativo estudar e compreender as causas da degradação/deterioração do mármore, e reconhecer os agentes responsáveis pela alteração destes materiais, de forma a que possam ser definidas estratégias eficientes para prevenção do seu declínio, contribuindo para a salvaguarda dos nossos bens patrimoniais.

Palavras-chave: Biodegradação/Biodeterioração, biofilmes, mármore, Convento das Maltesas, alteração de materiais pétreos.

I - INTRODUCTION

1. STONE MATERIAL

One of the oldest building material used by man is natural stone. The first stone monuments were constructed more than 5000 years ago. Stones are not only emblematic, that was used to symbolise cultural, aesthetic, and history but also the economic growth and evolution of man (A. Torok, 2010). Stone is one of the most tangible characteristics of cultural heritage and also one of the most commonly used material for construction or other pieces of art (Saiz-Jimenez, 2003). Stones are natural rocks that is shaped artistically to man's need and can be classified into building stones, decorative stones, stone used for sculptures and as aggregates for building (Winkler, 1997). Stone as an artistic medium as an expression of art and culture has been used from ancient times and this expression ranged from creating historical monuments to small statues (T. Warscheid, 2000). Stones are principally divided in two major categories – calcareous and siliceous stone (Fig.1.1 and Fig.1.2). Calcareous stones include limestone, marble, travertine and onyx (Fig.1.1). The main composition is calcium carbonate which make them very sensitive to either too acidic or alkaline and can dissolve without leaving behind any residue (Winkler, 1997).

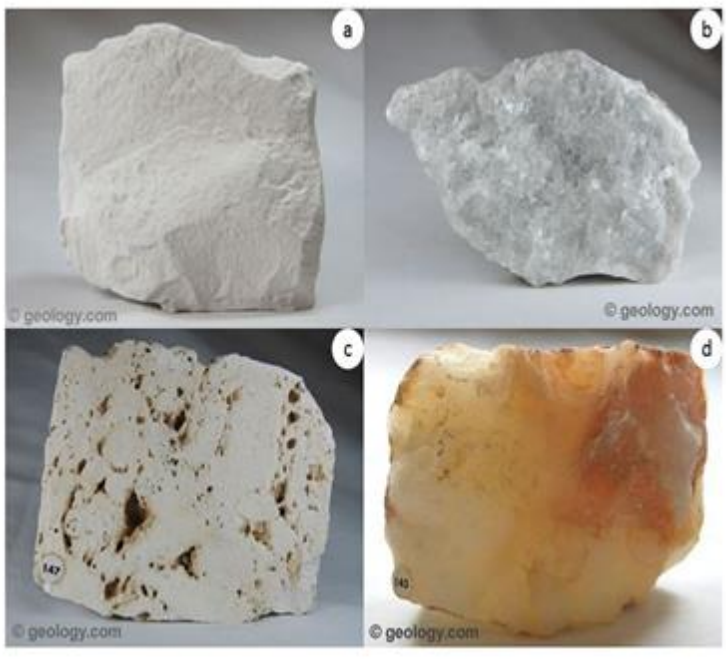


Figure 1. 1 Calcareous Stone: a) Chalk – Limestone, b) Marble, c) Travertine, e) Onyx (Adapted from: www.geology.com).

Siliceous stones include granite, slate, sandstone and quartzite (Fig.1.2). They consist mostly

of silica and quartz-like particles. Siliceous stones are more durable than calcareous stones. Sandstone consists mainly of quartz, feldspar and iron oxide and granite is essentially composed of quartz and feldspar.

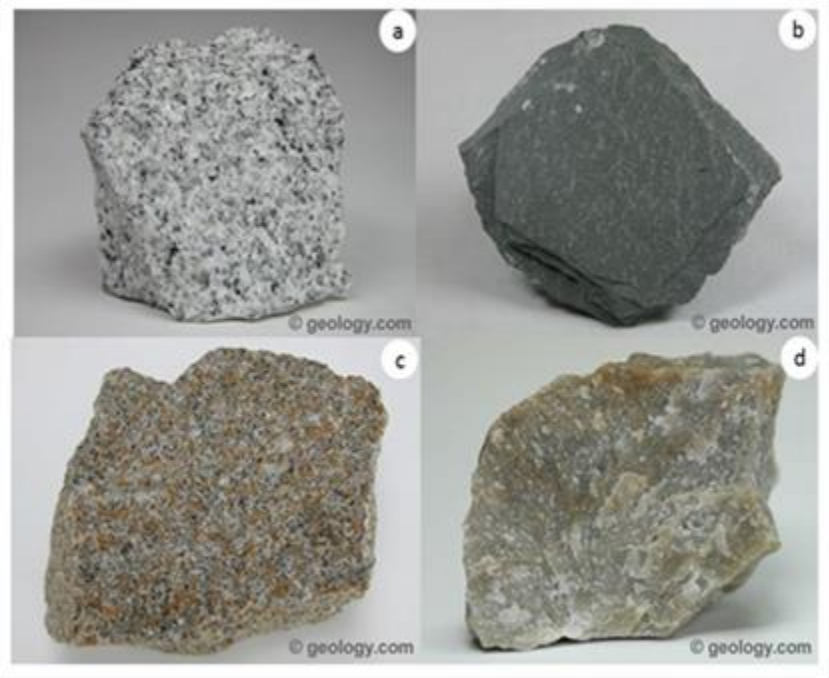


Figure 1. 2: Siliceous Stone: a) Granite, b) Slate, c) Sandstone, e) Quartzite. (Adapted from: www.geology.com).

However limestone and marble from the calcareous family, are the most commonly used material and, they are known to erode in small fractions, due to the rapid environmental changes of the recent times, their rate of erosion is also changing which will eventually result in complete vanishing of cultural heritage. Additionally, though stones play a really important part in a construction, we need to consider, that more often than not we use materials to stabilize the structures – mortars, plasters.

The primary focus for this thesis is the effect bio-deterioration on marble. The word marble is derived from the Greek word marmeirein or Latin word *marmor*, meaning stone of quality or white stone. Marble was considered superior and distinctive when compared to other stones. Owing to its softness, malleability and beauty, marble, since ancient times have been considered most suitable stone for expression of art and for construction. It is generally referred to as "cleaned" calcium carbonate as it is recrystallized metamorphic rock (Clara Urzi W. T., 1992). Marble, is a crystalline carbonated and metamorphic rock composed of calcite crystals (calcite marbles) or dolomite (dolomite marbles) resulting in recrystallization of limestone or dolomite (Fig.1.3). The recrystallization is the mark of separation between limestone and dolomite

(Marble The Divine Stone, 2015). During the formation of marble from limestone, it is generally white in colour. But marble can also be found in different colours – blue, grey, pink, yellow, green or black caused by impurities, like clay, iron oxides, or bituminous material, present in the marble. However "marble" is also sometimes referred to rocks that can be polished the term used for other derivatives of limestone, namely travertine, verde antique, serpentine. (https://geology.com/rocks/marble.shtml, n.d.). Marble is formed when limestone which is a sedimentary rock is subjected to high heat and pressure metamorphoses to marble.

Figure 1. 3: Transformation from Limestone to Marble (Adapted from: https://www.infoescola.com/geologia).

Microbial proliferation and biodegradation/biodeterioration is directly linked to environmental factors. The most important causes for biodegradation/biodeterioration that help in the growth of microbes are physical parameters like moisture, humidity, temperature, light and finally the chemical composition of the surface of the stone (Saiz-Jimenez, 2003). The degradation/deterioration of the stone starts since the stone is removed from the quarry to be used (T. Warscheid, 2000). However, we cannot take this cultural heritage for granted as the stones are slowly disappearing irreversibly (T. Warscheid, 2000), weathering processes are acting upon the stone which are eventually turning to soil (Stephanie Scheerer, 2009).

2. DETERIORATION OF STONE MATERIALS

Geological processes play an important role in the decay of stone weathering or degradation/deterioration (Winkler, 1997). The process of weathering is three folds first attacking the stability of the matrix and secondly by the means of chemical weathering through oxidation, hydration and the dissolution of carbonates. Finally the stone is also affected by human intervention in form of air pollution. All these forms of weathering and corrosion greatly accelerates any form of decay on the stone. Weathering, along with neglect from authorities of historic sites and unsuitable and inappropriate practices adopted by restorers can completely

destroy cultural heritage (T. Warscheid, 2000). Atmosphere, humidity, presence of salts being, are the primary physical cause of decay of stone (Winkler, 1997). Stone decay can also be attributed to natural disasters - earthquake, fire or flood, neglect, acts of vandalism and terrorism, treatments earlier used for conservation, chemical attack, salt attack, pollution, bio-deterioration to name a few. Limestone, marble and carbon cemented limestone are particularly susceptible to acidic pollution and staining from particulates found in the atmosphere.

It is of utmost importance to understand the reasons and the type of decay before the start of any consolidating work. It should be kept in mind that though the material might be the same, their chemical properties can vary greatly. This is so in the case of marble and limestone found in different parts of the world. There are multiple ways of understanding and studying different kinds of stones – percentage and relative ratio of pore-shaped and fissure shaped voids, degree of hygric swelling and its strength. Divisions are also made on the composition, texture and the homogeneity of the stone. Stone weathers gradually, at first glance it may look perfect but with subsequent examination, defect may come to light. There are also instances where there is surface blisters and sometimes the stone loses its integrity thereby completely crumbling (Eric Doehne, 2010).

2.1 MICROBIAL ECOLOGY OF OUTDOOR STONE SURFACES:

Stone monuments are subjected to decay by a very complex ecosystem of microflora that includes algae, bacteria, fungus, lichens and protozoa. Apart from microorganisms there is also infestations of small animals and plants. There are several ways that microorganisms can grow on stone surfaces (Fig.1.4):

a. Epilithic: when microorganisms grow on the surface of the stone.

b. Chasmolithic: when microorganisms grow in the crevices and fissures and are more protected.

c. Endolithic: when microorganisms grow within a couple of centimeters inside the stone surface. When the endolithic microorganisms are found in cracks it is called chasmoendolithic and when they are pores it is called cryptoendoliths.

Figure 1. 4: Microbial Ecology of Outdoor Stone - a) Epilithic, b) Chasmolithic and Endolithic (Adapted from: https://www.flickr.com/photos/henrymaofeng, (Susana E Jorge-Villar, 2011)).

It has been observed that the growth of microorganisms depend on the nutrients available, exposure to UV radiations and inorganic matter. Microorganisms in moderate climate generally colonise the surface of the stone, and in tropical and sub-tropical climates, in order to protect themselves from the sun, they penetrate deep into the rock substrate. It is also been noted that in several cases the micro-organisms can become endolithic, not only because they seek protection but also because of the availability of nutrients, a lack of space and a stable life for an extended period of time (Stephanie Scheerer, 2009).

2.2. AGENTS OF DECAY

2.2.1. ATMOSPHERIC CONDITIONS:

The atmosphere is made up of nitrogen, oxygen and carbon dioxide along with various impurities or pollutants like SO_2, SO_3, O_3, Cl_2, and other organic substances which settle on the surface of the stone. These are mostly due to industrial and automobile pollutants. Table 1 shows some source and effects of these pollutants in the environment. The chemical weathering

on stone is largely owing to water which is dissolved with impurities like carbon dioxide, sulphates or nitrates which cause acidic corrosion (Winkler, 1997).

These parameters induce differential thermal stress, stress from expansion, hygric stress, difference in wet and dry cycles, stress from different expansion rates of materials in pores in various stones. Various treatments, react with the stone surface differently and can result in cracks and stress which will eventually lead the stone to detach from the original surface, like in the case of flaking (Fig.1.5).

Table 1: Important Pollutants: sources and effects (Winkler, 1997).

	NATURAL SOURCES	**INDUSTRIAL SOURCES**	**RESULT OF ATTACK**
Particulates	Volcano, Forest Fires	Industrial exhasuts	Soot cover
CO_2	Volcanic, vegetation	Automotive, combustion	Acid Rain
SO_2	Volcanic, desert dust	Combustion of fossil fuels	Acid Rain,
SO_3		Oxidation of SO_2	Metals, plastics, acid rain
Cl	Volcanic, desert dust	Combustion, dry cleaning	Acid Rain
NO_x	Volcanic, vegetation, bacteria	Automotive combustion	Acid rain, photooxidation
O_3	Trace in natural amounts	Industrial, urban oxidation	Paints, plastics, metal corrosion, UV

Salt crystals generally form near on the surface of the stone consequently setting different ways of how the two parts of the stone – interior and exterior- react to environmental change.

Figure 1. 5: Evidence of flaking on marble building (Adapted from (V.Verges-Belmin, 2008)).

In case of intervention process it is important to understand and observe the compatibility of materials used during conservation. If the materials, used for conservation, are incompatible then this may produce more stress on the material. The thermal expansion of consolidants have to the similar to substrate that is being treated. “Intrinsic problems” (or “inherent vice”) is an expression that places the blame for stone decay squarely on the material, rather than the particular environment” (Eric Doehne, 2010).

Air pollution has been considered by many as one of the prime causes for stone decay. Sulphur oxides, nitrogen oxides and carbon dioxide, are generally considered as primary or traditional pollutants. This along with human interference has increased the amounts of pollution. All these are capable of dissolving in water and creating an acidic solution thereby affecting the calcareous materials. The effects of air pollution have been thoroughly studied in the 1970’s to 1990’s, however since the 1990’s more importance has been given to the effects of sulphur dioxide. In spite of the effects of air pollution being studied only from the 1970’s, Brimblecombe observed that this problem has been present since antiquity. The effect that air pollution has on calcareous stone is heavily dependent on the surrounding environment. It has been noted that if the stone is exposed to rain, the stone will gradually ebb and if the stone is not directly exposed the reactions will form black crusts on the surface of the stone (Eric Doehne, 2010).

The black crust (Fig.1.6) is essentially formed by the carbonaceous particles formed due to the burning of fossil fuels. These particles contain metal oxides that catalyse the sulphur dioxide oxidation which forms the black crust. It has also been suggested that the black crust is formed owing to biological factors in unpolluted areas. Ortega-Calvo has suggested that the ideal habitat for cyanobacteria is through the steady dissolution of the sulphates, which are formed due to microflora. Mansch and Bock have found that the nitrifying bacteria is also a great cause of stone decay that is associated with air pollution and the formation of black crusts. According to Schiavon, Chairi and Fabbri polycyclic aromatic compounds (organic compounds) are strong markers contributed by vehicular pollution on limestone. Carbon dioxide also plays an integral role in stone decay and is primarily due to climate changes. Nowadays climate change, though not considered by many, is a severe deterrent to cultural heritage especially in areas ridden with droughts, floods, and varying humidity cycles which lets microflora flourish on the stone surface and in turn increases the damages caused by crystallisation of salts (Eric Doehne, 2010).

Figure 1. 6: Black crust formation on marble statues (Adapted from: https://viajestic.atresmedia.com).

Sulphates too play an important and powerful corrosive agent which is present in acid rain which affects stone materials. Fossil fuels and coal combustions have the ability to release sulphur into the atmosphere. The SO_2 gas has the ability convert to sulphurous acid (H_2SO_3) in the presence of water in the atmosphere which affects the Relative Humidity. In urban industrialised area, smog also acts as an agent of degradation which SO_2 oxidises to SO_3 assisted by ozone and other organic compounds (Winkler, 1997). The role of natural and artificial pollutants is shown in Fig.1.7.

Sulphates play a dual role in attacking carbonate rocks – dissolution due to sulphuric or sulphurous acid and changing carbonates to calcium sulphate or calcium sulphite. Chlorine, is also an important component of the atmosphere which may convert to hydrochloric acid aiding in the dissolution of carbonate rocks (Winkler, 1997). The other atmospheric agents of decay can be moisture from rain water, ground water, leaking pipes and gutters, dusts, wind action, severe temperature changes, fog, relative humidty, repeated wetting and drying cycles, heating and cooling cycles and soluble salts (Rachel Douglas-Jones, 2016).

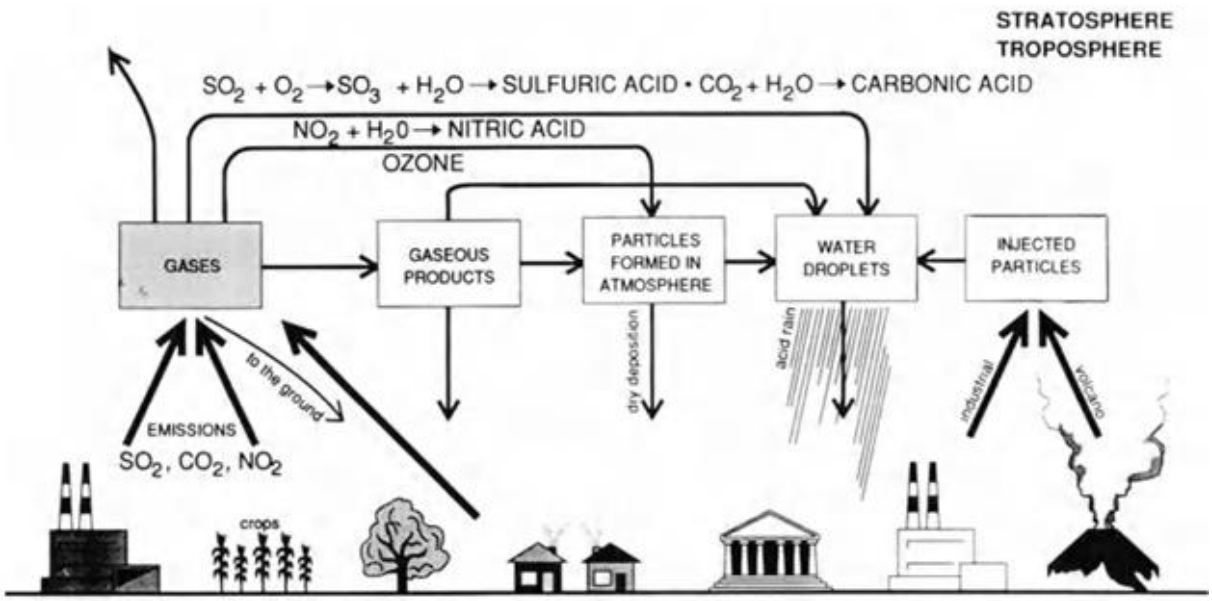


Figure 1. 7: Role of artificial and natural pollutants on materials decay (Adapted from (Winkler, 1997)).

2.2.2. BIOLOGICAL AGENTS

Microorganisms are one of the main causes for degradation/deterioration of stone materials. Microorganisms can flourish in two distinct environments – when the material is used as a nutrient for the microorganism (assimilative process) when the metabolites are formed by these microorganisms (non-assimilative process) (C.Gaylarde M. S., 2003). Microbial degradation/deterioration is dependent widely on climate and exposure and each and every case is different and need to be treated differently (Warschied, 2002).

There has always been a lot of controversy and discussion on whether biological growth is an important agent for decay of cultural heritage. The growth of microorganisms on the surface can be regarded both and a boon and curse to cultural heritage. Some studies suggest that after the initial physical and chemical weathering, the rock is attacked by the microorganisms as a result of the improvement in nutrients. The primary signs of biodegradation /biodeterioration is staining followed by the presence of biofilms. Biofilms are used by the microorganisms to protect themselves from changes in the environment, however they alter the mechanical abilities of the stone (T. Warscheid, 2000).

The biodeterioration/biodegradation of stone materials has been studied since the 1960's where the focus was mainly to study how these organisms changed the appearance on surface of the stone, the degradation/deterioration of the stone itself and the characterisation of the microorganisms. More recent studies show the effects of biofilm on the structures (Eric Doehne, 2010) and the influence of water on biodegradation /biodeterioration (T. Warscheid, 2000). The

microbial proliferation is also dependent on the material of the stone's porosity and permeability along with the environmental factors (T. Warscheid, 2000).

In more temperate climate with regular rainfall a mixture of microorganisms are found on the top layers of the building material i.e. stone, but this depends greatly on the porosity of the stone. In most circumstances this causes only discolouration and in more severe cases this leads to the formation of crusts, salts accumulations and oxidation. In case of semi-arid regions where the temperatures are higher with comparatively less rainfall the most common microorganisms founded are cyanobacteria, yeasts and lichens. The most varying microflora is found in arid climate where microorganisms like cyanobacteria and mineral oxidising fungi are found. Tropical climates which is marked with high humidity and temperatures allowing a diverse microflora to survive which are capable of extensive biofouling and biocorrosion. These microflora are extremely resilient and capable of sustaining itself under any kind of climate conditions and changes (Warschied, 2002).

According to the microbiota present and their interaction with environmental conditions, the development of microorganisms can be responsible by stains appearance, biofilms formation, fissures. Several studies has been focused on biofilms formation which seems to influence and change thermal, hygric and mechanical behaviour of the stone thereby assisting in its decay due to an increase in the surface wetting duration and providing a constant source of organic acids. Lichens can alter the surface of the structure it inhabits. The mechanical damage can be in the form of penetration of the hyphae into the stone and the contraction and expansion of the thallus due to the change of humidity. Chemical change is exhibited in three ways – secretion of oxalic acid, generation of carboxylic acid and generation of other acids that are capable of chelating ions such as calcium (Eric Doehne, 2010). It has been noted that the calcium oxalate forms a protective layer known as *scialbatura.* Scialbatura is generally yellow, red, brown or black patina, commonly found on white marble and at first glance is considered by many as dirt (Clara Urzi W. T., 1992). Scialbatura is most commonly found on Roman marbles that are exposed to the outside environment and the most famous example Trajan's column. There are various opinions on how scialbatura is formed. Some are of the researchers are of the opinion that scialbatura is formed as either a direct or indirect result due to some consolidations that were made on the stone. Other researchers are of the opinion that scialbatura the remainder of polychromy on marble. There are also others who believe that scialbatura is the work of lichen infestation as observed in the Parthenon and Colosseum (Marco Del Monte, A Study of Patina called 'Scialbatura' on Imperial Roman marbles, 1987). However, it has been observed that scialbatura doesn't form only on statues but on anything made of limestone or marble that has

been exposed to nature. The main composition of scialbatura is calcium oxalates – whewellite and weddelite and generally non-homogenous and also have an encrusted appearance. Nevertheless scialbatura is not an addition to the rock but rather a substitution of calcium by wheddelite and whewellite. This also explains the existence of lichens because the calcium oxalates are formed from the oxalic acids that are secreted from the lichens (Cristina Sabbioni, Oxalate Patinas on Ancient Monuments : the biological hypothesis, 1991). Analysis of oxalate patinas, has shown that calcium oxalates are formed when the oxalic acid attack the calcium carbonate in the substrate it has been seen that microbial reaction with organic materials complement the growth of calcium oxalate. The study of oxalate patinas also provides useful information regarding the impact of organic material on the formation of oxalate (Eric Doehne, 2010). On stone artefacts, man-made or artificial, the formation of patina has been observed. The most common type of microorganisms responsible for the degradation/deterioration of stone are sulphur-oxidising and nitrifying bacteria (C. McNamara R. M., 2015).

These binding material - mortars and plasters are commonly man-made and the compositions of these may vary during the manufacturing process and sometimes contain high levels of organic materials which are extremely vulnerable to biodeterioration (Stephanie Scheerer, 2009). According to Gorbushina and Krumbein any damage caused to stone, owing to microorganisms is referred to as bioweathering, sometimes better known as biodeterioration. However, it should not be surprising that any material of cultural heritage natural or man-made, will be subject to ageing, modification, alteration, decay and finally destruction (Clara Urzi W. T., 1992).

2.2.2.1 MACROORGANISMS

Apart from microorganisms, macroorganisms, too play an important role in growth of microorgnisms. Bird droppings, rat droppings, bat urine and mites are some of the major contributors.

2.2.2.1.1. Animals: Birds, bats and other smaller life forms

One of the most prevalent causes for aesthetic and chemical damage is due to bird droppings, particularly, pigeons. These bird droppings provide high nutrition to microorganisms especially chemoorganotrophic microorganisms which corrodes, due to discharge of acids, and the material is affected by these droppings. Apart from birds there are other smaller animals and insects that thrive on the acids secreted which also cause extensive damage. Some of the more

important insects that can cause damage are spiders, flies, mosquitoes, wasps, ants, mites and beetles (Clara Urzi F. D., 2001). Bats generally live near the ceiling area of the structures and bat faeces can sometimes lead to the collapse of buildings, and also create an area that becomes a breeding ground for other animals (Dennis Allsopp, 2004).

2.2.2.1.2. Plants: Mosses and Plants

In older structure, plant roots play an important destructive role. The plant roots climb onto and penetrate the structures, sometimes even supporting leaves and stems. These plants can cause mechanical damage in the forms of cracks, fissures and may cause severe aesthetic damage. With the vegetation covering the structures (Fig.1.8), water evaporation on these structures slow down, causing further damage. There is also an ionic change between roots and alkali cations of the stones, thereby causing chemical damage to the structures (Clara Urzi F. D., 2001). Vines and creepers are the most common plant life that can colonise structures. Repeated neglect does not help the case, either, as they in turn, harbour birds and animals, causing further damages. Trees can cause a huge problem too, mainly the growth of roots of bigger trees can lead to blockages of drains and destroy foundations of buildings. Tress can also cause a lot of indirect damages (Dennis Allsopp, 2004). Mosses, which are visible to the naked eye, cover the surface of the structures and are thick films that grow on the surface, causing considerable aesthetic changes on the stone materials. Additionally, they corrode the surface by releasing acidic metabolites, some which can dissolve surface of the material (Clara Urzi F. D., 2001).

Figure 1. 8: Macroorganisms – Trees and Mosses act as agents of deterioration (Adapted from: http://justfunfacts.com/interesting-facts-about-angkor-wat, http://sdhammika.blogspot.com).

2.2.2.2. MICROORGANISMS:

Microorganisms can induce damages in multiple ways – aesthetic, biogeochemical or biophysical. It is rare, if not impossible that only microorganisms are responsible for destruction of stone buildings. Similarly it is also not just one type of microorganism that is responsible for degradation. It is noted that initially the stone is subjected to physical or mechanical weathering making them susceptible for microbiological colonisation. The stone is first colonised by phototrophic bacteria that are light dependent. Phototrophic organisms allow the development of heterotrophic organisms, like fungi, to grow on the organic material which is gathered from the dead cells and debris. However, phototrophs organisms like cyanobacteria, algae and lichens are commonly detected on stone materials. Chemolithrophes derive utrition from inorganic chemicals. These organisms are sulphur oxidising and nitrifying bacteria and may thrive anywhere where there is even a little availability of sulphur and nitrogen respectively.

2.2.2.2.1 Light Dependent Growths – Phototrophs

2.2.2.2.1.1. Algae and Cyanobacteria:

Algae and cyanobacteria are commonly mistaken as dirt as these photosynthetic microorganisms, mainly discolour the surface that they cultivate on. These organisms are generally found on lower portions of the walls (Fig. 1.9), due to their capacity to retain water for longer. Cyanobacteria grow in a more or less homogenous pattern in tropical and humid climate (Dennis Allsopp, 2004). They are also known as green-blue bacteria, are endolithic organisms and can severely damage structures. They grow in a more or less homogenous pattern in tropical and humid climate. These microorganisms are oxygenic (May, 2010) photosynthetic bacteria that do not have a membrane-bound organelles, like chloroplasts.

Figure 1. 9: Cyanobacteria on interior and exterior walls (Adapted from: https://bakalchen.wordpress.com, right, https://www.qub.ac.uk).

In case of limestone, cyanobacteria completely dissolve the calcium carbonate, due to the production of organic acids. Soiling is probably the significant type of damage done by these sets of microorganisms. These microorganisms will grow in any place that has minimum amount of sunlight, hence they not only grow on stones but also on paintings or wood. They can grow very quickly, especially in tropics and sub-tropical areas that are very humid, and can be enormous patches of pink, green or brown. Cyanobacteria is extremely unaffected to shrivelling on dry surface owing to the pigments in the cell (Dennis Allsopp, 2004). The stains that cyanobacteria leave behind are dark brown and black patinas and sometimes can also be pink. They are capable of living in extreme temperatures due to their thick outer wall and the ability to produce pigments like scytonemin, which help protect them during extreme dryness. Cyanobacteria is responsible for most staining, once the area affected by these microorganisms absorbs more sunlight (Pinna, 2017).

Algae (Fig.1.10) are single-celled microscopic filamentous lacking vascular tissues like complex higher plants. These are found in areas of stonework where there is ample light and water or humidity (in cases of tropical regions). Algae and cyanobacteria cause mechanical damage by cracking, widening pre-existing cracks on the stone and is generally due to the action of frost. They generally grow inside the stone (endolithic organisms), destroying the stone completely. They are generally and most commonly found in wet and rainy areas and on archaeological materials like buildings, mosaics, frescoes, paintings. Algae form coloured patina of green, grey, black, brown and orange and have gelatinous layer. Microalgae largely promotes the growth of other organisms and provides nutrition for other heterotrophic microorganisms. Algae are also sometimes the cause of cracks and cavities on the structure as they leach away the calcium, silica and magnesium (Pinna, 2017). They are autotrophic and therefore can grow in artificial or natural light and damp areas (J.Karbowska-Berent). Algae are one of the main reasons for fouling which stain the stone like the red stains that are seen on marble, are caused by *Haematococcus pluvialisi*. Algae community grow on the heterotrophic bacteria which survive on trapped organic bacteria. Stone decay is prompted due the repeated wetting and drying cycles and thereby loosening the stone. Algae, especially, play an important role in the decay of marble and limestone due to water retention and facilitating the growth of microbes (May, 2010).

Figure 1. 10: Algae on exterior wall due to water leakage and humidity (Adapted from: http://www4.rgu.ac.uk, http://www.lefigaro.fr).

In spite of both algae and cyanobacteria are phototrophs, algae immensely differs from cyanobacteria with respect to cell structure, pattern of reproduction and resistance to environmental changes. Both, cyanobacteria and algae generally grow on the exterior of the structure, but in the long run can cause some serious internal damages too. Phototrophs are the first microorganisms to grown on the surface and then pave the way for heterotrophic bacteria, fungi and other microorganisms (Dennis Allsopp, 2004).

2.2.2.2.1.2. Lichens:

Lichens are composed of two parts – mycobiont fungi and photobiont algae or cyanobacteria (Fig.1.11).

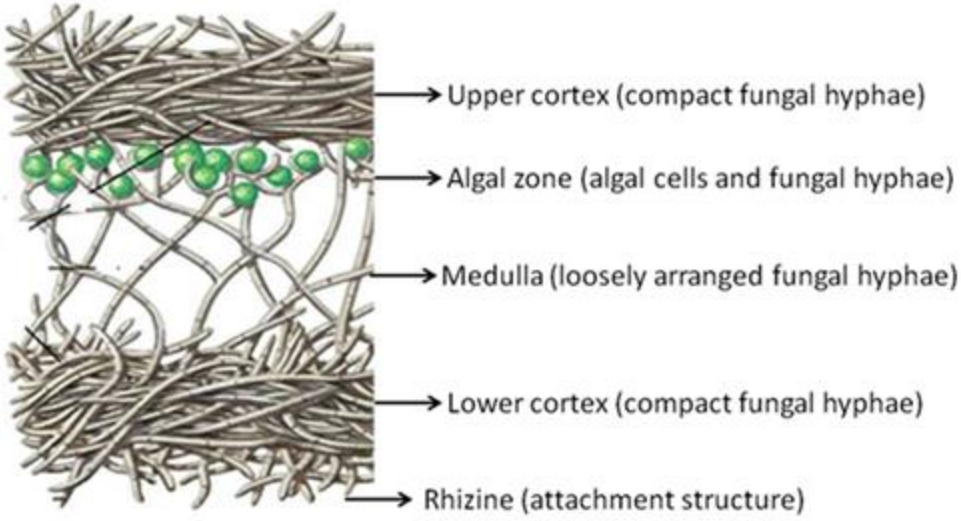


Figure 1. 11: Lichen Morphology (Adapted from**:** www.plantscience4u.com).

These two parts together produce a thallus (Fig.1.12). There are four different types of lichens that have been observed – crustose, foliose, fruticose and endolithic lichens (Fig. 1.12). Crustose form very close to the stone and are very difficult to remove. Foliose thalli breach/infiltrate less into the stone surface and have thread-like anchor devices and fruticose lichens are attached to the substrate by a button-like structure. Foliose and fruticose lichens are generally easy to remove without damaging the stone greatly. Endolithic lichens are found only on calcareous stones where they grow inside the stone and can be only observed when the fruiting body emerges from the stone.

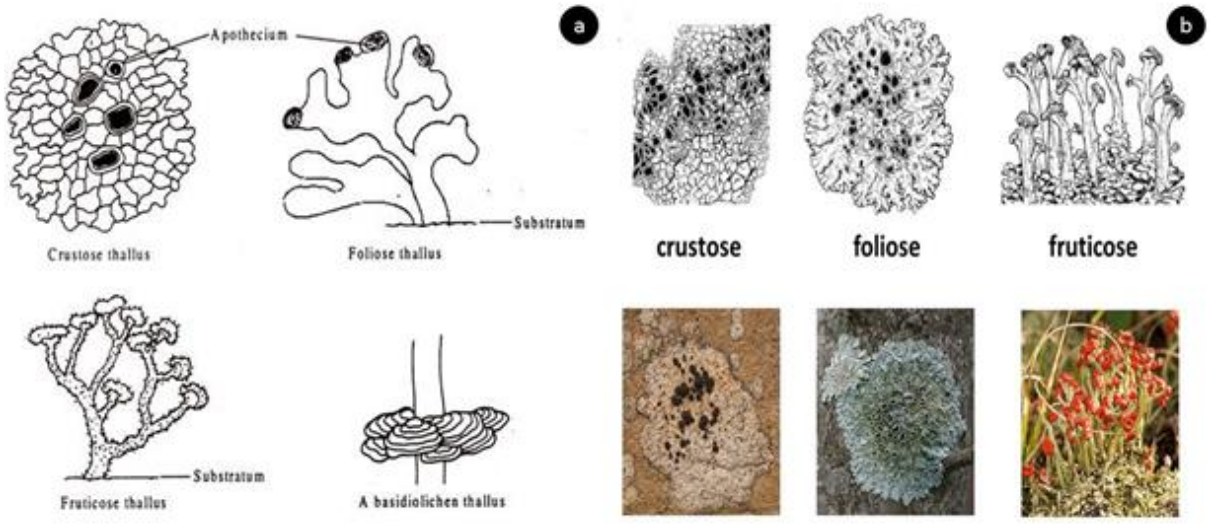


Figure 1. 12: a) Lichen thallus b) Different types of lichens (Adapted from: www.biologydiscussion.com, www.ohioplants.org).

Lichens are not restricted to particular temperature or humidity and can be readily found on any stone and hence are observed from tropical areas to polar areas. They are extremely strong withstanding prolonged periods of dehydration and then reabsorbing water and quickly rejuvenating. There are various lichens, which prefer both siliceous or calcareous rocks and some are found only on the surface or inside the rock (Fig.1.13). Lichens excrete organic acids – oxalic acid – as metabolic product and these remove the calcium and silicon from the stone. The root of the lichens are called rhizines which allow penetration of water, increasing erosion of the stone. Research suggests that the crustose lichens protect the stone from weathering and should only be removed after careful consideration. It has been seen that removal of lichens can leave the stone more porous and more inclined to chemical and biological deterioration. However, if the lichens are left on the stone, the stone will eventually degrade. Some of the common degradation observed when there is a lichen infestation is the flaking of the stone and

also a dirty appearance of the building which is due to the dark colonies created by lichens. Lichens are very sensitive to atomospheric and industrial air pollution and are more frequently observed in smaller towns than largae urbanised cities. With the increase in lichen infestations, sometimes the headstones observed in cemeteries are disfigured and the same effects are seen on carvings, inscriptions and niches in buildings in tropical areas. Lichens growth is heavily encouraged by wind action which acts as nutrient source for their growth. Monuments which are near large population of birds too are affected by lichens as the bird droppings act as a source of nutrient for lichens to survive (H.G.M. Edwards, 1997).

Figure 1. 13: Lichens colonising outdoor statues (Adapted from: lh3.googleusercontent.com).

Recently several biocides - quaternary ammonium salts, and herbicides, such as Diuron and dithiocarbamates - have been tested for the removal of lichens. It is of grave importance for the conservator to remember that the biocide used for the eradication of lichens should be slightly alkaline, if not pH neutral and not allow the formation of salts.

There are several instances when the growth of lichens and microorganisms have added a look of age to the cultural heritage which removed can leave the heritage looking bare and glaring, and the removal may also contribute substantially to the degradation/deterioration of these materials. This is very viable especially in the case of biofilm formation, where the biofilm actually forms a protective layer preventing degradation and stabilising the fragile surface of the stone (Eric Doehne, 2010).

2.2.2.2.2. Inorganic Material Dependent Bacteria – Chemolithotrophs

2.2.2.2.2.1. Sulphur – oxidising Bacteria and Nitrifying Bacteria

These bacteria convert inorganic sulphur into sulphate, which can cause damage in the form of sulphuric rain. The bacteria that generally attacks stone are *Thiobacillus thiooxidans*, *Thiobacillus thiosporus* and other thiobacilli bacteria (Fernanades, 2006). However it must be noted that sulphuric acid can also originate from atmospheric pollution so the exact role of sulphide in decay of stone, is still unknown.

Figure 1. 14: Effects of Sulphation on marble capitals (Adapted from: www.ysma.gr/en/conservation-degradation-phenomena, https://pubs.usgs.gov).

The action of heterotrophic bacteria is very controversial but it is know that they play an important role on the soiling process. It is suggested that they secrete calcium sulphate which breaks down the stone and these sulphate reducing bacteria in the soil produce hydrogen sulphide as a part of their metabolism and this is carried as a solution porous stone through the capillaries due to damp. Autotrophic bacteria is sulphur oxidising and use the sulphide that is oxidised as an energy source which produce sulphuric acid. This acid attacks the stone yielding calcium sulphate forming scales on the stone surface (Fig.1.14). Nitrosomonas and Nitrobacter are nitrifying bacteria also hampers the stone by dissolving the calcium (C.Gaylarde m. S., 2006). In a deteriorated stone, where sulphate levels are already low, they oxidize with the ammonia from the air forming nitrates react with the calcium carbonate of calcareous rock and form soluble calcium nitrate. This calcium nitrate is then leached with rain water leaving behind the loose silica powder, decaying the stone further. Nitrifying bacteria oxidise inorganic nitrogen for energy and use this to make acidic products like nitrous and nitric acid (Tiano, 2002) (Fernanades, 2006). There are two different types of nitrifying bacteria – firstly the bacteria that use ammonia to oxidise and create nitrous acid. The second type of bacteria uses the nitrous acid and oxidises it to form nitric acid secondly this is oxidised to nitric acid.

Nitrifying bacteria will only thrive if the substance has ammonia or nitrate. Ammonia can be fixed on the stone in form of dust of ammonium salts and nitrite is due to the pollutants from automobiles, soil or factories. Nitrifying bacteria can be found on stone if there is abundance of ammonia or nitrite and with increasing air pollution, these group of bacteria, play an aggressive role in stone decay (May, 2010).

2.2.2.2.3. Organic Material Dependant Growths – Heterotrophs

2.2.2.2.3.1. Fungi

The most characteristic feature of fungus is its tolerance towards light and change in temperature and humidity, allowing them to grow easily on damp walls and stone surfaces (J.Karbowska-Berent). Fungi can be found on stonework and are very resistant to dry conditions and high UV radiation. Genera like *Botrytis* sp., *Mucor* sp., *Penicillium* sp. and *Trichoderma* sp. are found with the secretion of citric and oxalic acid and can result in the dissolution of silicates and results in the degradation of a stone (Dennis Allsopp, 2004). The most common fungi that affects stones is Hyphomycetes (Milica V. Ljaljevic Grbic, 2009)and as they lack any sexual structures, usually common to other fungi are called "fungi imperfecti" and are commonly known as moulds. *Aspergillus* sp., *Fusarium* sp. *and Penicillium* sp. are also a part of Hyphomycetes. These strains can develop black, dark grey, or brown colonies and are also called dematiaceous fungi. These Hyphomycetes class of fungi can secrete organic acid – acetic, oxalic, formic, fumaric, glyoxylic, gluconic, tartaric – which breaks down weaker section of the rock. The strongest acid is oxalic acid and has the capacity of degrading minerals. The anion oxalate plays an important role in the transforming the mineral and result in dissolution of the mineral. The most common fungi that are found is *Aspergillus* sp., *Penicillium* sp., *Cladosporium* sp., *Aureobasidium* sp., *Alternaria* sp., *Chaetomium* sp., *Acremonium* sp., *Ulocladium* sp. and *Stachybotrys* sp.. Fungi is more hazardous than bacteria and dissolves rock phosphates by the chelating reaction of the oxalic acid (Gadd, 2007) .

Filamentous fungi are vastly responsible for flaking, degradation of exterior walls and discolouration (Christine Hallmann, 2011). Mostly they form dark pigments on exterior walls and are found along with phototrophic bacteria. The substrate plays an important role in the formation of colonies. In case of a fungal infestation, the walls get discoloured to grey. Fungal and cyanobacterial growth are generally attributed to the porosity of the walls, therefore the walls that retain moisture for long, are generally a very good places for fungal colonisation.

Filamentous fungi, along with phototrophs and bacteria (actinomycetes), slime moulds, protozoa and small animals (mites) are usually found in biofilms (Dennis Allsopp, 2004).
Fungi which is xerophilic can grow without depending on the RH whereas bacteria need a very high RH to survive, with an exception of salt-dependant bacteria. For a microorganism to gather/generate water in dry environment it either needs to acheive an osmotic equilibrium or use energy to pump water through its cell walls. It is in this environment that biofilms are created where the microflora is embedded within a slime layer. Biofilms are a protective layer formed It is of utmost importance to understand the reasons and the type of decay before the start of any consolidating work. It should be kept in mind that though the material might be the same, their chemical properties can vary greatlyover the microorganisms which balances out any change in humidity and temperature (Warschied, 2002).

2.2.2.3.2. Actinomycetes

Actinomycetes are filamentous heterotrophic bacteria, can be terrestrial or aquatic, with ability to colonise stone surfaces. They are very similar to fungi but the filaments are finer than the fungi. Actinomycetes also have the capability of producing various pigments and can survive in extraneous circumstances with low water and harsh sunlight. Actinomycetes can cause mechanical damage to the stone due to hyphae penetration. The myceli of the actimycetes give them the ability to spread over a larger surface and in turn gives them a greater area for penetration on the stone, which increase the surface of the biofilm. *Streptomyces* is a group of actinomycetes that are capable of causing great damage to limestone (May, 2010). Actinomycetes can be both terrestrial and aquatic (Stephanie Scheerer, 2009).

3. BIODEGRADATION / BIODETERIORATION OF STONE MATERIAL

Stone though is an extremely strong, robust and sturdy material, however colonisation by microorganisms, like bacteria, fungi and algae, lichens and plants play a very important role in its decay. Alsopp describes biodegradation as “the harnessing, by man, of the decay abilities of organisms to render a waste material more useful or acceptable”. Eggins and Oakely describe the biodeterioration as “a process that decreases the value of an object because it implies a damage to it”. Biodeteriogen is an organism which can cause damage to any material, though it is any organism that is discovered on various objects, whether the organism causes any damage to the substrate or not. When microorganisms utilise the substrate as a food source or for support for development, this evolution is called the process of bio-deterioration. Bio-

deterioration can cause visible changes to the substrate and cause severe physio-chemical damages to the substance or the material (Dennis Allsopp, 2004).

Biomineralisation is the process where microorganisms go through a metabolism process that causes precipitation which accumulates minerals on the substrate. The minerals produced by microbes and lichens consist of iron hydroxides, magnetites, manganese oxides, clays, amorphous silica, carbonates, phosphates, sulphates, sulphides and oxalates. Iron and manganese are removed and re-oxidised on the surface of the stone, forming patinas and crusts. If the metabolism of the microorganism depends on iron, the crusts formed on the stone surface will contain ferric oxides or oxyhydroxides (Pinna, 2017). The most common type of microorganisms associated with deterioration of structure or building materials are bacteria (autotrophic and heterotrophic), cyanobacteria, fungi and algae. The growth of these microorganisms depend on the availability of food and water and requirement is fulfilled by on the attributes of the material (substrate) already mentioned earlier (C.Gaylarde M. S., 2003).

These natural stones can include marble, limestone, sandstone, granite and artificial stones that comprise of ceramics, plasters, mortars, bricks and concrete. Natural stones differ from each other in terms of texture, porosity, pH, hardness, their chemical composition, and their reaction to microorganisms and biological growth. The method and means of how these stones react to biological growth, their vulnerability to this biological growth and to biodeterioration is called bioreceptivity. According to Guilitte, this concept can be defined in three parts: (1) bioreceptivity indicates the susceptibility of the stone to be colonised, (2) bioreceptivity is caused by the biotic and non-biotic factors and (3) bioreceptivity is caused by the nutrient that is contained in the stone itself - dead biomass, dust particles, animal faeces, water repellents and consolidants, biocides, etc (Guillitte, 1995)

Materials that have high porosity which leads to high absorption of water are likely to be more susceptible to biocolonisation. Surface roughness, too, plays an important role, - rougher surface will cause the stone to retain more water and moisture in micro-fissures leading to more biological activity. Moreover a rough surface provides sheltered areas where the micro-organisms can grow. Pore size is also important, as a higher amount of micro-pores in a certain area the higher the water retention and the rate of evaporation is lower, thereby making the micro environment perfect for microbes to flourish. Microbes are very susceptible to the pH of the environment. Microorganisms prefer a neutral pH most of the time and do not flourish in extreme pH, although it has been noted that some strains of fungus prefer acidic pH whereas cyanobacteria prefer alkaline conditions. Thus, bioreceptivity is very important to understand as it imparts information on how healthy stones are colonised and is generally studied in the

laboratory (Pinna, 2017).

A large host of microbial community is found in historic limestone and may contribute massively to biodegradation/biodeterioration. The microbial communities mostly found on limestone includes fungi, algae, cyanobacteria, heterotrophic and chemolithotrophic bacteria both on biofilms and on endolithic communities (C. McNamara T. P.-D., 2006).

There are two kinds of damage that generally take place on the stone surface simultaneously owing to damage caused by these microorganisms – aesthetic and structural. The damage generally starts with aesthetic changes and progresses to flaking, cracking and final dislocation of the stone (J.Karbowska-Berent). Degradation of calcareous stone is mainly due to dissolution of the matrix which takes place with physical, chemical decay and biodegradation. In calcareous lithotype, the material changes on the account of calcite leeching which changes the porosity and the mechanical stability of the stone. A conservator and restorer has to be extremely cautious while conserving stone building as artificial and synthetic consolidants can further damage the stone with change in colour, crust formation, growth of microorganisms and glossy appearance which is further compounded by the effects of air pollution (P.Tiano).

The changes on the stone surface is attributed to weathering and associated with the mineral composition of the stone. Weathering depends significantly on the composition of the stone – mechanical properties, solubility, porosity and the history of the stone (referred to as memory). Stones that have high porosity will degrade and weather faster as opposed to stones with low porosity (May, 2010) (Winkler, 1997).

It has been observed that the hydrocarbons in the air react with microorganisms to attach themselves to buildings. The microbes use the carbon from the hydrocarbon to produce corrosive acids. The primary causes for this is nitrogen dioxide and sulphur dioxide, from pollutants, which helps in the acceleration of biodeterioration. Nitric and sulphuric acid causes acid rain to degrade stone further converting carbonates to sulphates and soluble nitrates (Crabbe, 2009). This condition is not helped by the presence of other agents like dust, organic pollutants and remaining hydrocarbons. All these result in a black crust formation which is principally due to atmospheric particles – dust, heavy hydrocarbons, pollens and spores. Other kind of degradation that is observed is nitratation and sulfation damage. Pollutions due to nitrates, sulphates, crust formation, incrustations and patinas is responsible for weakening the stone matrix (May, 2010).

The optimal condition for microbes to colonise and develop, depends on the material used and environment and the climate has to be conducive for the microbes to grow. Climatic factors include temperature, humidity, rain, precipitation, shadowing, exposure to the sun and

pollutants. In case the damage is only on the exterior it is called aesthetic damage and if the damage penetrates deeper into the layers of the surface it can be classified as physical/mechanical damage or chemical damage (Clara Urzi F. D., 2001). Table 2 describes the factors that influence colonisation and biodeterioration.

Table 2: Factors influencing colonisation and biodeterioration (Clara Urzi F. D., 2001).

Climatic and Environmental Factors	Sun, shadow, rain, temperature
Inorganic and Organic Pollutants	C/N/S source as growth inhibitors
Surface Bioreceptivity	Nature of the material, conservation, length of exposure
Treatments	Biocides, surfactants, hydrophobic compounds

3.1 EFFECTS/CONSEQUENCES OF DEGRADATION/DETERIORATION ON STONE

3.1.1 WEATHERING AND EROSION

One of the most common problem plaguing marble is weathering. All natural elements – wind, rain and thermal changes play a part by affecting the polished surface. Erosion is also another commonly occurring problem affecting the marble. In erosion the marble surface, edges, carved details of the marble wears off with time giving the stone a very washed out grainy texture. Like weathering, this too, is caused due to natural agents and may lead to sugaring of the surface (Fig.1.15). However, there are many authors who do not separate these two kind of damage (V. Rives, 2006).

Figure 1. 15: Effects of weathering and erosion on stone (Adapted from www.marsnow.info, www.pinterest.com.mx).

3.1.2 CRACKING AND SUGARING

Cracking, is indicated by narrow fissures formed on the marble. These fissures leads to bigger cracks. Cracks can be owing to hard mortar, the corrosion caused by internal ferrous materials and structural issues. Marbles, depending on the impurities have natural veins in them. These veins maybe horizontal or vertical composed of clay, sand, silt or chert which is all present in the limestone before being metamorphosed to marble. These veins have various physical properties and weathering is not uniform and can lead to cracks. The marble crumbles leaving behind loose granules, and generally takes place when the binding material wears off due the dissolution by acidic water, leaving the marble surface looking granular and powdery (V. Rives, 2006). Apart from acidic water and salting, thermal changes are the reason for this damage, which permanently changes the volume, changing the calcite crystalline structure by fracturing it and dislocating the grains of the marble. This allows water penetration into the stone leading the loss of strength of the marble itself and can lead to complete dissolution (C.Gaylarde m. S., 2006). Examples of these effects are shown in the figure1.16.

Figure 1. 16: Effects of cracking and sugaring on stone **(**Adapted from: www.tutorgrafico.com, www.hirst-conservation.com).

3.1.3. SALTING

Soluble salts, along with air pollution, play an important role in the degradation of stone. Salts grow salt crystals inside the pores of the stones thereby destroying the stone, by creating mechanical stress within the stone and grind the stone to powder. Air pollution is a major reason for formation of salt crystals. Air pollution contains sulphates and nitrates. The soil is also partly

responsible for this condition, as salts may already be present in the soil used for construction. Other sources of salt formation are salts from sea water or from the winds in desserts, fertilizers, unsuitable cleaning materials, and unsuitable building materials and depending on the age of the buildings storage of salts for preservation of food supplies. Salts crystals generally grow owing to crystallisation which is generally caused due to the cooling of salts inside the stone itself. The most damaging salt formation is that of sodium sulphate which exists as an anhydrous salt thenardite (Na_2SO_4) or the decahydrate mirabilite ($Na_2SO_4.10H_2O$). The former increases it size three folds during its conversion to the later and this during dissolution and recrystallisation (Saidov, 2012). The decahydrate mirabilite, may further recrystallizes into metastable heptahydrate ($Na_2SO_4.7H_2O$). While studying stone decay with respect to salts, one must keep it mind that salt damages do not occur only when exposed to natural elements, namely outdoor surface but also due to the change in the hygroscopic action indoors. Salt formation is especially harmful if the stone is porous. In order to conduct a holistic study on the destruction of stone due to salts one has to not only study the amount of salt that is captured by the stone but also the rate of environmental fluctuations. Salt formation can at the end destroy the stone all together. The salt crystals settle inside the pores of the stone and exerts considerable pressure on the pore walls. If this pressure continues without the salt crystal being able to crystalise the stone will crumble. If the solution oozes out of the stone and squeezes out the crystallisation will not harm the stone. It must also be noted that salt crystallization is generally difficult to study because different stones have different pore sizes and the damage cannot be noticed until and unless these pores get full with the crystals and hence cannot be predicted. Secondly as the stone pores fill up with salt, these crystals have the ability to create more pores where salt crystallisation can occur. Salt crystallisation also is aided with the help of wind activities. This is also called alveolar weathering (Fig.1.17).

Alveolisation is a kind of differential weathering possibly due to inhomogeneities in physical or chemical properties of the stone. Alveolisation may occur with other degradation patterns such as granular disintegration and/or scaling. In arid climates large alveoles of meter size are frequently formed (e.g., Petra Jordan)" When this action takes place the salts deposits in the hollow area of the sheltered areas which is not washed away with rains and these give rise to endolithic microorganisms. It should also be noted that salt formation is capable in these areas as these areas also tend to dry the last and the moisture contents vary greatly in these cavities, thereby aiding the in the erosion in the deepest areas. This kind of erosion is helped by the nearby dust from nearby plays or sea salts.

Figure 1. 17: Effects of alveolisation on exterior walls of building (Adapted from: www.buildercorp.pl, www.wikipedia.com).

The location of these salts is greatly influenced by the rate of air exchange on the surface of the materials and the wind helps in the crystallization of these salts. This kind of salt weathering is also influenced by the rate of evaporation and the differences in the drying time. When the evaporation rate or drying time is short the salts generally accumulate on the surface. When the drying period is longer the salts collect in the sheltered pits, thereby resembling a honeycomb (Eric Doehne, 2010). We must remember that crystallisation is not the only way that salts can damage. Salts can also damage through different thermal expansion.

Salts also weather stones containing clay materials. Calcite is a porous material, and these salts can dissolve the calcite and alter biotite, quartz and feldspars. It is also noted that halophic bacteria are often present in this kind of weathering and sometimes aid the damage. Frost damages are very similar to salt damages (Eric Doehne, 2010). Damages due to salting is mostly physical in nature which leads to blistering, flaking, cracking, scaling and disintegration(Fig.1.18) (Stephanie Scheerer, 2009).

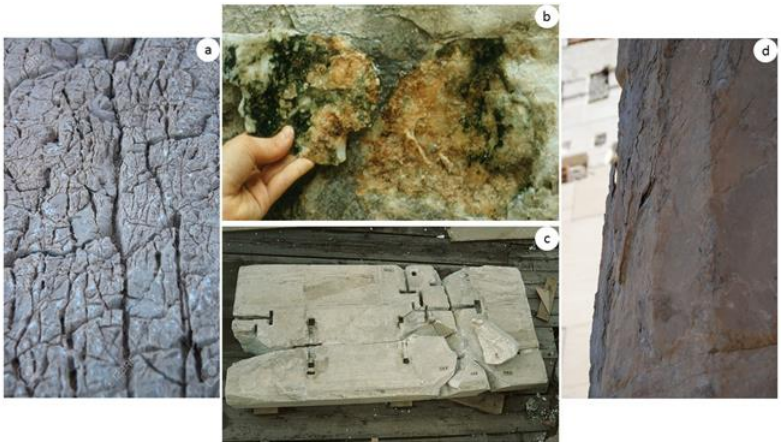


Figure 1.18: Effects of salting a) dislocation, b) breakage and c) flaking (Adapted from:

www.repository.acropolis-education.gr).

3.1.4. BIOFILM FORMATION

Biofouling or biosoiling is the alteration caused by a layer of microorganism on the stone surface. Biofouling can make the appearance of any structure look disagreeable or be unpleasing to the eye. The microorganisms have the ability to trap dust particles from the air which then soils the stones aids in the growth of plants. Biofouling is also responsible for damages on the stone surface. The effect of microorganisms can result in black patinas, stains, lack of adhesion, pitting, efflorescence and it is of utmost importance that the microorganisms responsible for this damage is recognised as it is important for conservation and preservation. Biofilms grow as colonies in a self-made extracellular polymeric substance (EPS) (Fig.1.19). With the development of these micro-colonies additional species called the secondary colonisers develop. Both autotrophic and heterotrophic microorganisms are found in the biofilm. The main interaction of the various species in the biofilm is cross feeding and metabolic exchange. Phototrophic microorganisms which grow with the help of photosynthesis in-turn sustain the growth of the heterotrophic microorganisms. Heterotrophic microorganisms help the growth of cyanobacteria. Biofilms are made up of EPS, multivalent cations, biogenic materials and inorganic particles. The main constituent of EPS is polysaccharides along with lipids, nucleic acids, proteins and humic substances. The matrix of the EPS is a three-dimensional, gel-like and inside it the microorganisms are charged (Pinna, 2017).

EPS acts as a physical barrier protecting the microorganisms from harmful substances like biocides and prevents the penetration of conservation materials. Biofilms helps the microorganisms grow by weakening the mineral lattice due to wetting-drying cycles and expansions and contractions. This can adversely affect the rock by changing pore-size, hardness of the surface, weight of the stone, alteration of thermal conductivity to name a few. In spite of this EPS can be advantageous as they form a protective layer around the stone surface (Stephanie Scheerer, 2009). EPS acts as an adhesive which glues the cell of the microorganisms to the structure, increasing enzymatic activities which forms a protective layer (J.Karbowska-Berent).

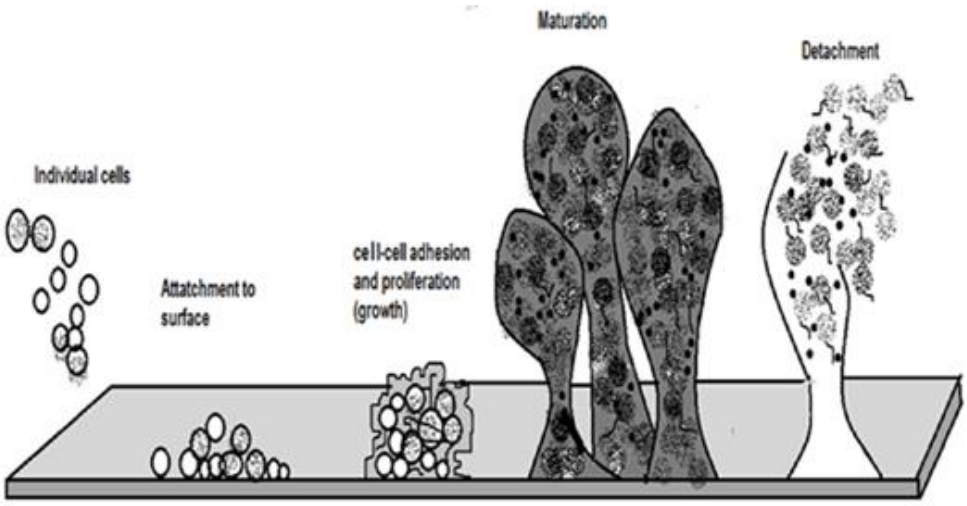


Figure 1. 19: Biofilm Life Cycles – attachment, growth of colonies and detachment (Adapted from: www.cell.com).

3.1.5. DISCOLOURATION

One of the most common problems owing to biodegradation/biodeterioration is discolouration and is mainly considered as an aesthetic problem. Discolouration maybe generated due to pigments, which maybe caused by the microorganisms like melanin, carotenes and other photosynthetic pigments. In sulphur polluted environments, the limestone can be converted to gypsum due to sulphur cycle bacteria. Darkening of areas can also be the work of fungi and cyanobacteria or airborne particles that are trapped in EPS. However discolouration can also be due to the amount of sunlight that is observed by a particular area. There can be increase in physical stress (expansion and contraction) by area that absorb more sunlight. Though we already know that black crusts are caused mainly due to vehicular exhausts, microorganisms may use the carbon as nutrients and thrive. Thin black crusts is mostly due to the action cyanobacteria in tropical and sub-tropical areas or fungal in moderate climates. Black crusts are especially harmful for porous stones as they might block these pores which can lead to water retention and finally break the stone (Fig.1.20) (Stephanie Scheerer, 2009).

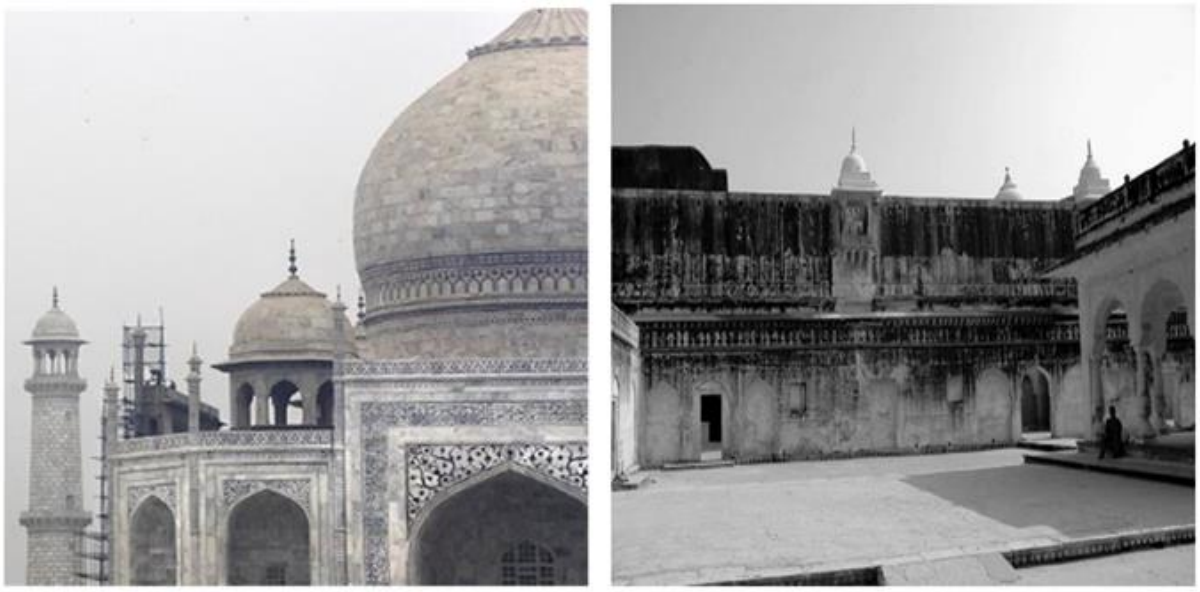


Figure 1. 20: Discolouration on monuments in humid climate (Adapted from (Stephanie Scheerer, 2009) www.washingtontimes.com).

3.1.6. REACTIONS OF ACIDS

Acids such as nitric acid, sulphuric acid, sulphurous acid, carbonic acid and nitrous acid can lead to the weakening of the matrix and this can take action till the depth of the stone (Stephanie Scheerer, 2009). Acids like oxalic, citric, acidic, gluconic, malic, succinic acids along with amino acids, nucleic acids, uronic acids react with stone, making it soluble along with salt formation and complexation (chelation). Microorganisms release acids, the most common ones being oxalic acid formed by fungus and lichens mainly as calcium oxalates – monohydrate (whewellite) and dehydrate (weddellite). However oxalic acid plays a dual role – dissolution in siliceous stone and protection in calcareous stones forming a calcium oxalate. These oxalates form yellow or brown patinas and sometimes this may be regarded as an additional, attractive feature. However oxalic acid is found to be disadvantageous and corrosive for siliceous stone (Stephanie Scheerer, 2009).

3.1.7. SOILING:

In case of soiling, the object is simply compromised due to the presence of the organism or its dead body, excreta or metabolic waste or in form of droppings. Microorganisms such as fungus and algae can be found on the surface of materials because they can use the surface dirt as food supply. In several cases fouling can also be a film of microorganisms and their products and this is called biofilms. These layers of biofilms are an accumulation of the organic products of

biological activity of these micro-organisms (Dennis Allsopp, 2004). Table 3 shows the microbial activity that affects the durability of stone.

Table 3: Microbial activities which affect the durability of stone (Clara Urzi F. D., 2001).

OBSERVED EFFECT	*MICROBIAL ACTIVITY*	*MAJOR MICROORGANISMS INVOLVED*
Discolouration	Presence of pigmented cells or products	Algae, cyanobacteria, fungi
Retention of water	Physical presence Production of slimes	All
Stimulation of growth of heterotrophic and higher organisms	Presence of live or dead cells or cell products	Algae, photosynthetic bacteria, including cyanobacteria
Disaggregation of material	Penetration into and growth within stone	Fungi, actinomycetes, cyanobacteria, algae (also lichens)
Formation of patinas	Oxidation of translocated Cations	Iron and manganese oxidizing bacteria, fungi, cyanobacteria
Degradation (‘corrosion’)	Acid production	Fungi, bacteria, cyanobacteria (also lichens)
Weakening and dissolution of structure	Mobilization and chelation of ions	All
Alkaline dissolution	Uptake of H+ ions by cells	Algae, cyanobacteria
Disruption of layered silicates	Liberation of polyols (e.g., glycerol, polysaccharides)	All

4. MARBLE AND ALENTEJO REGION

4.1 GEOGRAPHY OF ALENTEJO:

The South-Central area of Portugal is the Alentejo region (Fig.1.21). The word “Alentejo” is derived from alem and Tejo, literally meaning “Beyond the Tejo” or “Across the Tejo”. This name is fitting as the region extends from the left bank of the Tejo River, in the North and ends near the Algarve area, in the South. In the East it is bordered by Spain and on the West it is flanked by the Atlantic Ocean (Marble The Divine Stone, 2015).

The history of Alentejo is marked with the exploration and exploitation of marble, approximately in the 370 B.C, by the Phoenicians, Visigoths, Celtics and Arabs. However, the systematic exploitation began with the Romans, during the urbanisation of Lusitania. The Romans used the marble for construction. They preferred the white marble from Estremoz and Vila Viçosa and used them not only in Portugal but also in Spain. With the decline of the Roman Empire and the arrival of the Arabs, the marble was reused (a trait started by the Romans) for mosques and palace (Marble The Divine Stone, 2015).

4.1.1. GEOLOGICAL SETTING

Alto Alentejo region marble extends from the region of Sousel and Alandroal. The Alto

Figure 1. 21: Map of Alentejo region (Adapted from: http://www.costa-alentejana.pt, https://www.edimaps.com/map-of-alentejo-portugal).

Alentejo region is the primary marble district of Potugal. The region from Estremoz and Vila Viçosa is better known as the Estremoz Anticline. The anticline is about 40 kms long and 5-8 kms wide in the NW-SE orientation and the marble is formed in the south-eastern area of the anticline (Luis Lopes, n.d.).

The marbles from Estremoz, since antiquity, has been one of the most exploited sources of marble in the Mediterranean. Portugal, has several mining areas throughout the country, the North has rich reserves of igneous rocks, especially granites, the southernmost area, in the Algarve region is rich in limestone breccia. Schists are found in Porto and in Barrancos, Alentejo. The area between Sousel and Andoral in the Alentejo region, is where the most unique marble is found. Estremoz Anticline is an elliptical geological structure, located within the greater Ossa-Morena Zone, where Lower Palaeozoic marble are found with an outcrop of $27km^2$ (L.Lopes, 2015)

The marble was used for constructing a tombstone in 370 B.C., commissioned by a Carthaginain captain. This was discovered by Father Espanca in Terena, Alandoral. Subsequently the Romans used the marbles for adorning their construction and with the fall of the Roman Empire, these marbles were re-used for other functions repeatedly. With the advent of the middle ages, marble was used for the construction of palaces, castles and other important buildings. With the coming of the 15^{th} century, these marbles were also exported to India, Africa and Brasil. In Europe, the Portuguese marble has been used for the construction of Jerónimos Monastry, Escorial Monastry, monuments in Rome, Louvre and Versailles. With the advent of the 20^{th} century and with new techniques of exploitation, marble from Portugal has been exported all over the world. (L.Lopes, 2015).

As stated by Silva and Camarihas in 1957, the stratigraphy of Estremoz Anticline include:

Precambrian Ediacaran greywackes, shales and black cherts. These outcrops in two separate elongated NW-SEtrending ribbons and forms the core of the Estremoz Anticline (Fig.1.22 and 1.23) (Jorge F.Carvalho, 2008).

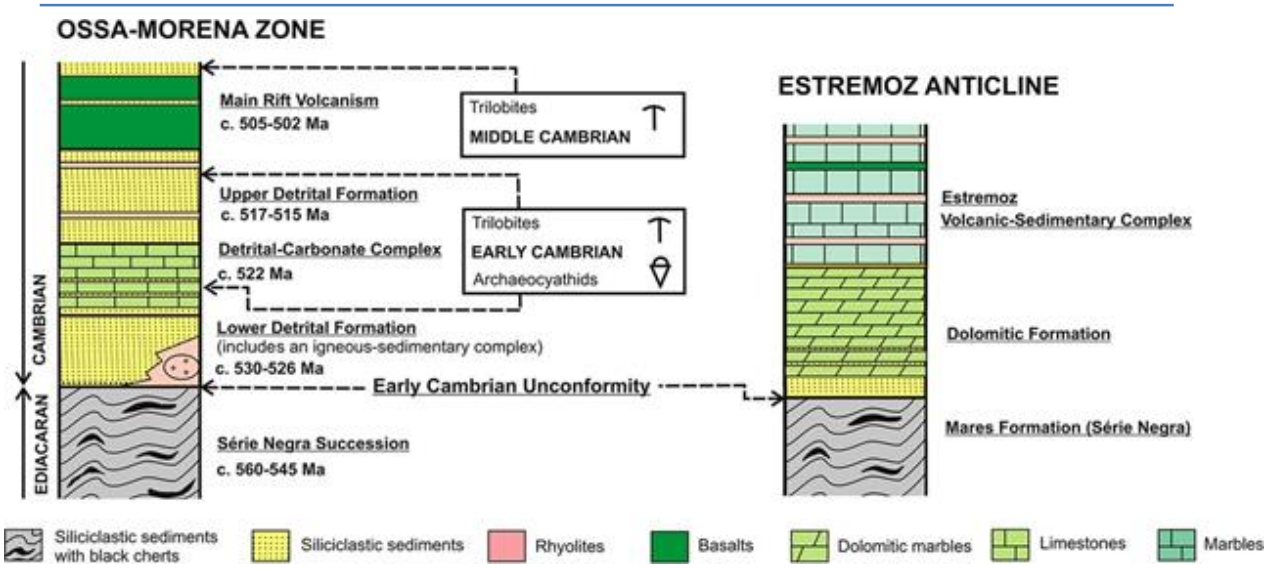


Figure 1. 22: Ossa – Morena Zone (Adapted from http://sp.lyellcollection.org/).

Cambrian arkosic sandstone at the base and on the top is the formation of dolomitic limestone A thick 50 mm-5 mm silica rich layer that sits on top of the dolomitic limestone. These are quartz and iron-rich, with pyrite forming iron-oxide crystals at the surface. Thick-bedded Cambrian-Ordovician limestone with interbedded basalts, rhyolites and shales. This succession is s the Voaalcanic-Sedimentary complex of Estremoz, used for creating ornamental objects of marble.

Shales, black shales and black cherts are the younger rocks from the Silurian – Devonian age, that surrounds the Estremoz anticline (L.Lopes, 2015).

The Volcanic-Sedimentary complex contains the Estremoz marbles along with calc-schists, metavolcanics, metapiltes, peltic schists and secondary metadolomites. The sequence underwent a regional metamorphism during the Hercynian Orogeny and were intruded by the late Hercynian plutons with NW-SE orientation. Intially the area around Estremoz had karsitic marbles with irregular marble outcrops which was covered with a thick layer of reddish soil – terra rossa (Devi Taelman aDevi Taelman, 2013).

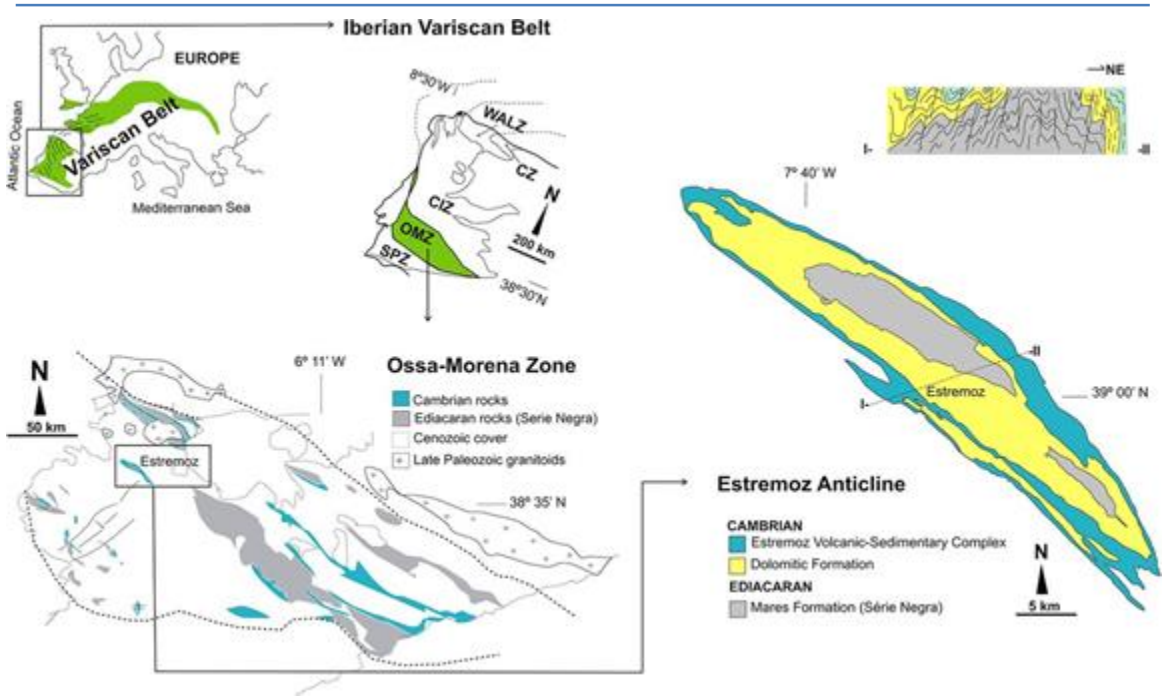


Figure 1. 23: Estremoz Anticline (Adapted from http://sp.lyellcollection.org/).

4.2 CONVENT OF THE MALTESAS

The Convent of Maltesas is located in Estremoz (Fig.1.24), a small municipality of Portugal in the Alentejo region. Estremoz, along with Borba and Vila Viçosa is internationally known for its marble trade which occurs in white, cream and pink. The marble from Estremoz has been used for the construction of Monastry of Jerónimos, Monastry of Batalha, Monastry of Alcobaça, the Tower of Belém, to name a few. Therefore it is not at all surprising to see the Convent of Maltesas, situated in Estremoz itself, is made from this marble (Crespo, 2ª Edição).

Figure 1. 24: Map of Estremoz (Adapted from http://www.vacances-location.net/).

Founded in 1501 – 02 by King Manuel I, the work on the Convent started around 1521 and that is probably when the work of the ground floor began. The church is situated on the eastern side of the main square (Rossio Marquês de Pombal) in Estremoz. Today the church is surrounded by the Church of San Francisco, Museum of Estremoz and the Câmara of Estremoz. The area is overseen by the Castle of Estremoz. The entrance of the church is marked with the Cross of Christ. The designated Maltese Convent, or Igreja de São João da Penitência, was instituted in 1519, being the only monastery of the Knights of Rhodes in Portugal it was subsequently incorporated into the Order of Malta (Fig.1.25).

Figure 1. 25: a) Present day aerial view of Convent of Maltesas, b) Layout of the cloisters (Adapted from http://www.monumentos.gov.pt).

The church is contemporary of 16th century structural design, boasting of Manueline architecture (Fig. 1.26). The arches in the lower register consist of twinned lanceolate arches. The interior spaces are covered by vaults of warheads, and edges with zoomorphic and anthropomorphic decoration. The area was enlarged in the 17th century with a second archway, supported by Tuscan pillars. The vaults are made of marble. Each wing has ten arches, subdivided into four twin arches and two simple ones. The stems (central part of the columns) are supported on square bases, whose capitals are indistinctly simple and smooth or with naturalistic motif. The church also brags of tiles which contain both geometric and vegetable motifs. However, unfortunately these tiles were vandalised and desecrated during the implementation of the Republic. The church was converted into a hospital between 1879 and1880. The main architecture and use of the church was changed immensely when it was turned into a misericordia. The Renaissance too left a mark on the convent, and this clearly

visible in form of the central tank in the garden, a quadrangular plant with a larger bowl of masks and a smaller one with heads of fauns.

In 1973, during the painting of the exterior elevations of the cloister, the primitive portal of the Room of the Chapter, of the Manueline period, was uncovered. It should have been entailed when building a new one in the smaller, eighteenth century. The inscription in Latin characters allusive to the episode of "Saint John in the Desert" that the eighteenth century work had covered up became visible again (Medreiros, 2001).

Figure 1. 26: Manueline architecture of Maltesas Convent (Adapted from http://www.monumentos.gov.pt).

The vast diversity of microorganisms involved in the biodegradation of cultural heritage makes difficult to understand clearly their particular role in the degradation process. However, it is explicitly acknowledged that different microorganism' species have different influences on the alteration' processes. The thesis project proposal aims to investigate microbial activity in architectural marble limestone monuments and contribute to the understanding of the role of microbial agents in the degradation of monuments built in marble (Medreiros, 2001).

The research comprises of two parts: material characterisation of the marble of Maltesas Convent by *in situ* examinations and analysis in the laboratory, and, biocolonisation assessment using traditional culture based techniques and Next Generation Sequencing.

In situ examinations were conducted to check chromatic alterations with the help of colorimetry, fibre optic reflectance spectroscopy and dinolite microscopy. Portable X-Ray Fluorescence was used for elemental analysis. Further examinations were carried out in the laboratory using X-Ray Diffraction, Raman Spectroscopy, ATR-Fourier Transform Infrared Spectroscopy and Scanning Electron Microscope coupled with Energy Dispersive X-Ray Spectroscopy.

II -MATERIAL AND METHODOLOGY

1. CASE STUDY – MARBLE MONUMENT

The monument used in this study is the 15th century church in Estremoz – the Maltesas Convent (Fig.2.1). Six areas were identified with different pathologies - saline efflorescence, yellow patina, red spots in porous zones, zones with fissures and red pigmentation, red and black zones. The following examinations were carried out in all the areas. These areas were chosen as they had the largest areas of uninterrupted growth of microorganisms and discolouration, which allowed easier access for studying and for larger areas for better sampling.

Figure 2. 1: Exterior shots of Convent of Maltesas, Estremoz (Adapted from: http://www.monumentos.gov.pt).

2. SAMPLING PROCESS

The sampling process was carried out using micro- and non-invasive methods, following the requirements for conservation purposes, minimising the structural and aesthetical impact of the stone pillars and walls, collecting the minimum amount of sample required for the different assays and sufficient to ensure the representativeness of the areas in analysis. Microsamples were collected in areas with different alteration signs, using sterile cotton swabs (Fig.2.2) placed in suspension of transport MRD medium (Maximum Recovery Diluent, Merck) for microbiological experiments, and with sterile scalpels and microtubes for stone microfragments

(100 mg) analyses, and six areas were marked with the pathologies mentioned earlier. The areas were marked as CM1 to CM6 (Fig.2.2). Samples were conserved at 4ºC until utilisation.

Figure 2. 2: Sampling process performed on Maltesas Convent (a) CM1; b) CM2; c) CM3; d) CM4; e) CM5; f) CM6).

3. *IN SITU* ANALYSIS:

Material characterisation was done with *in situ* analysis of Colorimetry, Dino-Lite Digital Microscopy and XRF and FORS spectroscopy. The material was further characterised using XRD, Raman spectroscopy, FTIR-ATR and SEM-EDS in the laboratory.

3.1. COLORIMETRY:

Colorimetry data was collected using a portable Datacolor Check II Plus spectrophotometer. This instrument has diffuse illumination 8º viewing in conformance with CIE publication No. 15.2 Colorimetry, SCE and Standard Illuminant/Observer D65/10^0. The aperture size used for the measurements were USAV (Ø5mm) which captures the smallest colour detail. The results were obtained in the CIE *L*a*b* chromatic space, which has been defined in 1976 by the International Commission on Illumination. The coordinates measured were *L** and this represents the lightness (0-100), *a** records the red/green axes and the *b** records the yellow/blue hue axes (0-100). The C* coordinate expresses the chroma which is the saturation or colour purity. This was derived from the previous measurements obtained (CIEL**C*h*). It

uses a pulsed xenon light source, a spectral range of 360-700 nm, an effective bandwidth of 10 nm and a wavelength bandwidth of 2 nm. It was calibrated using the accompanying white and black calibration accessories (M.Gill V. M., 2014) . Each sample was analysed three times. The goal of making colorimetric measurements were to evaluate the change in the colour of the marble due to growth of microorganisms and other environmental factors. Using a spectrophotometer is extremely useful to study colours even with slight differences, and help in the colour can be defined by plotting and quantifying these difference (Artioli, 2010).

3.2. DINO-LITE DIGITAL MICROSCOPE

After the use of Colorimetric analysis, further observations were made using visual and optical examination. Optical images were produced with a digital microscope (Dinolite, 430nm, Anmo Electronics Corporation) with a magnification between 45x – 75x in the reflected visible and UV light (M.Gill V. M., 2014).

3.3. FIBRE OPTIC REFLECTANCE SPECTROSCOPY (FORS)

FORS analysis was made using an ASEQ Instrument Instruments LR1-T v.2 compact spectrometer, with a spectral range of 300-1000 nm and a spectral resolution of < 1nm (with 50 µm slit). Measurements were taken using the ASEQ CheckTR software. Calibration was made using Whatman filter paper. Samples were analysed at an exposure of 100-200 ms, 5 scans and a BoxCar smoothing of 15. Each spot was measured for three times. FORS was used for analysis of colour changes in the marble induced by the environment (Bacci, 1995).

3.4. X-RAY FLUORESCENCE SPECTROSCOPY (XRF)

XRF analysis was carried out using a Bruker Tracer III-SD handheld X-ray fluorescence spectrometer. It has a 10 nm^2 XFlash® SSD; peltier cooled detector with a typical resolution of 145 eV at 100,000 cps. It has a Rh target X-ray tube and a maximum voltage of 40 kV. Analysis was made using 40 kV, 11 µA current and an acquisition time of 60s. Spectra was collected using the S1PXRF software and analysed using ARTAX.

4. LABORATORY ANALYTICAL SETUP:

After conducting analysis in-situ, the micro samples were further examined in the laboratory.

4.1. X-RAY DIFFRACTION (XRD)

X-ray micro-diffraction (μ-XRD) analyses were performed on a Bruker AXS D8 Advance with a DAVINCI design diffractometer, equipped with a Göbel mirror assembly and a LynxEye 1D detector. Cu Ka radiation and 0.3 mm diameter pinhole collimator were used for this study. The angular range (2θ) was scanned from 3º to 75º at a step size of 0.02º /s, with a working voltage and current of 40 kV and 40 mA, respectively. The XRD patterns deconvolution and matching were performed with Bruker EVA software using the International Centre for Diffraction Data Powder Diffraction Files (ICDD PDF). The semi-quantification of the identified crystalline phases was performed by a Rietveld quantification routine using the same software.

4.2. MICRO RAMAN SPECTROSCOPY

Raman spectra were acquired in a HORIBA Xplora Raman microscope, coupled to external power laser sources for specimen radiation: 638 nm (He–Ne) and 785 nm (diode laser). Samples irradiation was performed using a filter 10–50% to prevent any thermal damage of the material. Ordinary acquisition time was of the order of 10–20 s with 5 cm^{-1} of spectral resolution. The backscattered light is collected by the objective (10x or 50x), and then captured by a CCD (Charge Coupled Device) detector. Spectra were calibrated using the 520.5 cm^{-1} band of a silicon wafer. Spectra deconvolution was performed using LabSpec and the identification was made with Spectral IDTM 13.

4.3. FOURIER TRANSFORM INFRARED SPECTROSCOPY WITH ATTENUATED TOTAL REFLECTION (FTIR-ATR)

Infrared spectra were obtained on a Bruker ALPHA FTIR instrument, equipped with the attenuated total reflection (ATR—QuickSnap) set-up coupled with crystal diamond were used to complement the characterisation of the materials. To obtain a good signal-to-noise ratio, 128 scans were accumulated for each spectrum at a spectral resolution of 4 cm-1, between 4000 and 375 cm^{-1}. Spectral analysis was performed with OPUS 6.0 software.

4.4. SCANNING ELECTRON MICROSCOPY COUPLED WITH ENERGY DISPERSIVE X-RAY SPECTROSCOPY (SEM-EDS)

To assess the a) micromorphology and composition of the stone materials, b) the deterioration degree of the support and c) the signalisation of microbial contamination, microsamples of stone were used as such or coated with Au–Pd (Balzers Union SCD 030) during 30 s, and observed in an HITACHI S-3700N variable pressure scanning electron microscope (VP-SEM) with accelerating voltage of 10–20 kV. Microanalyses of the selected samples were performed using the same microscope coupled with a Bruker XFlash® 5010 Silicon Drift Energy-Dispersive Detector with 129eV spectral resolution at FWHM/Mn Kα to allow microstructural characterisation of the mortars and elemental composition (point analysis and 2D mapping). EDS analyses were performed at 20 kV.

5. ASSESMENT OF MICROBIAL COMMUNITIES:

5.1. ISOLATION AND CHARACTERISATION OF MICROBIAL POPULATION

Samples collected with sterile cotton swabs were mechanically shaken for 1 h and inoculated (100 µL), under aseptic conditions, in different culture media, specific to each microorganism like: NA (Nutrient Agar) for bacteria (Appendix I, Table A.I), MEA (Malt Extract Agar) (Appendix II, Table A.II) and CRB (Cook Rose Bengal) (Appendix II, Table A.III) for filamentous fungi, ASM1 (Appendix II, Table A.IV) and F2 (Appendix II, Table A.V) for lichens and cyanobacteria identification. The cultures were incubated at 30ºC for 24-48 hours for the development of bacteria, and for 4-5 days at 28ºC for fungal growth. To detect slow growing microbial population, plates stayed in incubation at the same temperature for longer period of time. Each different colony observed was picked up to obtain pure cultures, incubated at the temperatures previously mentioned, subsequently stored at 4ºC and periodically peaked to maintain the cultures active.

5.2. CHARACTERISATION OF MICROBIAL ISOLATES

The microbial isolates obtained were characterised based on the macroscopic features of the colonies (texture and colour) and micro-morphology of the hyphae and reproductive structures (in the case of spore isolates). Fresh preparation for fungal isolates characterisation were stained with lactophenol blue (10x) and observed with a 10x and 50x objective with an optical microscope Leica DM 2500P and digitally recorded by a Leica DFC290HD camera. The

bacterial isolates were carried out with Gram staining and observed in the same optical microscope with a 100x objective lens.

5.3. MICROBIAL DIVERSITY IDENTIFICATION

Metagenomic DNA was extracted from marble samples using E.Z.N.A.® Stool DNA Kit (Omega Bio-tek, Norcross, Georgia, USA), with slight modifications from the manufacturer's instructions. Bacterial and fungal communities were characterised by Illumina Sequencing technology for the 16S rRNA V3-V4 region and Internal Transcribed Spacer 2, respectively. Generally, metagenomic DNA was amplified for the hypervariable regions with specific primers and further reamplified in a limited-cycle PCR reaction to add sequencing adaptor and dual indexes. The prokaryotic population was characterised, using the 16S V3 forward primer 341F 5′-CCTACGGGNGGCWGCAG-3' and 16S V4 reverse primer 805R 5′-GACTACHVGGGTATCTAATCC-3' (Herlemann D P, 2011) (Klindworth A, 2013). Eukaryotic diversity was assessed using a pool of forward primers: ITS3NGS1_F 5'-CATCGATGAAGAACGCAG-3', ITS3NGS2_F 5'-CAACGATGAAGAACGCAG-3', ITS3NGS3_F 5'-CACCGATGAAGAACGCAG-3', ITS3NGS4_F 5'-CATCGATGAAGAACGTAG-3', ITS3NGS5_F 5'-CATCGATGAAGAACGTGG-3', and ITS3NGS10_F 5'-CATCGATGAAGAACGCTG-3' with the reverse primer ITS3NGS001_R 5'-TCCTSCGCTTATTGATATGC-3' (Tedersoo L, 2014). Besides the 16S and ITS target-specific sequences, the primers also contain adaptor sequences allowing uniform amplification of the library with high complexity ready for downstream NGS sequencing on Illumina Miseq. Amplicon PCR reactions were performed for each sample using 2X KAPA HiFi HotStart Ready Mix, and the amplification involved 3 min of denaturation at 95ºC, followed by 35 cycles (prokaryotes)/45 cycles (eukaryotes) of 95ºC for 20 s, 55ºC for 30 s and 72ºC for 30 s and a final extension at 72ºC for 5 min. Negative controls were included for all amplification reactions. Electrophoresis analysis of the Amplicon PCR products was undertaken on a 1% (w/v) agarose gel and the ~500bp V3-V4 and ~400 bp ITS2 amplified fragments were purified using AMPure XP magnetic beads (Agencourt, Beckman Coulter, USA) according to manufacturer's instructions. Attachment of dual indexes and sequencing adaptors to both ends of the amplified target region was performed 2x KAPA HiFi HotStart Ready Mix ,using 5 µl of the previous amplicon PCR product purified, 5 µl of Illumina Nextera XT Index Primer 1 (N7xx), 5 µl of Nextera XT Index Primer 2 (S5xx). Index PCR amplification involved the following steps: 3 min denaturation at 95ºC, followed by 8 cycles of 95ºC for 30 s, 55ºC for 30 s and 72ºC for 30s and a final extension at 72ºC for 5 min. The metagenomic libraries/ Index

PCR products were analysed by agarose gel electrophoresis (1%, w/v), purified by AMPure XP magnetic beads (Agencourt, Beckman Coulter, USA) and quantified by fluorimetry with Quantus Fluorometer ONE dsDNA quantitation kit (Promega, Madison, USA).

Libraries were normalized and pooled to 4 nM. Pooled libraries were denatured and diluted to a final concentration of 10 pM with a 15 % PhiX (Illumina) control. Sequencing was performed using the MiSeq Reagent nano Kit V2 in the Illumina MiSeq System. Samples sequencing was performed using a 2 x 250 paired-end (PE) configuration; image analysis and base calling were conducted by the MiSeq Control Software (MCS) directly on the MiSeq instrument (Illumina, San Diego, CA, USA). The Eukaryotes are studied under BaseSpace.

III -RESULTS AND DISCUSSION

Marble has always been a preferred material since antiquity for construction. In this study, marble materials inserted on the Maltesas Convent, with visible alteration signs and exposure to different environmental conditions (sun, humidity, wind) were analysed in order to understand the impact of environmental and biological agents. The research work were divided into three broad categories of pathology – salting, staining and biofilm formation.

The first examinations were conducted in situ to check chromatic changes on the marble and to understand the materials composition. For analysing chromatic changes three methods were used, like Dino Lite Microscopy, Colorimetry and FORS. Six areas were selected and divided according to the pathologies exhibited – CM1 and CM2 indicating salting, CM3 and CM4 exhibiting signs of staining, and, CM5 and CM6 representing biofilm formation (Fig. 3.1).

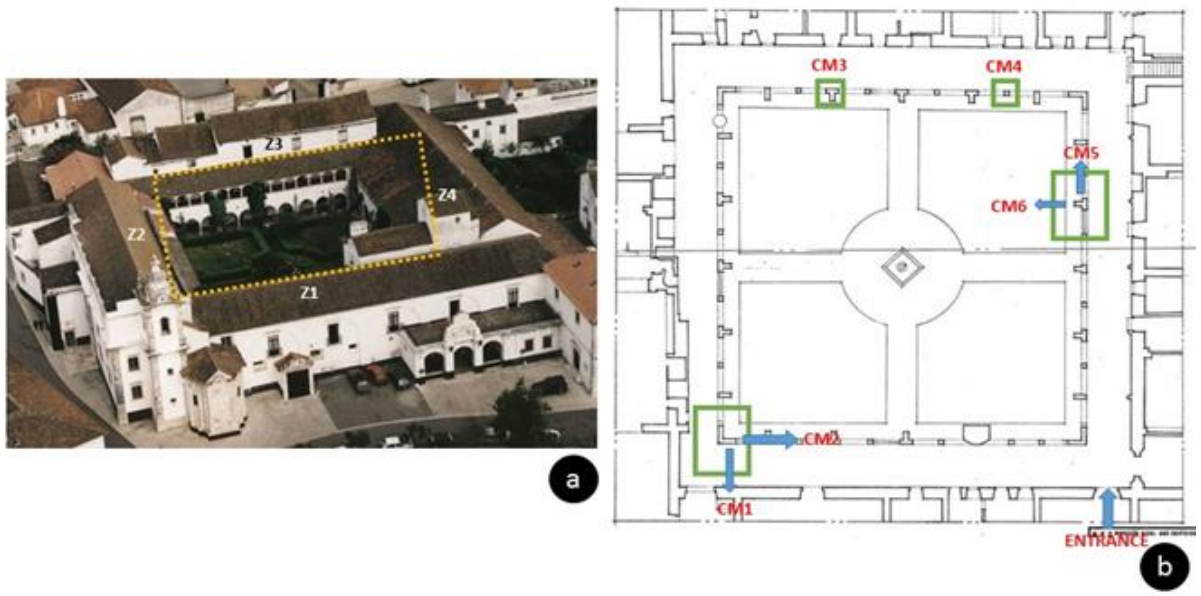


Figure 3. 1: a) Ariel view of the Maltesas Convent in Estremoz, b) Map of the convent and the sample areas (Adapted from http://www.monumentos.gov.pt).

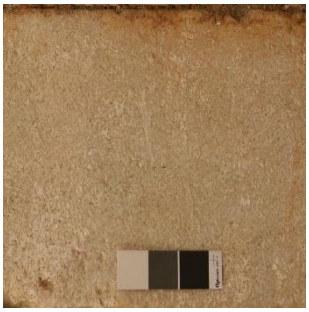
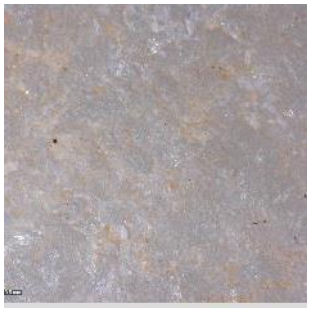
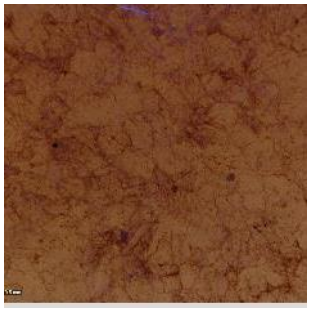
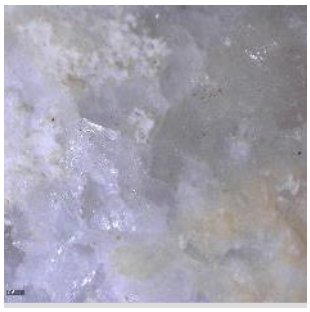
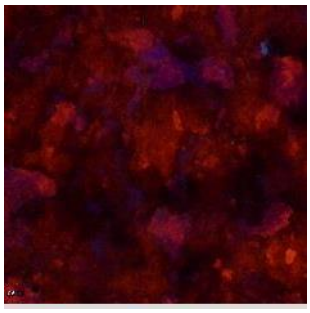
3.1 CHROMATIC ALTERATIONS

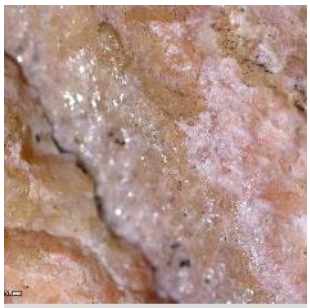
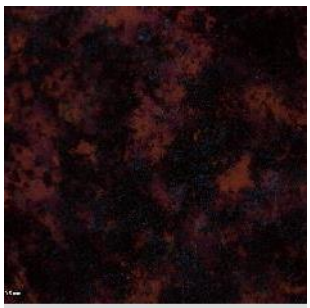
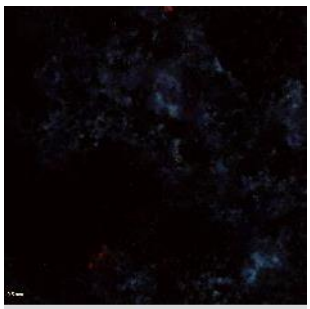
The first alterations that are generally visible before any kind of examination to the naked eye are changes in colour. These chromatic alterations can be caused owing microorganisms (biological patina) or atmospheric reasons. These chromatic alterations can lead to different types of patina formation (Franco Palla, 2007). Colour changes can also be the result of an increase in light (chroma) in form of fading and bleaching and a decrease of light in form of

darkening or in a change in hue which is staining and yellowing (Berns, 2005).

DinoLite Microscopy was the first *in situ* examination that are conducted. Table 4 show some digital microscope analysis of non-altered and altered marble stones from Maltesas Convent. In some cases it is possible to observe fluorescence areas which can be correlated with microbial proliferation, but also with some organic constituent. The analysis on non-altered stone were performed in order to compare with altered areas. There is some fluorescence in CM1, CM5 and CM6 probably due to material deposition and biological cells.

Table 4: Digital microscope analysis of non-altered and altered marble stones from Maltesas Convent.

Area I.D.	Area detail	Digital Microscope 430 nm	
		Vis	UV
Non altered			
CM1			
CM2			

Area I.D.	Area detail	Digital Microscope 430 nm	
		Vis	UV
CM3			
CM4			
CM5			
CM6			

3.1.2 COLORIMENTRY AND FIBRE OPTIC REFLECTANCE SPECTROSCOPY (FORS)

Colorimetry is used for measuring colour (Svahn, 2006). Colorimetry replaces “vague” non-colour specific terms to an objective numerical value (Alison Gilchrist, 1999). Colour measurement is an important tool for stone conservation and prevent soiling and re-soiling. This method can also be used to identify pigments (Svahn, 2006). Colorimeters and spectrophotmeters are used to measure reflected and transmitted visible light on an object. The main objective of a colorimeter is to measure the light emission using receptors of primary

colours – red, blue and green. A spectrophotmeter measures the light in 380-780 nm wavelength. The colour is plotted onto a curve by joining the values obtained from each wavelength, whereas colorimetry result is a numerical value of the colour (Svahn, 2006). The spectral reflectance along with colorimetric calculations have been used for studying chromatic differences. This is important in case of further conservation work (Daniel Vazquez, 2014).
The colorimetric measurements on Maltesas Convent were performed in marble areas with alteration patterns and without signs of change. Table 5 summarise colorimetric measurements indictating the chromatic difference observed, according to the CIELab system.

Table 5: Colorimetric measurements indictating the chromatic difference as observed through colorimetry. The measurements were conducted against non-altered stone (NA)

	CIE L* a* b* parameters			
Sample	L*	a*	b*	ΔE
NA	72.74 ± 4.64	0.6 ± 1.34	5.33 ± 3.01	-
CM1	91.52 ± 2.41	1.84 ± 0.58	6.40 ± 2.04	18.85
CM2	66.98 ± 3.95	8.69 ± 3.53	25.00 ± 6.03	22.02
CM3	41.58 ± 8.57	10.94 ± 1.16	17.21 ±2.69	34.31
CM4	63.82 ± 5.49	8.77 ± 1.73	16.50 ± 2.13	16.46
CM5	36.05 ± 4.33	13.46 ± 3.02	15.55 ± 2.21	40.19
CM6	27.64 ± 1.68	1.82 ± 0.51	4.25 ± 1.34	45.12

Acoording to CIE L*a*b system, the colour is determined by three coordinates – L*, a*, b* (A. Ozguven, 2014). The L* was plotted on z-axis and measures the greyscale, the various grade of lightness from 0 to 100 where 0 is black and 100 is white and is called the neutral axis. a* measures the green-red axis (the red is plotted on the positive values and the green on the negative values) and is plotted on x-axis. b* is plotted on y-axis and plots blue/yellow colours, where the blue is plotted on negative values and yellow on positive values . The hue is the value of a* and b* and saturation is the absolute value of these coordinates (Kevin Beck, 2015). The ΔE is used to study colour variation, which was compared with the non altered marble (Table 5). When ΔE = 1 the change in colour is not visible to the naked eye, ΔE = 2 refers to a direct comparison and Δ5 ≥ refers to a strong colour difference. (F. Persia, 2010). The results revealed that, for all the samples, ΔE are above 5 which shows a strong colour difference. CM5 and CM6 which a thick biofilm formation shows the maximum difference in ΔE and this can be appointed as a sign of biodegradation (Choudhury, 2010). Additionaly, FORS results show a difference between the non-altered stone against marble samples with alteration. Figure 3.2 exhibits the

main spectral differences between the marble samples. This is an indication that there is a chromatic alteration and that the original colour of the marble has been changed.

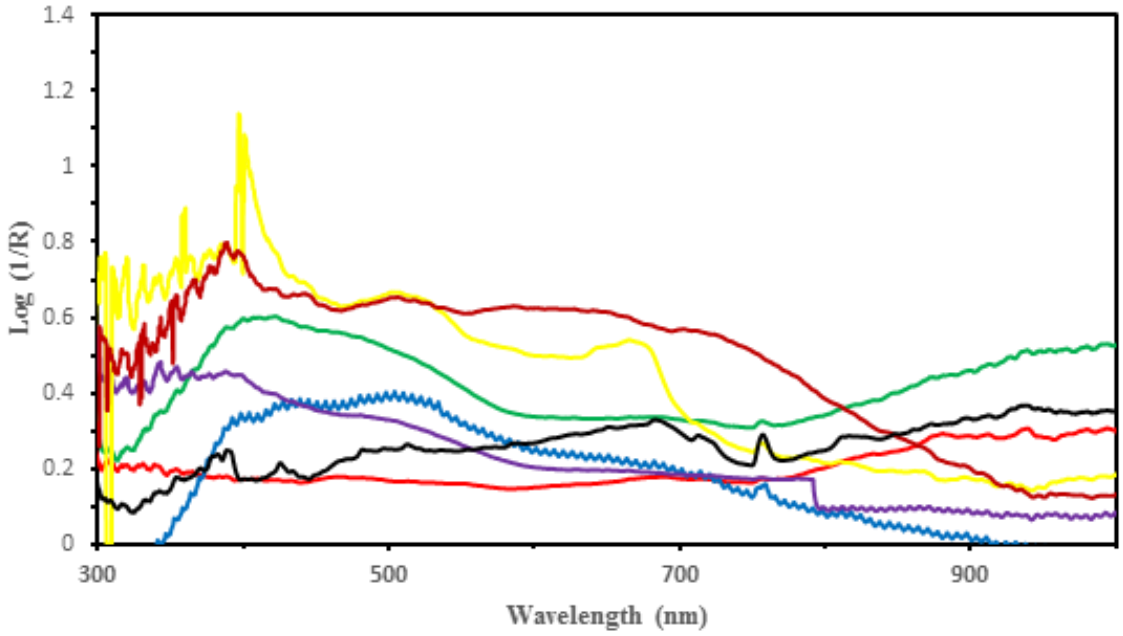


Figure 3. 2: Chromatic observations as observed by Fibre Optic Reflectance Spectroscopy. NA —, CM1 —, CM2 —, CM3 —, CM4 —, CM5 —, CM6 —

Three main pathologies were observed in Maltesas Convent. These pathologies were salting, pigmentation and discolouration, and, biofilms formation.

3.2. SALT EFFLORESCENCE

Samples CM1 and CM2 exhibit signs of salting (saline efflorescence) and both the samples are located in the inner zone of the Cloister walls. CM1 neither receive direct sunshine nor is it subjected to the splash of heavy rain. There is also no visible signs of water damage or leakage. However CM2, is always partially exposed to direct sunshine and rain.

XRD analysis allowed the detection of calcium oxalates like wheddelite and whewellite in the CM1 and CM2 (Fig. 3.3) respectively is a suggestion of calcium degradation a common occurrence on limestone and calcium monuments (Cristina Sabbioni, Oxalate patinas on ancient monuments : the biological hypothesis , 1991). With the formation of oxalates the surface looks encrusted or stratified and as suggested by Del Monte and this is noted on the sample area too. (Fig 3.4).

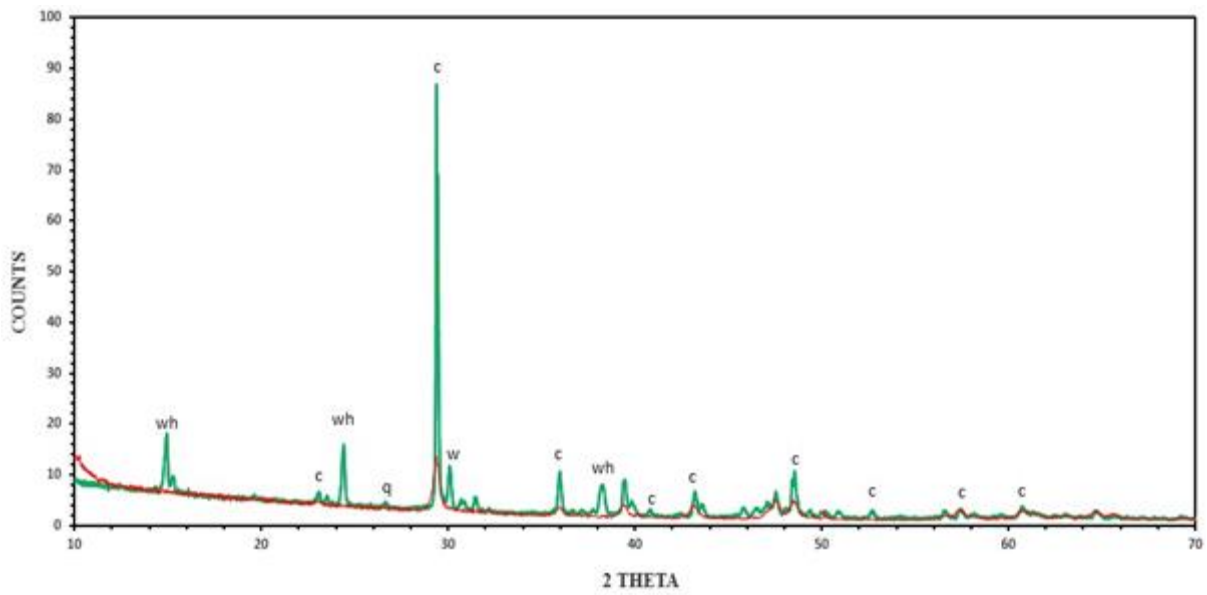


Figure 3. 3: XRD Diffractograms (CM1 —, CM2 —) Calcium oxalate – wheddelite and whewellite as observed on XRD. [c – calcite ($CaCO_3$), wh – whewellite ($CaC_2O_4.H_2O$), w – weddellite ($CaC_2O_4.2H_2O$), q - quartz (SiO_2)].

Marco and Sabbioni suggests that this is one of the effects of scialbatura or patina formation and calcium oxalates suggest a degradation of calcium carbonate (Marco Del Monte, The study of the Patina called "Scialbatura" on Imperial Roman Marbles, 1987). It has also been observed dark black spots with white patches and are likely organic matter and a source of nutrient for the microbial colony which increases the chance of decay (Borreli, 1999).

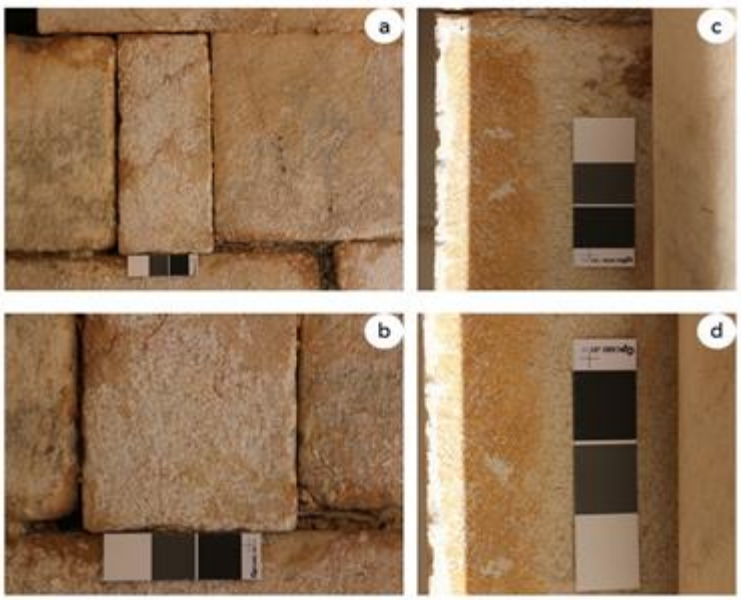


Figure 3. 4: 1 – a) CM1 area showing encrustation and stratification, b) magnification of CM1 area as shown in area a, c) CM2 area showing encrustation and stratification, d) magnification of CM2 area as shown in area c. The images also exhibit a chromatic alteration.

Calcium carbonate has three anhydrous phases in nature – calcite which is followed by aragonite and vaterite, the most stable being calcite (Christos G, 2000). Calcium oxalate formation on stone is a common phenomenon and is found commonly in the Mediterranean Basin and hence sometime is also referred as the "Mediterrenean patina" (D. Benedetti, 2007) (M.Garcia -Valles, 1998). The formation of these films are based on the presence of calcium oxalate ($CaC_2O_4.nH_2O$) – weddelite ($CaC_2O_4.2H_2O$), and whewellite ($CaC_2O_4.H_2O$) (Rihana Terzu, 2016), gypsum ($CaSO_4 \cdot 2H_2O$), calcite ($CaCO_3$) and silicates (SiO_2).

The colour of these films range from pink, yellow, ochre and brown. The oxalates are widely found on various stone structures – calcareous stones and siliceous stones alike over a range of temperature conditions. The formation of the oxalates can be attributed to either biological or chemical origin (Rampazzi, 2004). XRD along with the chromatic alterations detected by colorimetry shows the formation of yellowish patinas (Borreli, 1999).

In CM1 there is presence of magnesium but the small veins can be owing to the lime washing that is clearly visible on the building surface. Quicklime has been repeatedly used as a quick preservation process for the Convent. The major component of quicklime is CaO but calcinated lime always contains small amounts of magnesium oxide in them, therefore it can be a possibility why magnesium was observed. Calcium nodules were observed on CM1 area (Fig 3.5) and were visible by the naked eye.

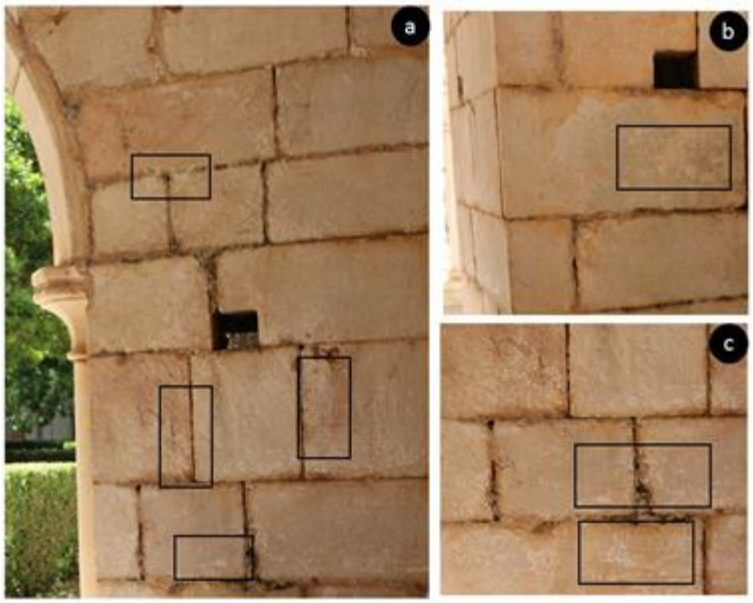


Figure 3. 5: Evidence of lime nodules on the pillars an effort to conserve the convent in CM1

On the other hand, there is also signals of a small amount of sulphur on the rock surface, detected on the microfragment analysis by SEM-EDS (Fig. 3.6a), which could be an indicative

of air pollution which is possible as the area studied was exposed to the natural environment (M.Gill R. V., 2003).

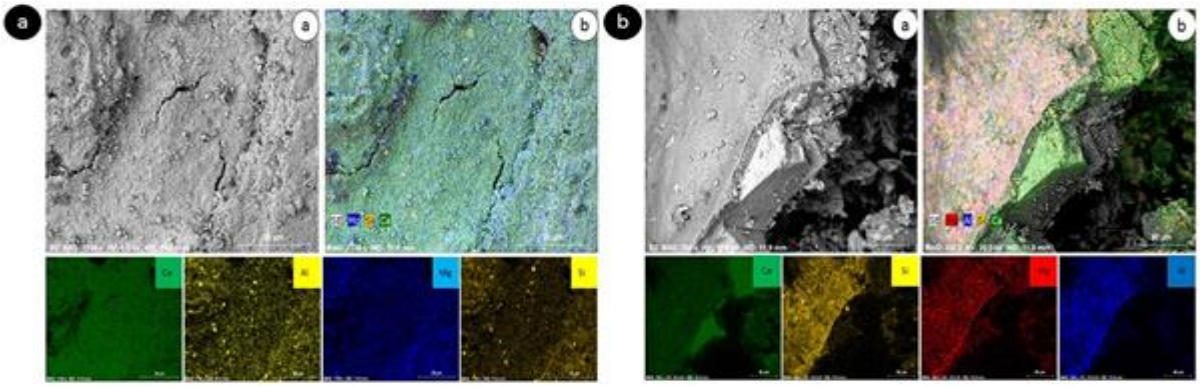


Figure 3. 6: Scanning electron microscopy (SEM) in back-scattered mode (a) and EDS two dimensional(2D) elemental maps of (a, dark circle) CM1 showing the Mg presence due to lime wash and (b, dark circle) CM2 showing the presence of Al, Si confirms the presence of quartz in in the sample in Maltesas Convent.

The elemental composition was studied with the help of SEM-EDS (Fig. 3.6b) and the spectra (Fig. 3.7) also demonstrate small amounts of Al, K, Na, Si in both samples and this can be co-related to the XRD diffractograms as a parameter for quartz and this hold true as there is evidence of quartz in XRD and the peak is very small in CM2.

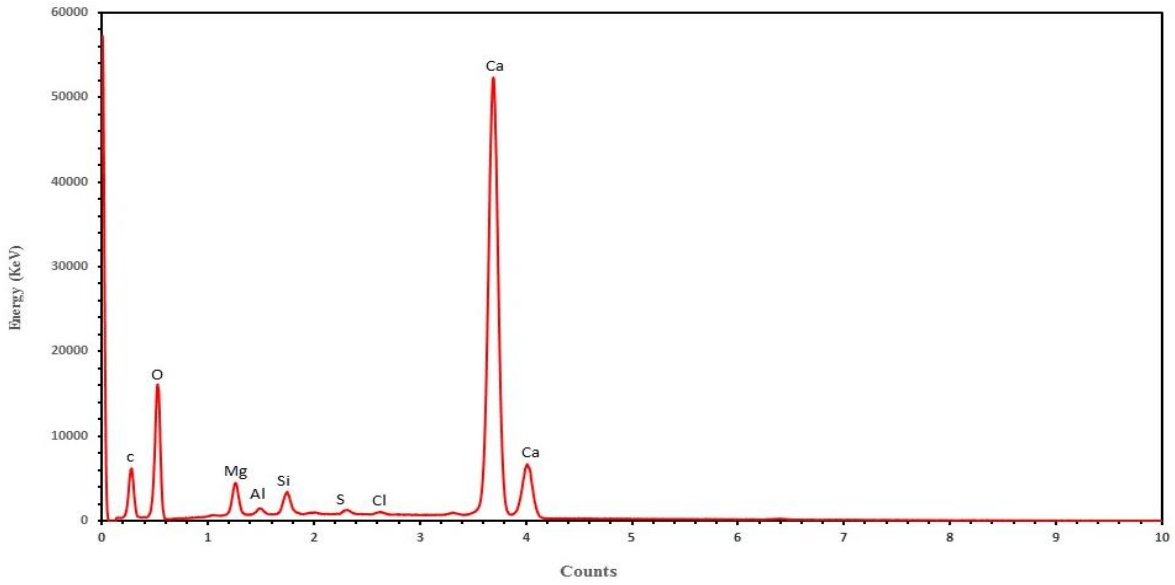


Figure 3. 7: X-ray spectra from SEM-EDS with a small peak of sulphur suggestion of gypsum caused by air pollution.

Formation of oxalates on monuments which are formed due to the burning of hydrocarbons in industrial urban areas as sulphur dioxide (SiO_2) and when this reacts with calcium carbonate it

results in the production of calcium sulphate or gypsum (C. Saiz-Jimenez, 2004). Apart from the aforementioned forms of calcium oxalate, it can also be found as caoxite ($CaC_2O_4.3H_2O$). Calcium oxalate formation can also sometimes be beneficial to the stone monument and can slow the aging and degradation process.

Raman spectroscopy confirms the oxalates present in CM1 and CM2 (Fig. 3.8 and 3.9).

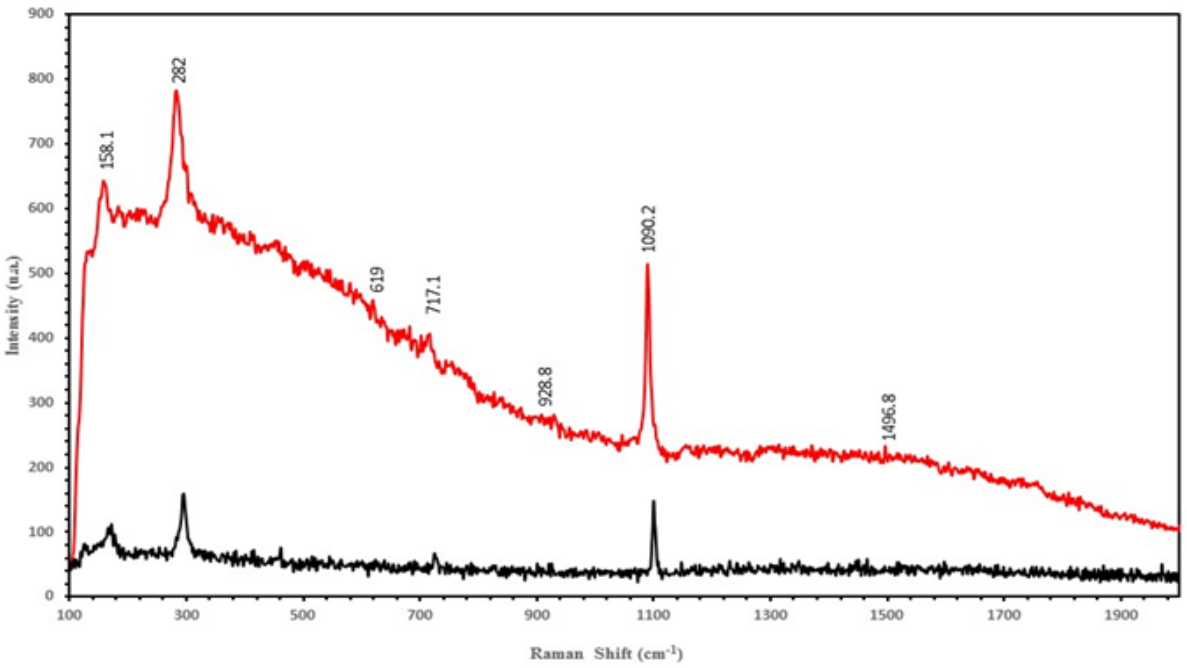


Figure 3. 8: Raman spectra of oxalate presence, identified on CM1 sample (CM1 —, NA —).

The Raman spectra for wheddelite is found between 455 cm^{-1} to 950 cm^{-1} and 1440 cm^{-1} and the characteristic peak of whewellite is at 945cm^{-1} (Tania Rosado M. G., 2013). CM1 records the oxalate peaks at 171.1 cm^{-1}, 928.8 cm^{-1} and 1496.8 cm^{-1} (Fig. 3.8) and CM2 records oxalates at 14141.1 cm^{-1} and 1475.71 cm^{-1} (Fig.3.9). There is also evidence of calcite in 1092.2 cm^{-1} and 1090.7 cm^{-1} for CM1 and CM2 respectively.

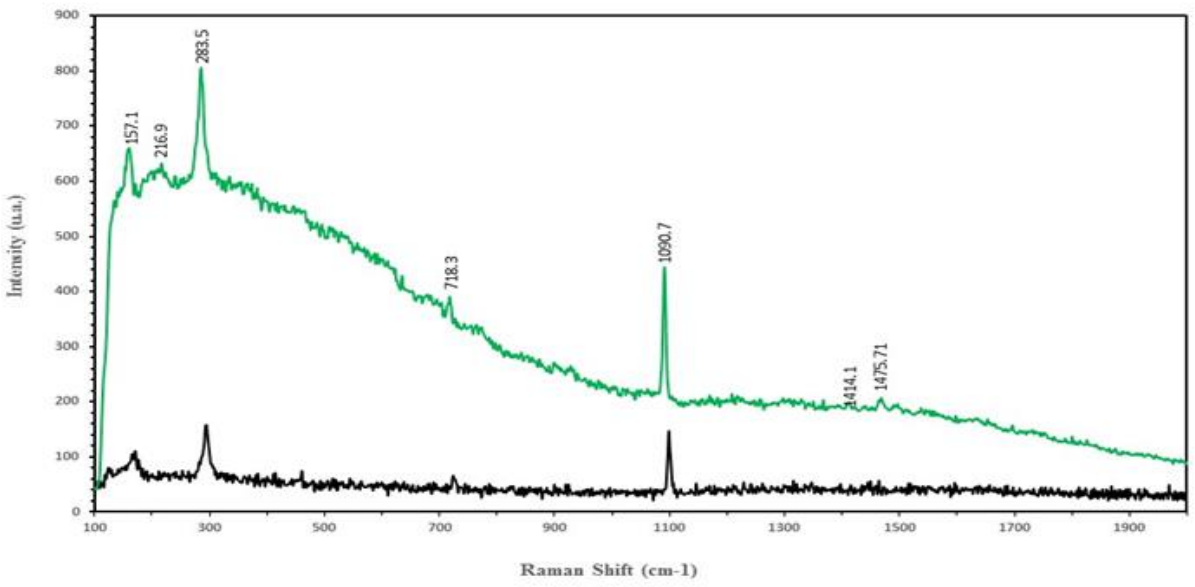


Figure 3. 9: Raman spectra of oxalate presence, identified on CM2 (CM2 —, NA —).

FTIR results yield calcite and stretching in area around v 1420 cm^{-1} to v 1418 cm^{-1} and bending δ875 – δ712 cm^{-1} area, corroborating the Raman evidences. There is also a peak for oxalate on CM2 around 780 cm^{-1} and 1313 cm^{-1}. In CM1 there is a very strong silicate band. (Fig.3.10).

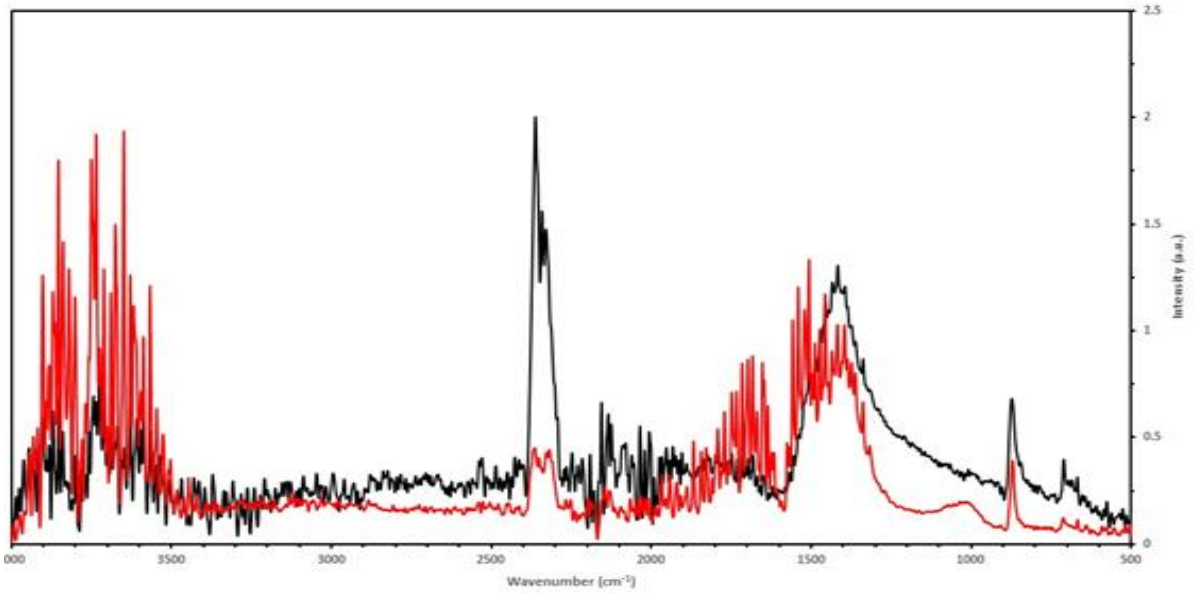


Figure 3. 10: Silicate and calcite detection in CM1 microfragments by fourier transform infrared spectroscopy attenuated total reflection analysis (CM1—, NA—).

This is corroborated with the SEM-EDS results which shows trace amounts of magnesium due to lime washing and as lime has other clay materials in them, it is not revelation to find silicate band. This may be due to clay minerals present in the limewash which includes Si, Al, and Fe all confirmed by SEM-EDS. It is known aluminosilicates have Si,Fe and Al (A. Calia, 2011).

FTIR of CM2 shows oxalates and calcite (Fig.3.11).

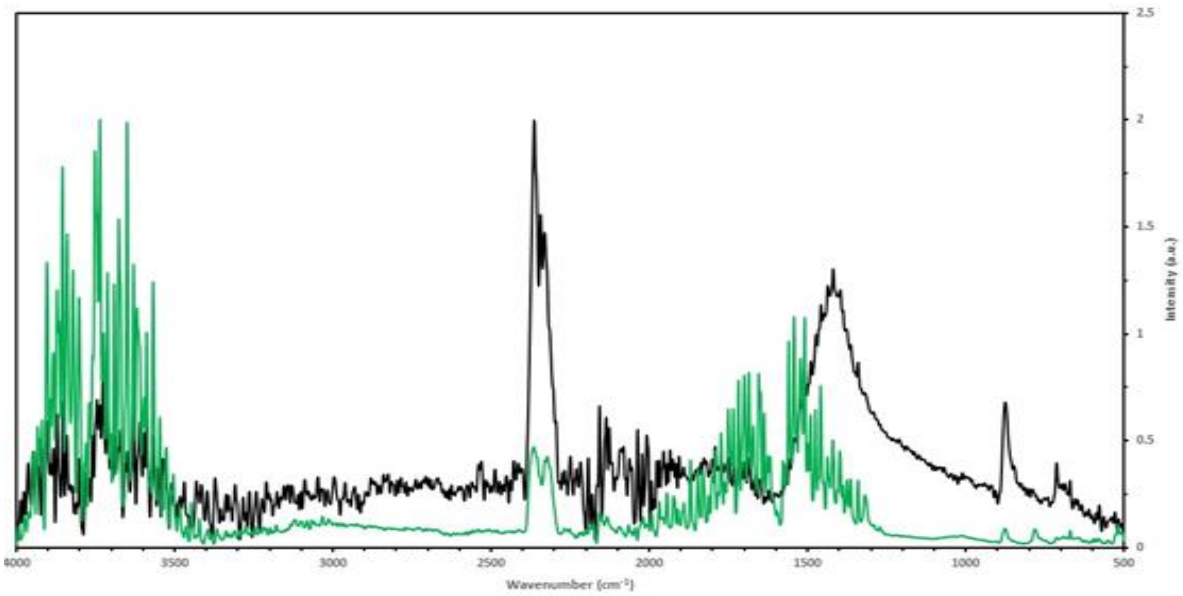


Figure 3. 11: Silicate and calcite detection in CM2 microfragments by fourier transform infrared spectroscopy with attenuated total reflection analysis (CM2 —, NA —).

The chemical origins of oxalates can be related to the degradative oxidation of organic materials. The oxalate formation is due to the reaction between oxalic acid and the calcite leading to the formation of weddelite ($CaC_2O_4.2H_2O$), and whewellite ($CaC_2O_4.H_2O$) depending on the thermodynamic conditions. To study oxalates, one needs to align themselves to the cause and effect of atmospheric weathering and biocolonisation (Marco Del Monte, The study of the Patina called "Scialbatura" on Imperial Roman Marbles, 1987). Several polysaccharides, proteins and lipids are applied for conservation purposes and these can undergo photo-oxidation which aids in the formation of oxalates. Calcium oxalate is found on calcium carbonate penetrating deeply in the inter-granular position along the micro fracture as oxalate is less soluble in water when compared to calcium carbonate and the film acts as a barrier and prevents acid attack

3.3 STAINING:

Change in colour, pigmentation on the surface is one of the most common types of aestheic changes encountered. This discolouration can present a biogenic origin and takes place when the microorganism tries to protect itself from harmful UV light and chemicals (Claudia Schabereiter-Gurtner, 2001).

This pathology was observed on samples CM3 and CM4. Both the samples are from the same

area. Both the samples neither receive direct sunlight nor are they affected by water and there is no indication of any water seepage. Mutually these areas were affected by staining, nevertheless the staining is of two different kinds. The stain observed in CM3 is characterised as the red spot in a porous area. This area was chosen as there is a sudden occurrence of a red spot on the wall. CM4 is characterised by the red zone in the fissures. In order to characterise the red spots in, XRF, SEM, Raman Spectroscopy and FTIR-ATR were used to check the material composition and products alteration detection. NGS along with traditional methods of isolations was complementary used to verify the role of microorganisms in the staining areas. In order to understand the causes of red spots in CM3, XRF (Fig.3.12) and SEM-EDS (Fig. 3.13) analysis were performed.

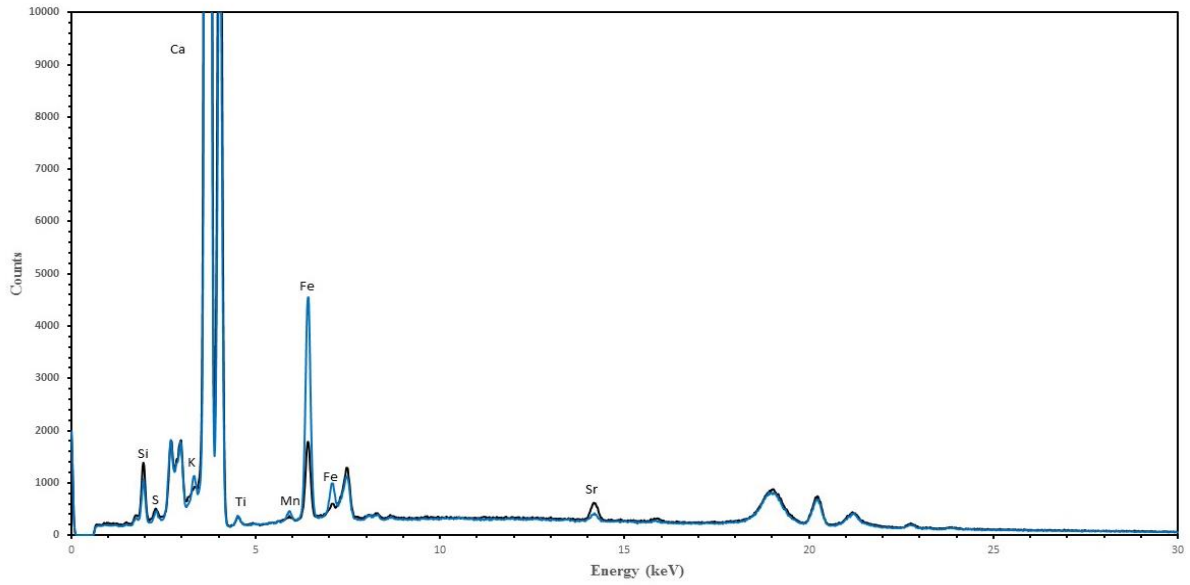


Figure 3. 12: XRF peaks indicating elemental analysis of CM3 showing a high presence of iron (CM3—; NA—).

The red spot on CM3 can be suggested that it was owing to the degradation of the iron existing in the marble as an impurity (Fig. 3.12). This phenomenon is observed on the marble at Maltesas Convent which results in exuding a pinkish hue to the marbles.It also can due limestone degradation and can be caused due to the Terra Rossa. Terra Rossa is a formation owing to chemical degradation of limestone, predominantly in the Mediterranean region. Terra Rossa is also called “Red Soil” which is generally found in karst terrain (S.K.Haldar, 2014).

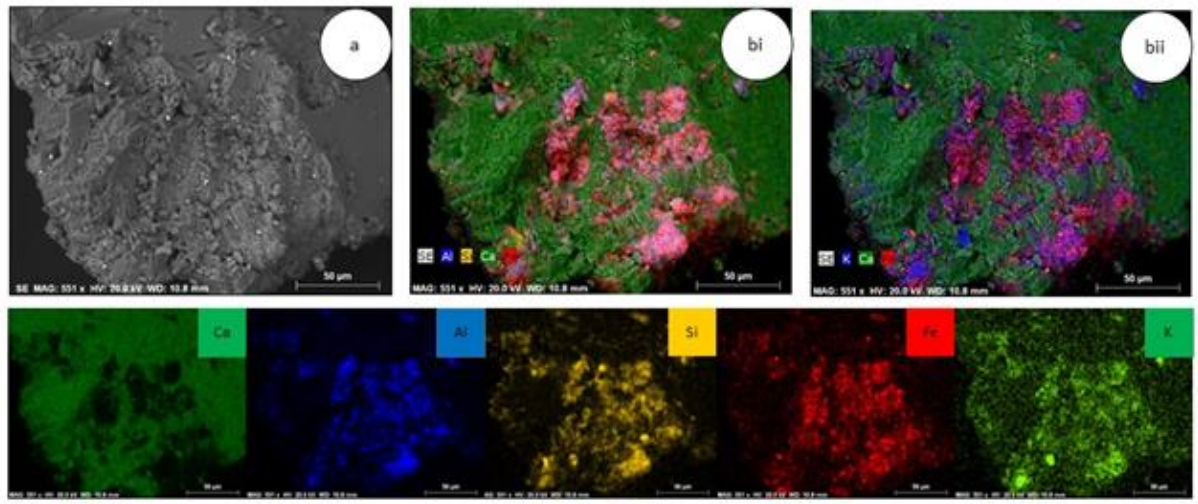


Figure 3. 13: Scanning electron microscopy (SEM) micrograph in back-scattered mode and EDS two dimensional(2D) elemental maps of CM3 showing abundance of iron (bi) CM3 showing Al, Si Fe, Ca, and (bii) CM3 showing Al, Si, Fe, Ca in the sample in Maltesas Convent

Terra Rossa marks the transition of brown earth to red laterite soil and is a reddish clay residue resulting from the dissolution of limestone, generally overlies on top of the limestone (Olsen, 1980) and the dolomitic layers and forms a discontinuous layer in varying thickness from a few centimeters to a few meters (Durn, 2003).The Terra Rossa region is made up of mica, quartz and clay minerals (S.K.Haldar, 2014). All these elements are visible on the SEM-EDS (Fig 3.13). This is also evident from the marble slab that was recovered from the Convent (Fig 3.14) which clearly shows iron in the veins of the marble.

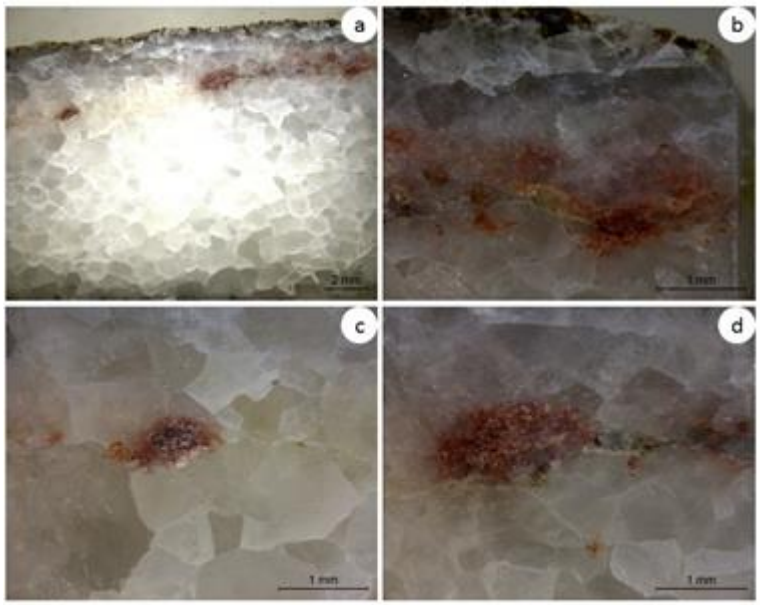


Figure 3. 14: Presence of iron inside the iron as observed on Optical Microscopy. a) The complete image of the iron degradation b), c), d) Magnification of iron degraded areas from image a.

CM4, too, has reddish – pink pigmentation around fissures and because the XRD, XRF and SEM results proved inconclusive Raman spectroscopy and FTIR was used to identify the reason for its cause. Microorganisms have the ability to irreversibly alter the appearance of the substrate (Guadalupe Pinar, 2009). The SEM-EDS results also exhibit of Al, Si and Fe along with Ca which suggests the composition of the mortar along with siliceous aggregates, which are evident from FTIR results. There is also presence of carotenoid which is exhibited in the Raman spectra (Fig. 3.15).

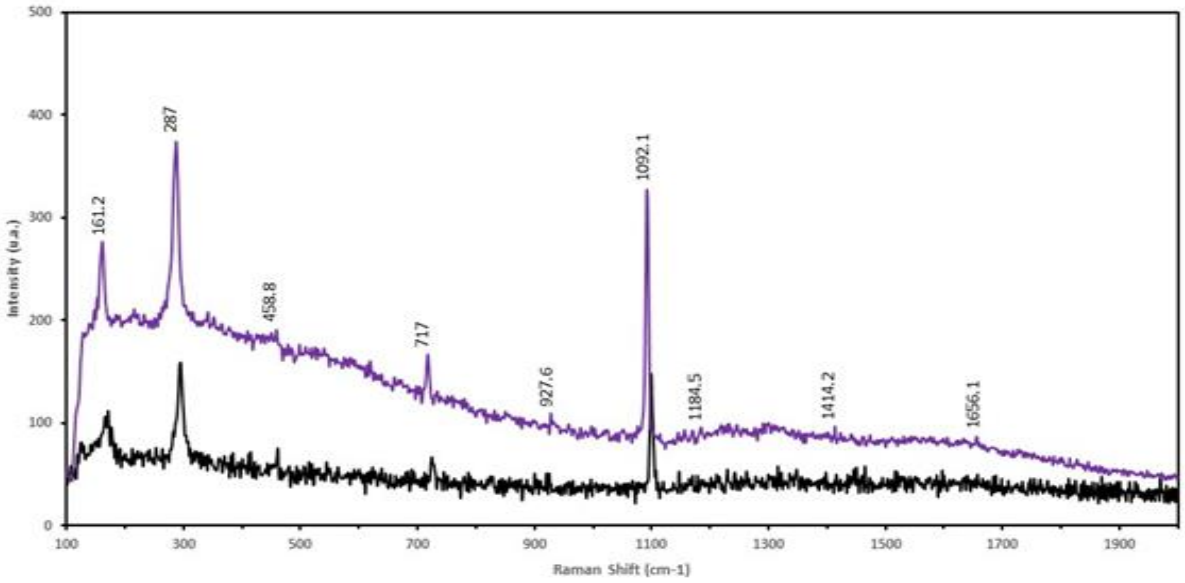


Figure 3. 15: Carotenoid compound detection in CM4 microfragments by Raman spectroscopy (CM4 ▬, NA▬).

There is also evident a strong calcite band at 1034 cm^{-1}, 917 cm^{-1} and 1017 cm^{-1} for silicates at 1035 cm^{-1}, 1018 cm^{-1} and 917 cm^{-1} (Fig. 3.16).

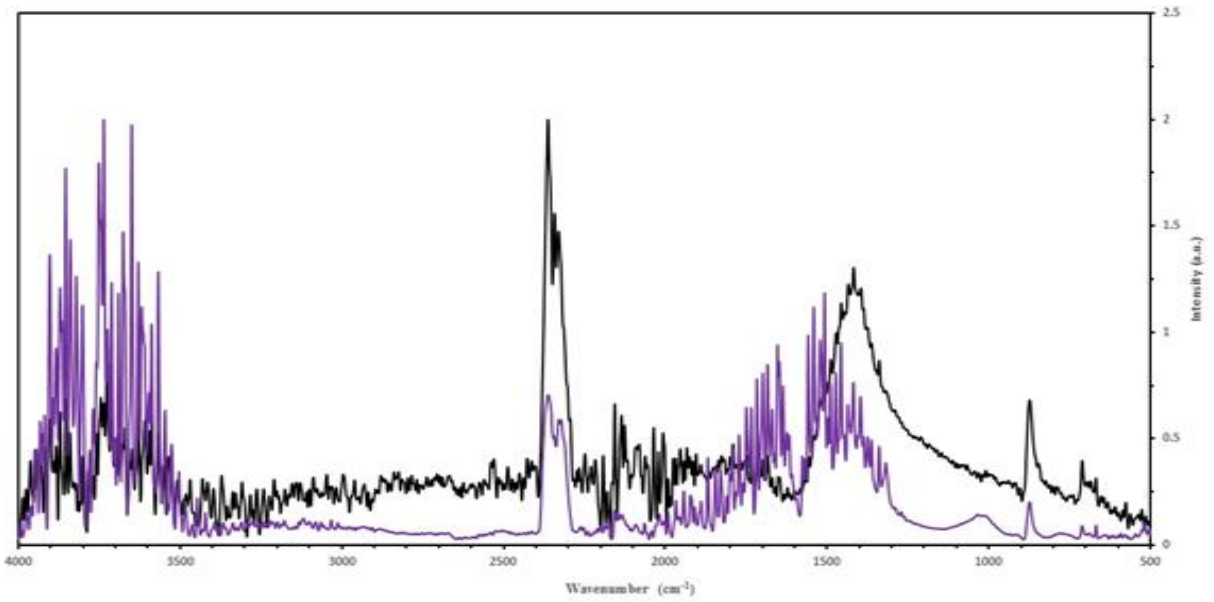


Figure 3. 16: Silicate and calcite detection in CM4 microfragments by fourier transform infrared spectroscopy attenuated total reflection analysis (CM4 —, NA —).

3.4. BIOFILMS FORMATION

There was an indication of severe microbial proliferation on area CM5 and CM6. Both CM5 and CM6 were taken from the buttress from the cloisters from the garden. The area from where both these samples were taken were subjected to severe direct sunlight and rain and there is visible indication of water seepage. The chromatic differences are obvious as the microbial population red and black patina for CM5 and CM6 respectively. Microfragments collected from these two altered areas were analysed by SEM-EDS (Fig. 3.17 and 3.18).

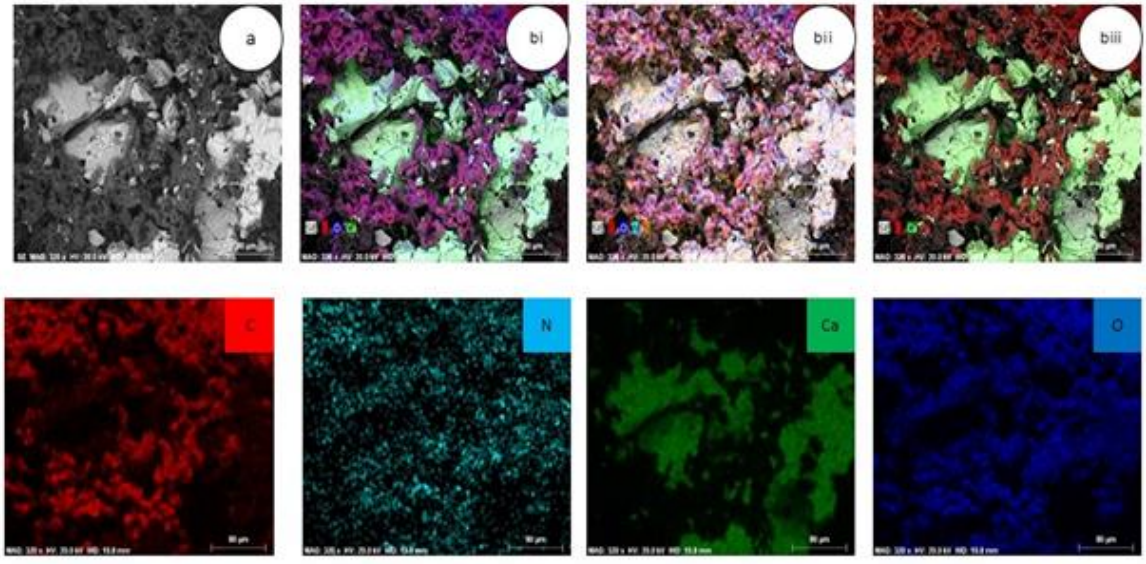


Figure 3. 17: Scanning electron microscopy (SEM) micrograph in back-scattered mode and EDS two dimensional(2D) elemental maps of the sample CM5 exhibiting microbial proliferation on the sample. Presence of C,N,O which are indicators for organic remains in Maltesas Convent.

The area of CM5 had a microbial proliferation which altered the colour to red. The biofilm formation has been observed by SEM-EDS (Fig. 3.17 and Fig. 3.18). The detection of carbon (C), oxygen (O) and nitrogen (N) signalise the presence of organic material on the rock surface which can be associated to biological contamination.

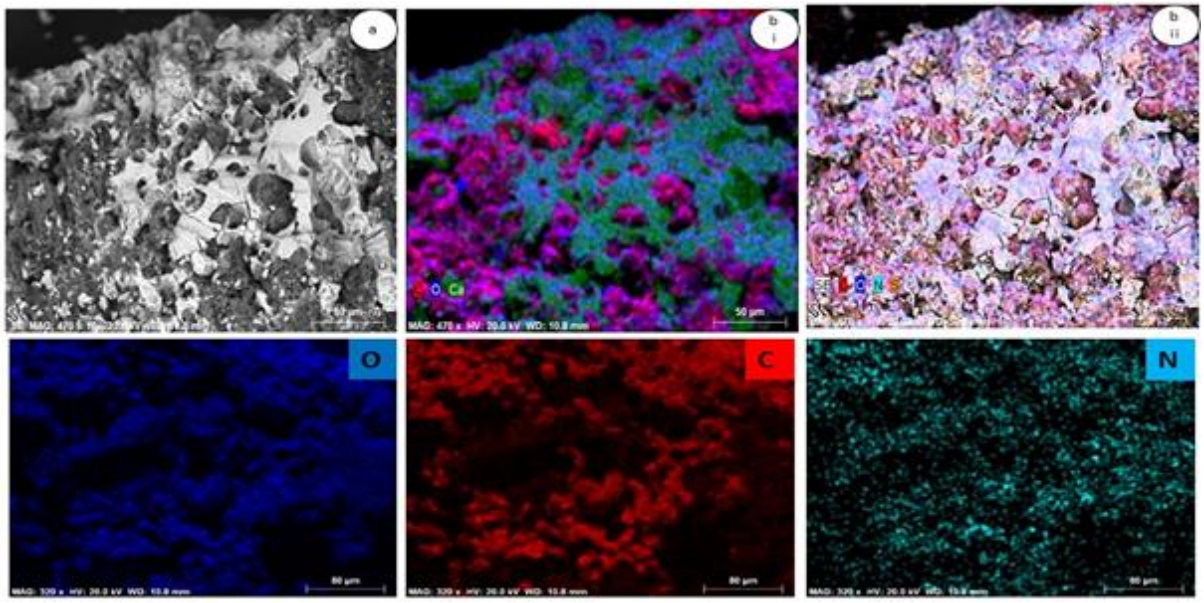


Figure 3. 18: Scanning electron microscopy (SEM) micrograph in back-scattered mode and EDS two dimensional(2D) elemental maps of the sample CM6 exhibiting microbial proliferation on the sample. Presence of C, N, O are indicators for organic remains in Maltesas Convent.

On the other hand, Raman spectra for both, CM5 and CM6, show no peaks maybe because they are covered due to biofilm formation (Fig 3.19). When there is a severe case of microbial proliferation no other peaks are observable. This is the case in both these samples as no other peaks are visible, not even for calcite which is the dominant material.

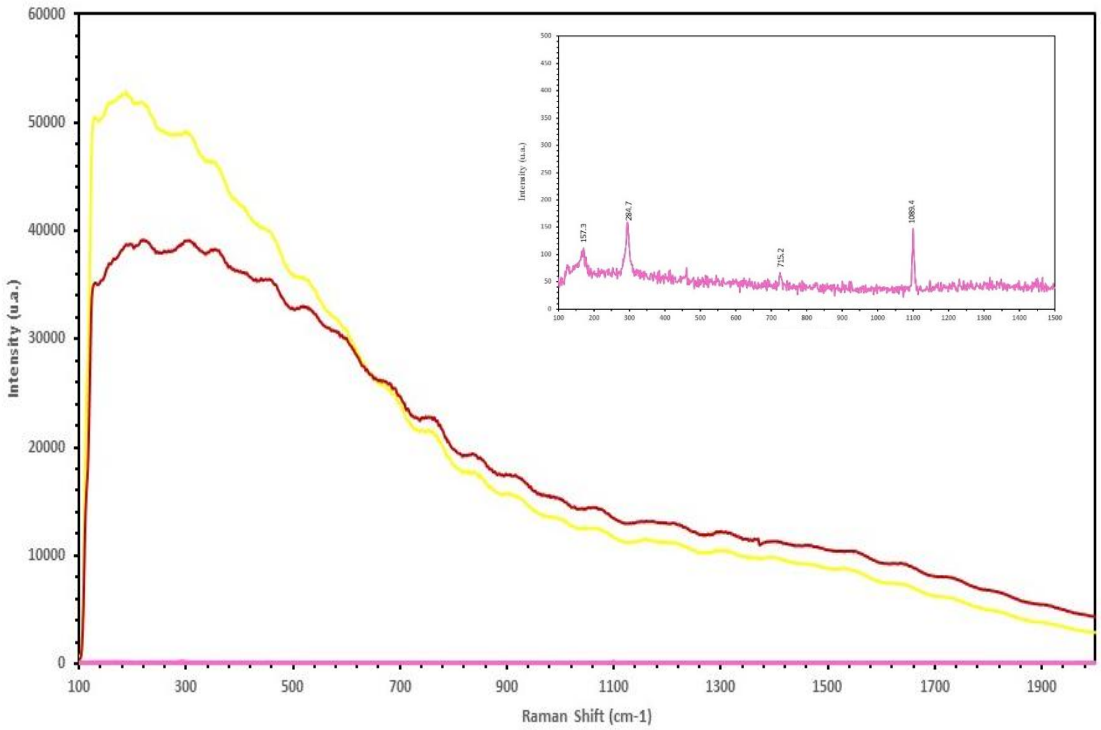


Figure 3. 19: Microbial proliferation detection in CM4 and CM5 microfragments by Raman spectroscopy (CM5 —, CM6 —, NA —. Insert magnification of N.A. area).

SEM micrographs also confirm the presence of microbial proliferation on samples CM5 (Fig 3.20) and CM6 (Fig. 3.21). In the CM5 area it was possible observe the ability of filamentous fungi to cover the marble surfaces (Fig. 3.20), evidencing the destructive effect of these type of microorganisms.

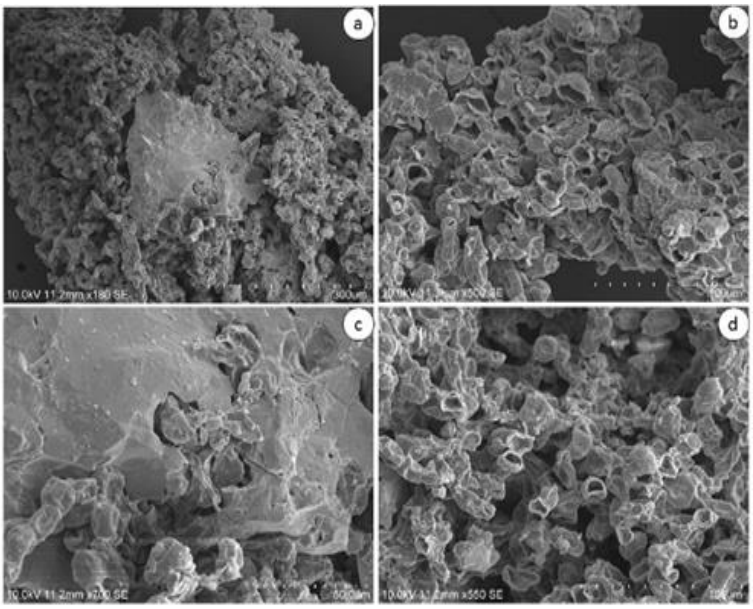


Figure 3. 20: Scanning electron microscopy (SEM) micrograph CM5 exhibiting microbial proliferation on the sample (a) Microbial proliferation – filamentous fungi surrounding the substrate. (b.c,d) Magnified area a showing filaments of the fungal proliferation.

On the other hand, in the figure 3.21 (CM6 area) it was encountered diatoms, microalgae but also filamentous fungi proliferating on the black biofilm, wich suggest a very complex community.

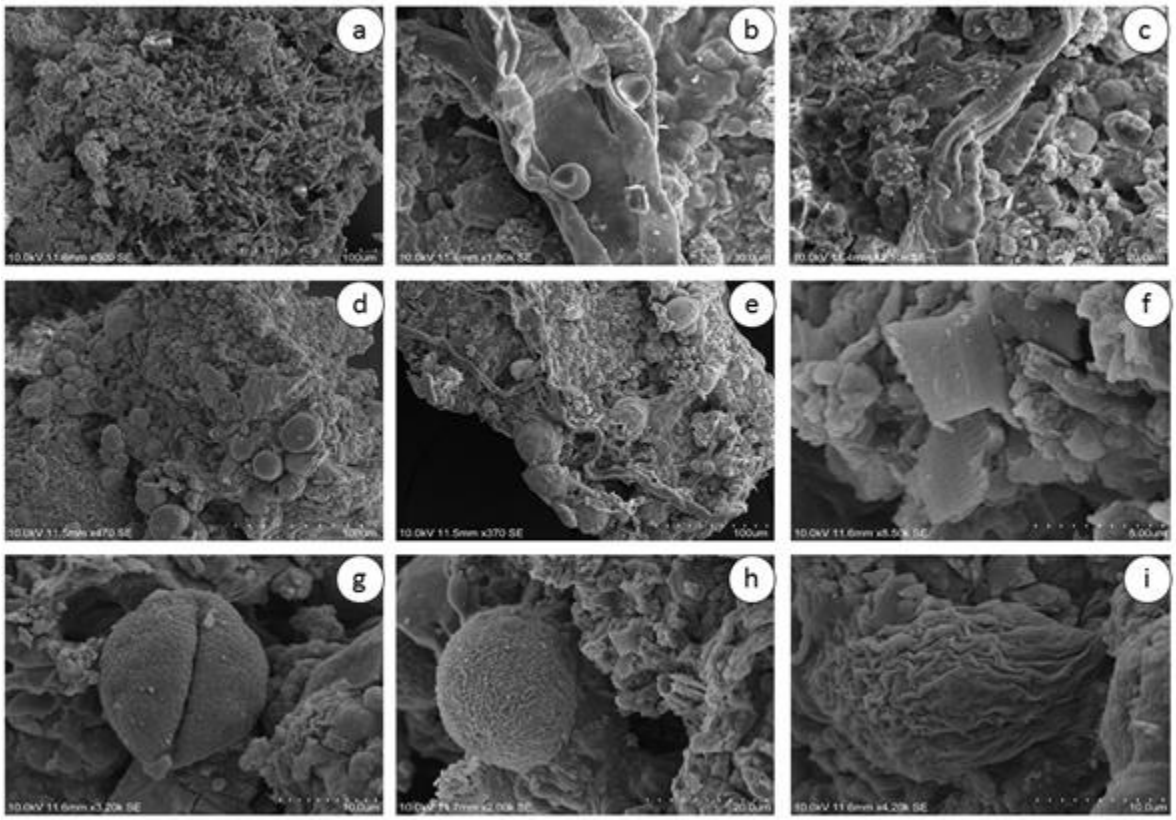


Figure 3. 21: Scanning electron microscopy (SEM) micrograph of CM6 (a-e) exhibiting different types of microbial proliferation on the sample,(f)Presence of diatom, (g-i) unidentified structures in the black biofilm.

3.5 MICROBIAL DIVERSITY IDENTIFICATION

Microorganisms contribute a great deal in deterioration of stone along with the deterioration of other material used as building matter – concrete, mortar, paints (Katja Sterflinger G. P., 2013). In several cases microbial colonisation of algae, fungi and bacteria leads to severe sturctural and aesthetic damages (Claudia Schabereiter-Gurtner, 2001).

This approach allowed to identify the cultivable predominant groups of filamentous fungi present on the Maltesas like *Aspergillus* sp., *Mucor* sp., *Penicillium* sp., *Alternaria* sp. and *Cladosporium* sp. and yeasts like *Rhodotorula* sp. were encountered along with a number of variety of black yeast (Appendix III, Table A.V). While studying the bacteria we encountered coccus and bacillus Gram-positive and Gram-negative (Appendix IV, Table A.VI). Lichens or cyanobateria was not observed using Culture Dependent Methods but were detected by NGS

results (Fig. 3.22 and 3.23)), by the presence of *Dirina* sp..

Krumbein was the first to suggest the role of fungi in the detrerioration of carbonate. Fungi has the ability to excrete acids like acetic, oxalic citric, formic fumaric, glyoxylic acid as excreta which hamper the carbonate. The mostly commonly found microbes are *Penicillium* sp., *Cladosporium* sp. are responsible for soiling (Sterflinger, Fungi : Their role in deterioration of cultural heritage, 2010) All these species have been found on the marbles studied from Maltesas Convent. It must be also noted that fungi can erode silicates. Grote in 1986 has suggested that *Alternaria* sp., *Cladosporium* sp., *Fusarium* sp. and *Penicillium* sp. have the ability to oxidise iron and manganese on rock (Sterflinger, Fungi as a Geologic Agent , 2000) and it is evident from the samples collected that there is evidence of both manganese and iron and the above mentioned microbial species have been found on the samples studied. Fungi plays a destructive role as it is extremely corrosive on the stone. In moderate climate *Alternaria* sp., *Cladosporium* sp., *Epicoccum* sp., *Areobasidum* sp. and *Phoma* sp. are found (Sterflinger, Fungi : Their role in deterioration of cultural heritage, 2010). All these species of microorganisms have been found from the samples from Maltesas Convent. With reference to CM1 calcium oxalate can be caused owing to surface microflora like bacteria, fungi, algae and lichens which have the ability to survive on any stone surface despite the differences in the provenance, previous treatments and other dead or decaying microorganisms which colonises the stone (Rampazzi, 2004). There are some microorganism that excrete oxalic acids, there are some other microorganisms that use this oxalic acid to grow (Benedetti, 2007). Oxalic acid is a by-product of metabolism by lichens and the remains of the reproductive system has been found to be one of the causes for these patina, however not all diversities of lichen have the ability to make oxalic acid. The oxalic acid reacts with calcareous stone which forms a thin membrane of calcium oxalate. Fungus, *Sporotichum* spp. is one of the main culprits for the cause of oxalate films on monuments (Benedetti, 2007).

Next Generation Sequencing (NGS), massively parallel or deep sequencing are related terms that describe a DNA sequencing technology which has revolutionised genomic research (Sam Behjati, 2013). The release of metabolic acids is one of the best-known biogeochemical destructive mechanisms at rock surfaces (Liz Karen Herreraa, 2004).

Unlike, the Culture Dependent Method (CDM), NGS studies can give a more detailed panorama of the microrganisms that are present in the substrate. However, sometimes microbes identified by NGS cannot be cultivated in the laboratory. With the help of NGS we have been able to identify a number of black yeast like *Coniosporium* sp., *Capnobotryella* sp., *Exophiala* sp., *Knufia* sp. and *Trimmatostroma* sp., also reported by (Katja Sterflinger G. P., 2013). The

growth of black fungi on rock surfaces leads to selective absorption of solar radiation hampering the crystalline formation. They are also responsible for the rock surface alteration (Katja Sterflinger H. P., 2001)**.** The black patina can also be caused by fungi *Pencillium* sp. and *Curvularia* sp. both present in the samples, as described by (Liz Karen Herreraa, 2004) in all samples. Fungi are one of the most important microorganisms of endolith category to populate stone surface and result in soiling. The common fungi population found in temperate climate include generaly microrganisms of the genera *Alternaria* sp, *Cladosporium* sp, *Epicoccum* sp, *Aureobasidium* sp and *Phoma* sp (Sterflinger, Fungi: Their role in deterioration of cultural heritage, 2010) (Fig.3.22).

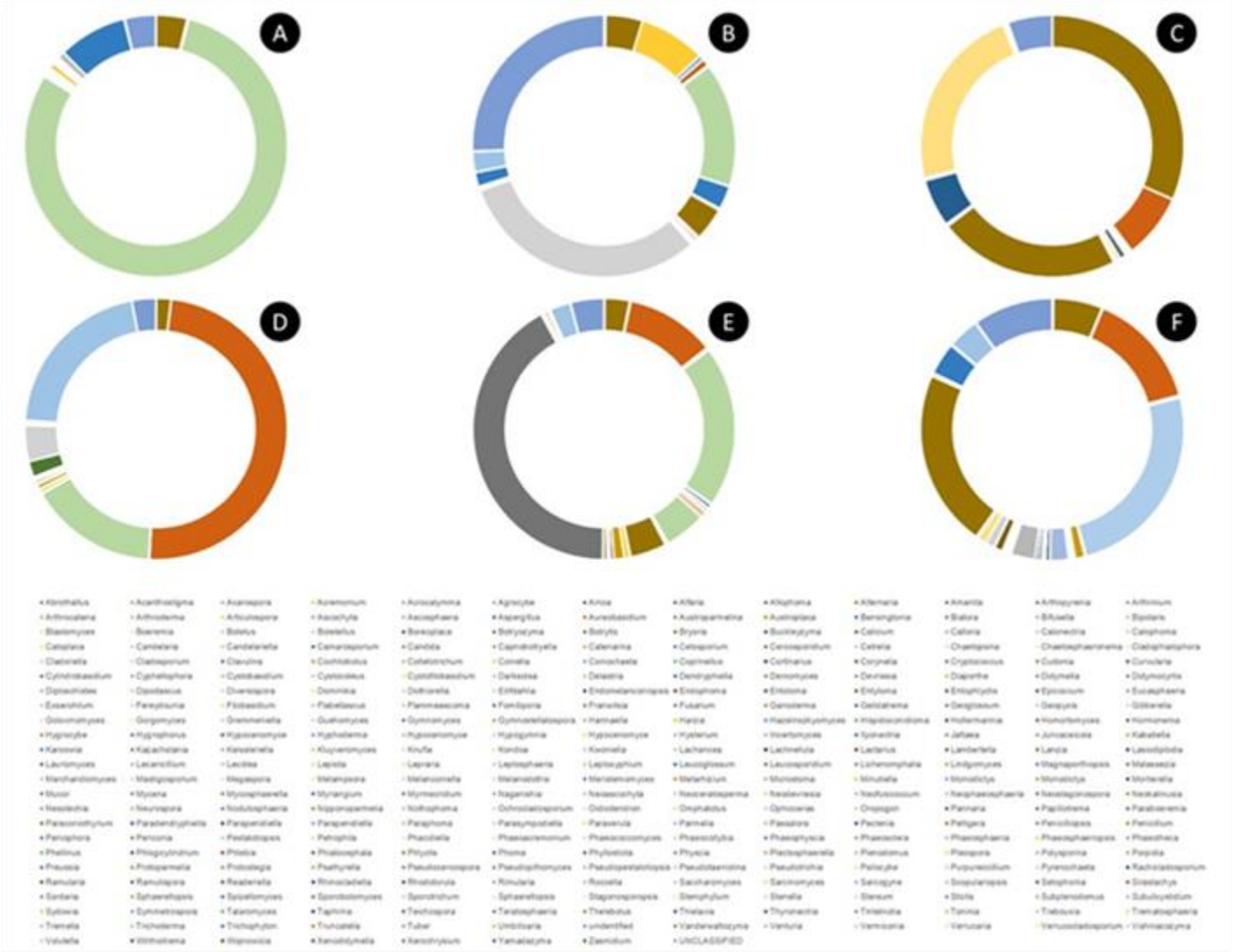


Figure 3. 22: NGS results of Eukaryotes present on Maltesas Convent [(A)CM1, (B) CM2, (C) CM3, (D) CM4, (E)CM5, (F)CM6].

Prokaryotes like *Rubrobacter* sp. (Claudia Schabereiter-Gurtner, 2001) and *Staphylococcus* sp. (Liz Karen Herreraa, 2004) were found in the samples that impart a rosy and yellowish discolouration respectively (Fig.3.23).

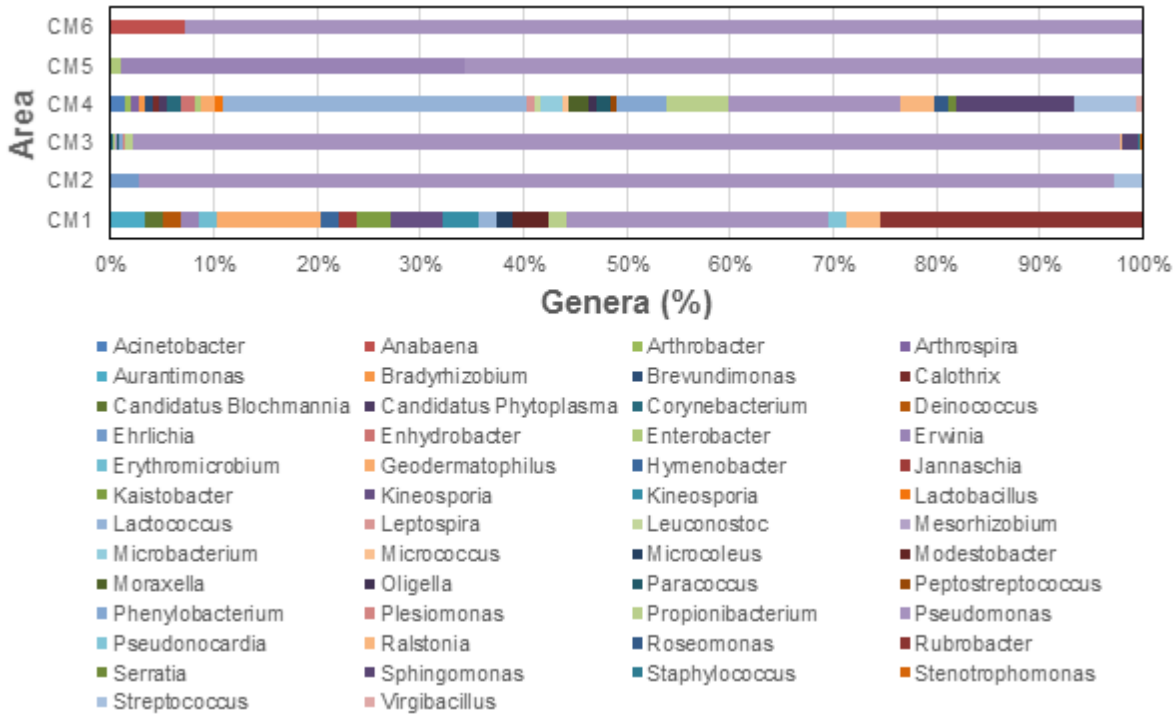


Figure 3. 23: NGS results of Prokaryotes proliferating on Maltesas Convent.

The study of microbial proliferation is not a stand-alone study, there are several factors that aid the growth of these microbes. The atmospheric conditions is one of the main reasons for this. While studying the microorganisms from Maltesas Convent we have encountered several pathologies that have resulted due to the microbial community. There are several instances that help us identify these microorganisms before running in-depth analysis, like chromatic alterations which in many cases are visible by the naked eye. To study their growth a much consolidated approach needs to executed, which will help future conservators and restorers to get a very concise picture for mitigating the issues. This analysis conducted during this thesis aims to do this by first studying the chromatic changes leading to characterising the material and then studying the microbial communities that affect Cultural Heritage.

IV -FINAL REMARKS

Marble samples from Maltesas Convent, located in Estremoz, with several damaging signals were studied to assess biocontamination presence and understand the role of microbial proliferation on the alteration process of these materials. Several *in situ* and laboratory analysis, using non-invasive and non-destructive techniques, were carried out to study these materials and to try detect alteration products. Subsequently, in order to study the microbial population CDM and NGS approaches were used. To study chromatic alterations DinoLite Microscope, FORS and Colorimetry were used. *In situ* XRF was also used to study the elemental analysis of the material. Complementary techniques like XRD, SEM and SEM-EDS were applied to overcome the drawbacks on the material analysis and biocolonisation assessment. Raman spectroscopy and FTIR-ATR were used to support and improve the characterisation of products alteration.

Three different types of pathologies were observed on the marble materials of Maltesas Convent – salt efflorescence formation, staining and/or pigmentation and the development of biofilms. Some Chromatic alterations detected *in situ* by portable Microscopy was correlated with microbial proliferation, colorimetry and FORS results also corroborate the biogenic origin of these alterations.

The possible formation of scialbatura due to the presence of calcium oxalates – wheddelite and whewellite, and also a small amount of quartz, detected by XRD and Raman spectroscopy show the presence of degradation products in some zones of the monument. The presence of these compounds can be a result of air pollution, sunlight and rain exposure once the marble materials are on outdoor area of Maltesas Convent. It was also been observed through SEM-EDS some amount of magnesium and this may be due to some thoughtlessly conservation work that was done by applying lime wash.

Samples CM3 and CM4 exhibit pathologies of staining. The red discolouration, as suggested by XRF results, seems in some cases to be attributed to the presence of iron and its degradation. This is also supported by the finding from SEM-EDS which shows a high presence of iron in the correspondent samples. On the other hand, the red pigmentation observed on the fissures of CM4 area seems to be due to the presence of carotenoids, according to Raman spectroscopy and FTIR-ATR results.

The most obvious aesthetic damages observed on Maltesas Convent was the biofilms formation on CM5 and CM6 areas. These zones were subjected regularly to strong sunlight and rain water. SEM-EDS analysis signalise biocontamination on these surfaces and SEM observation shows

the presence of filamentous fungi, proliferation of fungal hyphae on the stone materials and their capacity for in depth dissemination. It was also detected microalgae, diatoms and several unidentified structures. On the other hand, Raman spectroscopy shows a profile of biocontamination and the absence of peaks characteristic from marble materials, which seems to be masked by the biofilm layer that covered several areas of Maltesas Convent.

CDM approaches allowed the identification of prominent cultivable filamentous fungi like *Aspergillus* sp., *Mucor* sp., *Penicillium* sp, *Alternaria* sp. and *Cladosporium* sp. and yeasts like *Rhodotorula* sp. and black yeasts. While studying bacteria we encountered coccus and Gram-positive and Gram-negative bacillus. Next Generation Sequencing, corroborating and complementing the CDM findings. This approach allowed identify a number of black yeasts *Coniosporium* sp., *Capnobotryella* sp., *Knufia* sp., *Exophiala* sp., and *Trimmatostroma* sp., which plays an important role in cultural heritage deterioration. Bacteria like *Rubrobacter* sp. and *Staphylococcus* sp. were found in the samples that impart a rosy and yellowish discolouration respectively, supporting the patina formation and carotenoid staining on the marbles. But mainly, Next Generation Sequencing, a high-throughput screening method, enabled a more complete characterisation of the microbiota present on Maltesas Convent, allowing access to a dynamic of microbial populations.

Cultural Heritage is an important manifestation of the people of the past – aesthetic, cultural and economic ways of life, that contains the visible and tangible traces from antiquity, being very important their preservation and protection. A substantial part of cultural heritage is built and forms a concrete and noticeable form of expression and thought processes. Marble, has been a form of expression since antiquity and was used as tool to exhibit social and economic status. It is therefore very important save and preserve these monuments because the marble used for any construction will change in their physical and chemical properties from when the leave a quarry till they complete disintegrate.

This study highlights the importance of the biocontamination on deterioration process of marble materials and consequently of cultural heritage assets, due to the ability to adapt itself to the environmental conditions and the supply of nutrients and slowly but steadily destroy these expression of mankind. Thus, the destructive action of these microorganisms, needs to be controlled to avoid material disintegration and promote cultural heritage safeguard.

To further increase the knowledge about biodeterioration in Maltesas Convent, and better understanding of marble alteration processes, it would still be interesting to do:

- Raman mapping to assess material composition and alteration produts distribution on the marble surfaces. Application of Spatially Offset Raman Spectroscopy (SORS) to study and characterise the materials below the areas with thick biofilm formation. Surface Enhanced Raman Spectroscopy (SERS) help to study the pigmentation that takes place on the stone due to the presence of microorganisms.
- In-depth study of lichens and phototrophic microorganisms like cyanobacteria and algae, involved in the process.
- Comparative Denaturating Gradient Gel Elecrophoresis (DGGE) analyses of the total DNA and RNA from microbial communities, in order to estimate the biodeteriogenic potential in a fast screening.
- The application of Fluorescence *In Situ* Hybridisation (FISH), with specific probes for biodeteriogenic agents, can be taken into account to perform their monitorisation.

V -BIBLIOGRAPHY

A. Calia, M. G. (2011). Cultural Heritage Study : Microdestructive techniques for detection of clay minerals on the surface of historic buildings. *Applied Clay Science* , 525 - 531 .

A. Ozguven, Y. O. (2014). Effects of high temperature on physico-mechanical properties of Turkish natural building stones. *Engineering Geology*, 127-136.

A. Torok, R. (2010). Current Methods and future trends in testing durability analyses and provenance studies of natural stones used in historical monuments. *Engineering Geology*, 139-142.

Alison Gilchrist, J. N. (1999). Colorimetry, Theory. *Encyclopedia of Spectroscopy and Spectrometry* , 337-343.

Artioli, G. (2010). *Scientific Methods and Cultural Heritage: An introduction to the application of materials science to archaeometry and conservation science.* Oxford University Press .

Bacci, M. (1995). Fibre Optics Application to work of Arts. *Sensors and Actuators B Chemical* .

Benedetti, D. (2007). Transformation in calcium carbonate. *Phase Transition*.

Berns, R. S. (2005). Rejuvenating the Appearance of Cultural Heritage Using Color and Imaging Science Techniques. *AIC Colour 05 - 10th Congress of the International Colour Association* .

Borreli, E. (1999). *Conservation of Architectural Heritage, Historic Structure and Materials - Salt* . Rome: ICCROM.

C. McNamara, R. M. (2015). Microbial deterioration of historic stone . *Frontiers in Ecology and the Environment* .

C. McNamara, T. P.-D. (2006). Microbial Porcesses in the Deterioration of Maya Archeological Buildings in Southern Mexico.

C. Saiz-Jimenez, P. B. (2004). *Damages caused to European monuments by air pollution: assessment and preventive measures.* (C. Saiz-Jimenez, Ed.) London: Taylor and Francis Group.

C.Gaylarde, M. S. (2003). Microbial Impact on Building Materials : An Overview. *Materials and Structures* , 342-352.

C.Gaylarde, m. S. (2006). Microbial Impact on building materials : an overview. *Materials and Structure* , 342-352.

Choudhury, A. (2010). Visual Measures of COlour. In M. Gulrajani, *Colour Measurement : Principles, Advances and Industrial Applications* . Woodhead Publishing Limited.

Christine Hallmann, J. R. (2011). Microbial diversity on a marble monument: a case study. *Environmental Earth Sciences* , 1701-1711.

Christos G, K. N. (2000). Calcium carbonate phase analysis using XRD and FT-Raman spectroscopy. *The Analyst*.

Clara Urzi, F. D. (2001). Biodeterioration of Cultural Heritage in Italy: State of Art.

Clara Urzi, W. T. (1992). On the Question of Biogenic Changes of Mediterranean Monuments (coating,crust,microstromatolite,patina,scialbatura,skin rock varnish). *2md International Symposium on : The Conservation of Monuments in the Mediterranean Basins.* Geneva.

Claudia Schabereiter-Gurtner, G. P. (2001). Rubrobacter related bacteria associated with rosy discolouration of masonry and lime wall paintings. *Archives of Microbiology* , 347-354.

Crabbe, R. H. (2009). Environment, Pollution and Effects. In R. H. Watt, *The Effects of Air Pollution on Cultural Heritage* (pp. 1-27). Boston: Springer Verlag.

Crespo, M. (2ª Edição). *Estremoz e o seu termo "regional".* Vila Viçosa: Gráfica Calipolense.

Cristina Sabbioni, G. Z. (1991). Oxalate Patinas on Ancient Monuments : the biological hypothesis. *Aerobiology*, 31-37.

Cristina Sabbioni, G. Z. (1991). Oxalate patinas on ancient monuments : the biological hypothesis . *Aerobiology*, 31-37.

D. Benedetti, E. B. (2007). Transformation in calcium carbonate stones. *Phase Transition*.

Daniel Vazquez, A. A.-B. (2014). Spectral and Colorimetric Measurements for Cultural Heritage. *Colour and Space in Cultural Heritage session at the Denkmäler 3D Conference, "From low-cost to high-tech.* Dortmund.

Dennis Allsopp, K. J. (2004). *Introduction to Biodeterioration.* Cambridge University Press.

Devi Taelman aDevi Taelman, M. E. (2013). Roman marble from Lusitania: petrographic and geochemical characterisation. *Journal of Archeological Sciences*, 2227-2236.

Durn, G. (2003). Terra Rossa in the Medeiterrranean Region : Parent Materials, Composition and Origin . *Geologica Croatica* , 83-100.

Eric Doehne, C. A. (2010). *Stone Conservation An Overview of Current Research.* Getty Conservation Institute.

F. Persia, L. C. (2010). Study of Ageing Effects on Treated Marbles by Colorimetry and Laser Induced Fluorescence. *International congress on Science and technology for the safeguard of cultural heritage in the Mediterranean basin* , (pp. 239-334). Cairo.

Fernanades, P. (2006). Applied Microbiology and biotechnology in the conservation of stone cultural heritage materials. *Applied Microbiology Technology*, 73-291.

Franco Palla, E. T. (2007). Chromatic Alteration on Marble Surfaces Analysed by Molecular Biology Tools. *Conservation Science in Cultural Heritage* , 111-121.

Gadd, G. M. (2007). Geomycology: biogeochemical transformations of rocks, minerals, metals and radionuclides by fungi, bioweathering and bioremediation. *Mycological Research*, 3-49.

Guadalupe Pinar, K. R. (2009). The micro-biota of a sub-surface monument the medieval chapel of St. Virgil (Vienna, Austria) . *International Biodeteriorationa nd Biodegradation*, 851-859.

Guillitte, O. (1995). Biorecptivity : A New Concept for Building Ecology Studies. *The Science of the Total Environment* , 215-220.

H.G.M. Edwards, N. M. (1997). Calcium Oxalate in lichen biodeterioration studied usin FT-Raman spectroscopy . *Spectrochimica Acta Part A*, 99-105.

Herlemann D P, L. M. (2011). Transitions in bacterial communities along the 2000km salinity gradient of the Baltic Sea. *ISME J* , 5:1571-1579.

https://geology.com/rocks/marble.shtml. (n.d.). Retrieved from https://geology.com/rocks/marble.shtml.

J.Karbowska-Berent. (n.d.). Microbiodeterioration of Mural Paintings : A Review.

Jorge F.Carvalho, P. H. (2008). Decision criteria for the exploration of ornamental-stone deposits: Application to the marbles of the Portuguese Estremoz Anticline. *International Journal of Rock Mechanics and Mining Sciences*, 1306-1319.

Katja Sterflinger, G. P. (2013). Microbial deterioration of cultural heritage and works of art — tilting at windmills? *Applied Microbiology and Biotechnology*, 9637-9646.

Katja Sterflinger, H. P. (2001). Molecular taxonomy and biodiversity of rock fungal communities in an urban environment (Vienna, Austira) . *Antonie van Leeuwenhoek* , 275-286.

Kevin Beck, S. J.-B.-M. (2015). Non-destructive diagnosis by colorimetry of building stone subjected to high temperatures. *European Journal of Environmental and Civil Engineering*, 643-655.

Klindworth A, P. E. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. . *Nucleic Acids Res* , 41.

L.Lopes, D. (2015). Global Stone Heritage : Estremoz Marbles, Portugal. In B. S. D.Pereira, *Global Heritage Stones : Towards International Recognition of Building and Ornamental Stones.* London: The Geological Society.

Liz Karen Herreraa, C. A. (2004). Biodeterioration of peridotite and other constructional materials in a building of the Colombian cultural heritage. *International Biodeterioration and Biodegradation* .

Luis Lopes, R. M. (n.d.). *Marbles from Portugal*. Retrieved from www.naturalstone-online.com: https://www.naturalstone-online.com/fileadmin/NatursteinDaten/Anzeigenseite_neu/portugal_marble1.pdf

M.Garcia -Valles, M.-S. J. (1998). Interaction of rocks and atmosphere : Patinas and Mediterranean monuments. *Environmental Geology*.

M.Gill, R. V. (2003). Color essays: an insight look of Alentejo traditional limewash paintings and colored lime mortars. *Conference of Historical Mortars, Lisbon, LNEC,* . Lisbon.

M.Gill, V. M. (2014). Material and Diagnostic Characterization of 17th Century Mural Paintings by Spectra Colorimetry and SEM-EDS: An Insight Look at Jse de Escovar Workshop at the CONVENT of Na Sra da Saudacao (Southern Protugal) .

(2015). *Marble The Divine Stone.*

Marco Del Monte, C. S. (1987). A Study of Patina called 'Scialbatura' on Imperial Roman marbles. *Studies in Conservation* , 114-121.

Marco Del Monte, C. S. (1987). The study of the Patina called "Scialbatura" on Imperial Roman Marbles. *Studies in Conservation, Vol. 32, No. 3*, 114-121.

May, E. (2010). Stone Biodeterioration. In C. R.Mitchell, *Cultutral Heritage Microbiology: Fundamental Studies in Conservation Studies.* Washington DC: ASM Press.

Medreiros, J. F. (2001). *Património Religioso de Estremoz.* Estremoz: Câmara Municipal de Estremoz - Gráfica Eborense.

Milica V. Ljaljevic Grbic, J. B. (2009). *Role of Fungi in Biodeterioration Process of Stone in Historic Buildings.* Belgrade, Serbia: Institute of Botany and Botanical Gardens .

Olsen, C. e. (1980). The Terra Rossa Limestone Contact Phenomenon in Karst , Southern Indiana. *Soil Science Society of American Journal*, 1075-1079.

P.Tiano, S. S. (n.d.). Biomeditated Calcite Precipitation for the Reinforcement of Monumental Stones.

Pinna, D. (2017). *Coping with Biological Growth on Stone Heritage Objects - Methods, Products, Applications and Perspectives.* Apple Academic Press.

Rachel Douglas-Jones, J. J. (2016). Science, value and material decay in the conservation of historic environments. *Journal of Cultural Heritage* , 823-833.

Rampazzi, L. (2004). Analytical investigation of calcium oxalate films on marble monuments. *Talanta*.

Rihana Terzu, e. a. (2016). Analythical study of marble consolidation of Oxalate precipitation using density, FTIR, and Powder XRD Measurements. *Journal of Eng. and Processing Managment*.

S.K.Haldar, J. (2014). Sedimentary Rocks, Chapter 5. *Introduction to Mineralogy and Petrology*, 121 - 212.

Saidov, T. (2012). Sodium sulphate heptahydrate in weathering phenomena of porous material; PhD Thesis. The Netherlands: Eindhoven University of Technology Library.

Saiz-Jimenez, C. (2003). *Biodeterioration: An Overview of the State of the Art and Assessment of Futire Directions.*

Sam Behjati, P. S. (2013). What is Next Generation Sequencing? . *Archives of Disease in Childhood* , 236-238.

Stephanie Scheerer, O. O.-M. (2009). *Microbial Deterioration of Stone Monuments - An Updated Overview*. Elsevier Inc.

Sterflinger, K. (2000). Fungi as a Geologic Agent . *Geomicrobiology Journal* , 97-124.

Sterflinger, K. (2010). Fungi : Their role in deterioration of cultural heritage. *British Mycological Society*, 47-55.

Sterflinger, K. (2010). Fungi : Their role in deterioration of cultural heritage. *British Mycological Society* , 47-55.

Sterflinger, K. (2010). Fungi: Their role in deterioration of cultural heritage. *Fungal Biology Reviews*, 47-55.

Susana E Jorge-Villar, H. E. (2011). Raman spectroscopic analysis of arctic nodules: Relevance to the astrobiological exploration of Mars. *Analytical and Bioanalytical Chemistry*, 2927-2933.

Svahn, H. (2006). *Final Report for the Research and Development Project : Non Destructive Field Tests in Stone Conservation Literature Study*. Stockholm: Riksantikvarieambetet.

T. Warscheid, J. (2000). Biodeterioration of Stone : A Review. *International Biodeterioration and Biodegradation*.

Tania Rosado, M. G. (2013). Oxalatae Biofilm Formation in mural paintings due to microoraganisms - A Comprehensive study . *International Biodeteriorationa dn Biodegradation* , 1-7.

Tania Rosado, M. S. (2016). A first insight on the biodegradation of limestone: the case of the World Heritage Convent of Chirst. *Applied Physics Material Science and Processing*.

Tania Rosado, M. S. (2017). Microorganisms and the integrated conservation-intervention process of the renaissance mural paintings from Casas Pintadas in Evora - Know to act,act to preserve. *Journal of King Saud University* .

Tedersoo L, B. M. (2014). Global diversity and geography of soil fungi. *Science* , 346:1078.

Tiano, P. (2002). *Biodegradation of cultural heritage: decay mechanisms and control methods.* Lisbon: New University of Lisbon, Department of Conservation and Restoration.

V. Rives, J. G. (2006). Decay and Conservation of Building Stones on Cultural Heritage Monuments. In P. M. Vilarinho, *Advances Material Science Forum* (pp. 514-516). Trans Tech Publications .

V.Verges-Belmin. (2008). *Illustrated Glossary on Stone Deterioration Patterns.* Champigny/Marne, France: ICCMOS International Scientific Committee for Stone (ISCS).

Warschied, T. (2002). The Evaluation of Biodeterioration Process on Cultural Objects and Approaches for their Effective Control . In V. H.-F. Robert J. Koestler, *Art, Biology and Conservation Biodeterioration of Works of Art.* The Metropolitan Museum of Art.

Winkler, E. (1997). *Stone in Architecture - Properties and Durabiltiy* . New York : Springer - Verlag Berlin Heidelberg .

VI -APPENDICES

APPENDIX I

NUTRIENT AGAR (NA) – for identifying Bacteria:

Table A. I : Composition for NA for studying bacteria (for 1L).

COMPOSITION	QUANTITIY (g)
Peptic Digest Animals	5
Beef Extract	1.5
Yeast Extract	1.5
Sodium Chloride	5
Agar	15

For making Nutrient Broth we do not add Agar

APPENDIX II

MALT EXTRACT AGAR (MEA): for identifying yeast and fungus:

Table A. II: Composition for MEA for studying yeasts and fungi (1L).

COMPOSITION	QUANTITIY (g)
Malt Extract	30
Peptone Mycologic	5
Glucose	20
Agar	15

For making Malt Extract we do not add Agar

COOK ROSE BENGAL (CRB): for identifying yeast and fungi

Table A. III: Composition for MEA for studying yeasts and fungi (1L).

COMPOSITION	QUANTITIY (g)
Peptone	5
Glucose	10
K2HPO4	1
MgSO4	05
Rose Bengal	0.05
Chloramphenicol	0.1
Agar	15.5

APPENDIX III

ASM1 – for identifying lichens

Table A. IV: Composition for ASM1 for lichens.

Solution Stock A:

COMPOSTION	QUANTITY FOR 1 L/100mL
$NaNO_3$	8.5/0.85
$MgCl_2.6H_2O$	2.05/0.205
$MgSO_4.7H_2O$	2.45/0.245
$CaCl_2.2H_2O$	1.45/0.145

Solution Stock B:

COMPOSITION	QUANTITY FOR 100 mL
$KH_2PO_4.3H_2O$	0.87
$Na_2HPO_4.12H_2O$	1.78

Solution Stock C:

COMPOSITION	QUANTITY FOR 100 mL
H_3BO_3	2.48
$MnCl_2.4H2O$	1.39
$FeCl_3.6H2O$	0.65
$ZnCl_2$	0.335
$CoCl_2.6H2O$	0.019
CuCl	0.0014

Solution Stock D:

COMPOSITION	QUANTITY FOR 100 Ml
$Na_2.EDTA.2H_2O$	1.86

Prepare Solutions A, B, C, D and sterilse.

Prepare the medium with H_2O to sterile + A, B, C, D sterile

F2 – for identifying lichens

Table A. V : Composition for F2 for studying lichens.

Solution Stock A: Primary Solution – make separately

COMPOSTION	QUANTITIY FOR 1L/100mL (g)
$NaNO_3$	75/7.5
Na_2HPO_4	5/0.63

Solution Stock B: Trace Metal Solutions

COMPOSITION	QUANTITY FOR 1L/100mL (g)
Na_2.EDTA	1.86/0.186
$FeCl_3.6H_2O$	3.15/0315
BASIC METAL SOLUTION	1 (of each of 5 solutions)

Solution Stock C: Basic Metal Solution (separated)

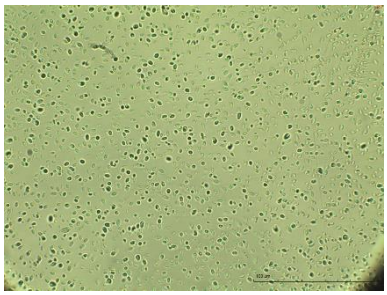
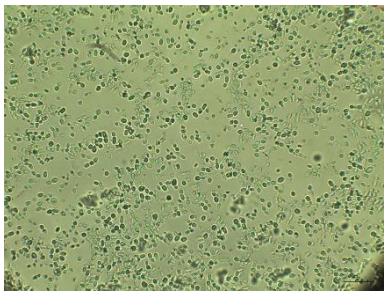
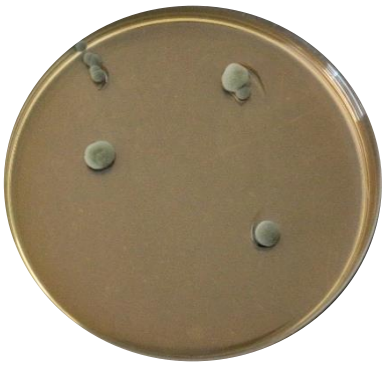
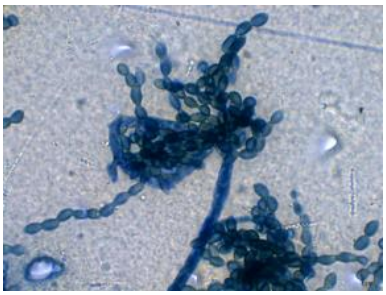
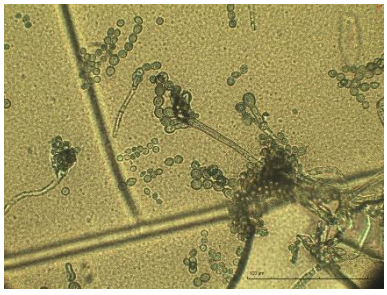
COMPOSITION	QUANTITY FOR 100mL (g)
$MnCl_2.4H_2O$	1.8
$NaMoO_4.2H_2O$	0.63
$ZNSO_4.7H_2O$	2.2
$CoCl_2.6H_20$	1
$CuSO_4.5H_2O$	1

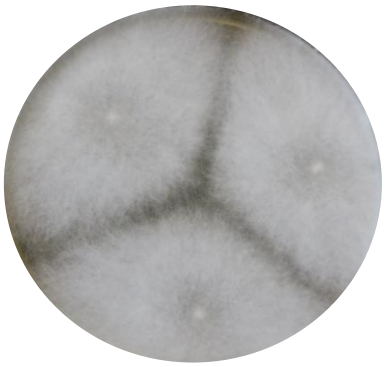
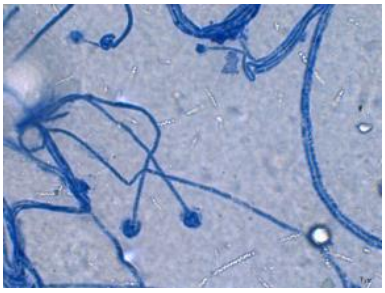
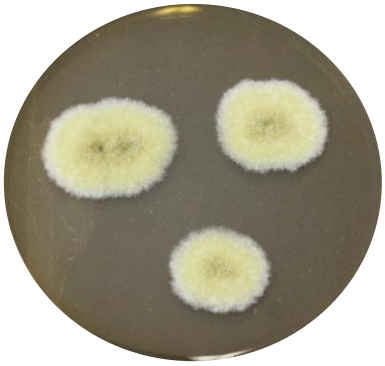
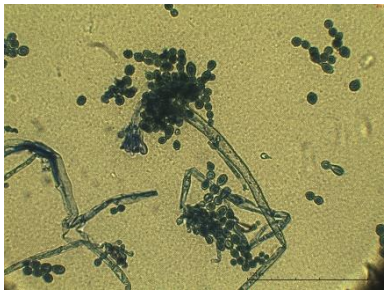
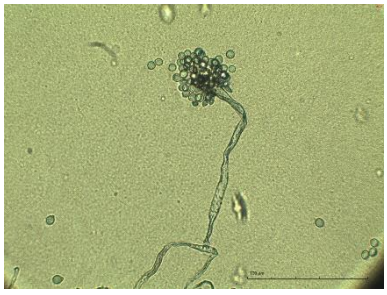
Solution Stock D: Vitamins

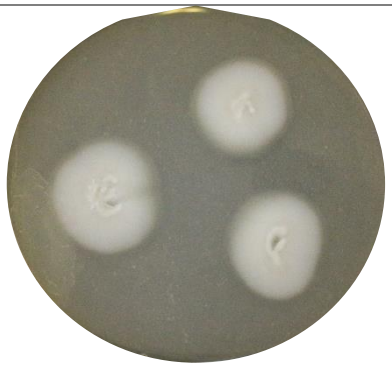
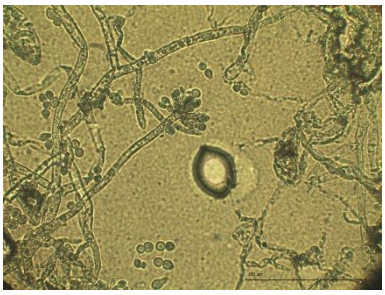
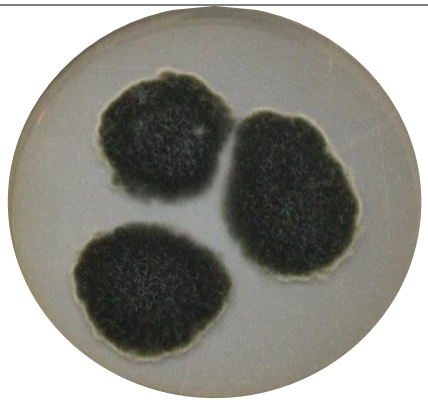
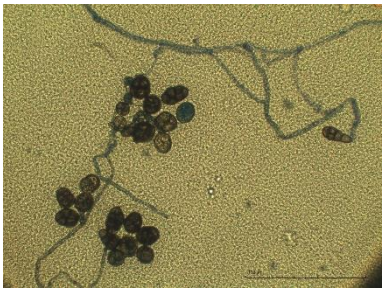
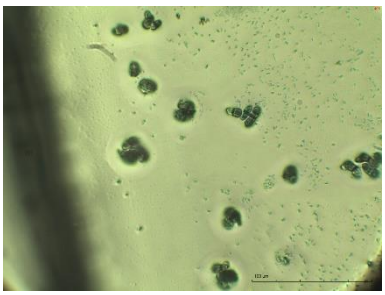
COMPOSITION	QUANTITY FOR 1L/100mL (g)
Biotina	10/1
B_{12}	1/0.1
Tiamina HCl	0.1/0.02

APPENDIX IV

Table A. VI: Macroscopic and microscopic view of isolated fungi

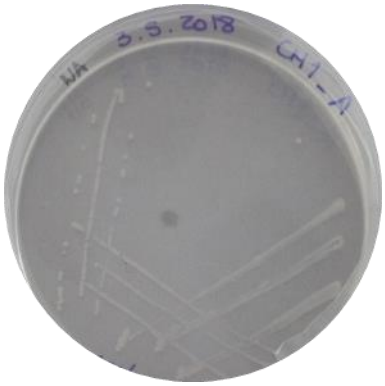
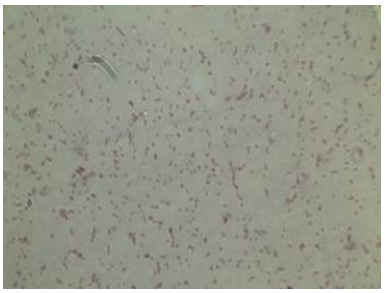
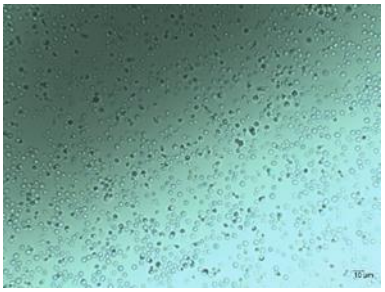
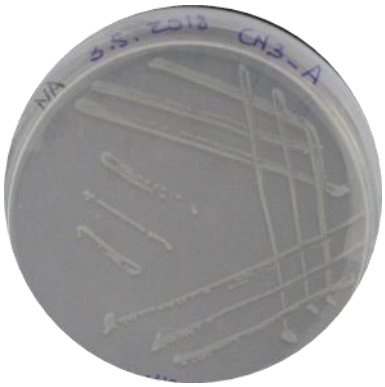
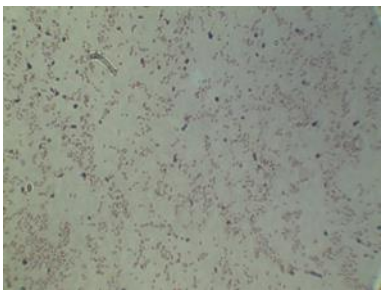
SAMPLE	MACROSCOPIC FEATURES	MICROSCOPIC FEATURES	IDENTIFICATION
Yeast CM1_1			
CM3_1			*Rhodotorula* sp.
CM3_2			*Cladosporium* sp.
CM3_3			*Aspergillus* sp.

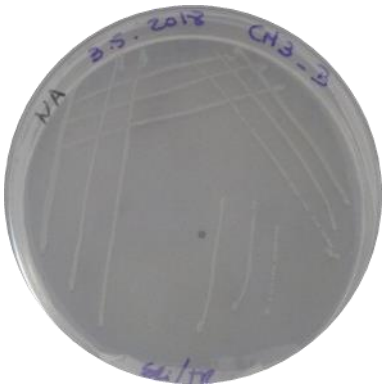
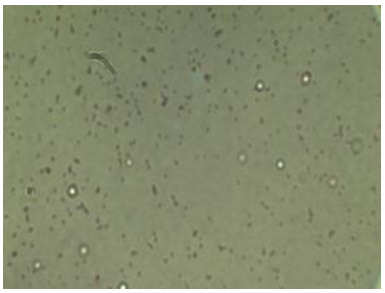
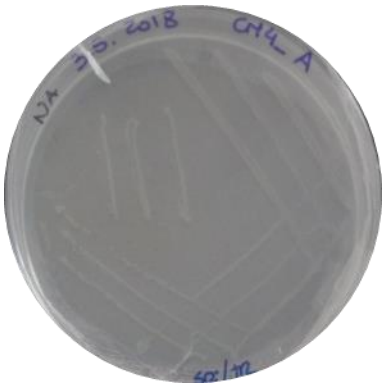
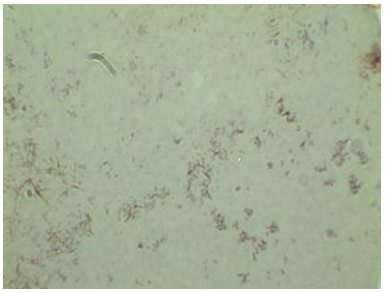
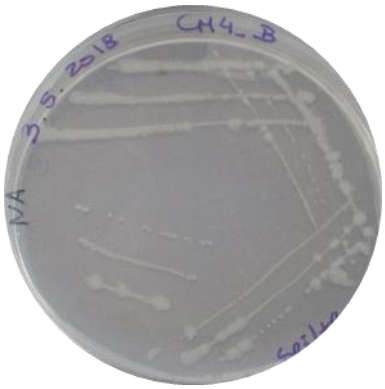
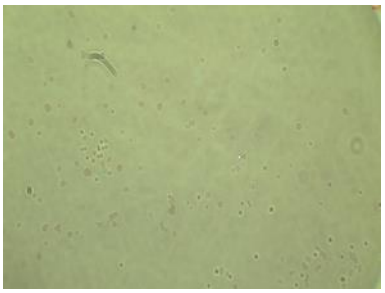
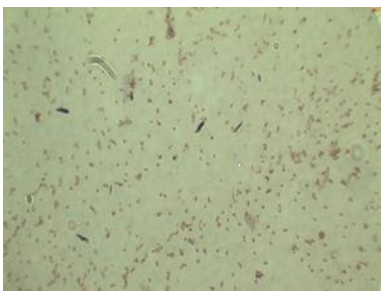
CM3_4			*Mucor* sp.
CM4_3			*Aspergillus* sp 1
CM4_5			*Aspergillus* sp.1

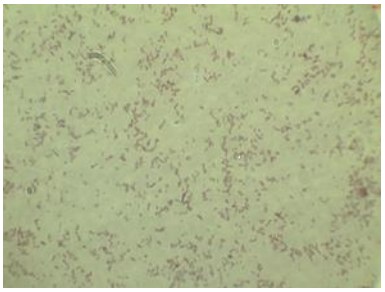
CM4_6			Mycelium
CM5_5			*Alternaria* sp.
Black Yeast CM6_1			

APPENDIX V

Table A. VII: Macroscopic and microscopic view of isolated bacteria.

SAMPLE	MACROSCOPIC FEATURES	MICROSCOPIC FEATURES	IDENTIFICATION
Coccus CM1_A			Gram-positive
Bacillus CM2_A			Gram-positive
CM3_A			*Bacillus* sp. Gram-negative

Coccus CM3_B			Gram-positive
CM4_A			*Bacillus* sp. Gramn-negative
Coccus CM4_B			Gram-positive
Coccus CM5_A			Gram-negative

Coccus **CM6_A**			Gram-positive