New methodologies for the characterisation and biodegradation assessment of mural paintings

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"Science is like a succession of closed doors that we open in stages of conquests"

Albert Einstein

Aos meus pais e irmão

To my parents and brother

"Afinal, há é que ter paciência, dar tempo ao tempo, já devíamos ter aprendido, e de uma vez para sempre, que o destino tem de fazer muitos rodeios para chegar a qualquer parte."

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Abstract

Mural paintings are an ancient art form, with historic and cultural value, whose preservation is imperative.

These artworks have suffered degradation, promoted by several agents, however, the contribution of the microorganisms on the paintings alteration has been undervalued.

This work aimed the development of innovative strategies that allow to identify and characterise the role of the microorganisms in the degradation/deterioration of mural paintings.

Complementary methodologies, including culture-dependent methods and molecular approaches were used, combining with microanalytical techniques to material characterisation. This enabled the development of novel analytical protocols for microbial population assessment.

Following the characterisation of the microbial diversity, the metabolically active population were assessed by enzymatic markers and viability assays, in order to signalise the main biodeteriogenic agents involved in the biodeterioration of these heritage assets.

Through simulation assays, using high cells density from the microbial isolates, complemented with *in situ* tests, it was possible to detect the presence of several alteration products namely oxalates, *plattnerite* and carotenoids, attributed to specific biodeteriogenic agents. Mitigation strategies, directed to the identified biodeteriogenic agents, were also developed.

Keywords

Mural paintings, Aesthetic and structural damages; Biodegradation/Biodeterioration; Microbial diversity assessment; Biodeteriogenic agents; Microorganisms metabolically active; Pyrosequencing; Mitigation strategies

Novas metodologias para Caracterização e Avaliação da Biodegradação de pinturas murais

Resumo

A pintura mural é uma ancestral forma de arte, com enorme valor histórico e cultural, cuja preservação é imperativa.

Estas obras de arte têm sido alvo de degradação, provocada por diversos agentes, no entanto, a contribuição dos microrganismos para o processo de alteração das pinturas tem sido pouco valorizada.

Este trabalho teve como objetivo o desenvolvimento de estratégias inovadoras que permitam identificar e caracterizar o papel dos microrganismos no processo de degradação/deterioração de pinturas murais.

Metodologias complementares, incluindo métodos de cultura e abordagens moleculares, foram usadas em combinação com técnicas micro-analíticas de caracterização material, permitindo o desenvolvimento de protocolos analíticos inovadores para avaliação da população microbiológica.

Após a caracterização da diversidade microbiológica avaliou-se a população metabolicamente ativa recorrendo a marcadores enzimáticos e testes de viabilidade celular para sinalizar os principais agentes biodeteriogénicos envolvidos na biodeterioração destes bens patrimoniais.

Através de ensaios de simulação laboratorial, utilizando elevadas densidades celulares de isolados microbianos, complementadas com ensaios *in situ*, foi possível detetar a presença de diversos produtos de alteração nomeadamente oxalatos, platenerite e carotenoides, atribuídos a agentes biodeteriogénicos específicos. Foram ainda desenvolvidas estratégias de mitigação direcionadas para os agentes biodeteriogénicos identificados.

Palavras-chave

Pintura mural; Danos estéticos e estruturais; Biodegradação/Biodeterioração; Avaliação da diversidade microbiológica; Agentes biodeteriogénicos; Microrganismos metabolicamente ativos; Pirosequenciação; Estratégias de mitigação

List of Publications

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Rosado T, Reis A, Mirão J, Candeias A, Vandenabeele P and Caldeira AT (2014) Pink! Why not? On the unusual colour of Évora Cathedral, International Biodeterioration & Biodegradation, 94:121-127.

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Rosado T, Caldeira AT, Martins M R, Dias C, Gil M, Carvalho L, Mirão J and Candeias A E (2014) Material characterization and biodegradation assessment methodology of mural paintings – application to the renaissance frescoes from Santo Aleixo church, southern Portugal, International Journal of Architectural Heritage, 8:1-18.

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Rosado T, Gil M, Mirão J and Candeias A and Caldeira AT (2013) Oxalate biofilm formation in mural paintings due to microorganisms - a comprehensive study, International Biodeterioration & Biodegradation, 85:1-7.

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Abbreviations

μ-XRD	Micro X-Ray Diffraction
$Cu_3(CO_3)_2(OH)_2$	Azurite
2D	Two-dimensional
$2PbCO_3.Pb(OH)_2$	Hydrocerussite
$2PbCO_3 \cdot PbOH_2$	Lead white
A	Adenine nucleotide
AFLP	Amplified Fragment Length Polymorphism
Al	Aluminium
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
APS	Adenosine phosphosulfate
(aq)	Aqueous
ARDRA	Amplified Ribosomal DNA Restriction Analysis
ATP	Adenosine triphosphate
Au-Pd	Gold-Palladium
B.C.	Before Christ
BBM	Bold's Basal Medium
BLAST	Basic Local Alignment Search Tool
BSE	Back-scattered mode
C	Carbon
C	Cytosine nucleotide
Ca	Calcium
$Ca(OH)_2$	Calcium hydroxide
CaC_2O_4	Calcium oxalate
$CaC_2O_4.2H_2O$	W*eddellite* (calcium oxalate di-hydrate)
$CaC_2O_4.H_2O$	*Whewellite* (calcium oxalate mono-hydrate)
$CaCO_3$	Calcite
$CaSO_4$	*Anhydrite* (Calcium sulphate)
CCD	Charge Coupled Device
CO_2	Carbon dioxide
CRB	Cook Rose Bengal
Cy3	Cyanine 3
Cy5	Cyanine 5
DGGE	Denaturing Gradient Gel Electrophoresis
DMF	Dimethylformamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
dsDNA	Double stranded Deoxyribonucleic acid
e.g.	For example
EDX	Energy Dispersive X-ray Spectroscopy
EPS	Extracellular Polymeric Substances
Fe	Iron
Fe^{2+}	Iron (II) ion
Fe_2O_3	*Hematite*
FeO(OH)	*Goethite*
FISH	Fluorescence *In Situ* Hybridisation
FTIR-ATR	Fourier Transform Infrared spectroscopy-Attenuated Total Reflection

(g)	Gas
G	Guanine nucleotide
H	Hydrogen
$H_2C_2O_4$	Oxalic acid
H_2O	Water
HCl	Hydrochloric acid
HDS	High Deteriorated Sites
He	Helium
INT	2-(p-Iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride
INTF	Iodonitrotetrazolium formazan
ITS	Internal Transcribed Spacers
K_2HPO_4	Dipotassium hydrogen phosphate
LB	Lilly and Barnett medium
LDS	Low Deteriorated Sites
MALDI-TOF	Matrix-Assisted Laser Desorption Ionization/Time-Of-Flight
MEA	Malt Extract Agar
MEYE	Malt Extract Yeast Extract Medium
Mg	Magnesium
$MgCl_2$	Magnesium chloride
$MgSO_4$	Magnesium sulphate
MnO_2	Manganese dioxide
MS	Murashige and Skoog medium
MTS	[3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]
MTT	[3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]
MUB	Modified Universal Buffer
NA	Nutrient Agar
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
Ne	Neon
O	Oxygen (element)
O_2	Oxygen
OTU	Operational Taxonomic Units
P	Phosphorus
Pb	Lead
Pb^{2+}	Lead (II) ion
Pb_3O_4	Red lead
Pb^{4+}	Lead (IV) ion
$PbCO_3$	Cerussite
PbO_2	*Plattnerite*
PbS	Galena
$PbSO_4$	Anglesite
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
p-NP	p-Nitrophenol
PNS	p-Nitrophenyl sulphate
PO_4^{3-}	Orthophosphate ion
PPi	Inorganic pyrophosphate
p-PNP	p-Nitrophenyl Phosphate
R2	Reasoner´s medium

RAPD	Random Amplified Polymorphic DNA
rDNA	Ribosomal Deoxyribonucleic Acid
RDP	Ribosomal Database Project
RFLP	Restriction Fragment Length Polymorphism
RH	Relative Humidity
RNA	Ribonucleic Acid
rRNA	Ribossomal Ribonucleic Acid
(s)	Solid
S	Sulphur
SCP	*Santa Clara* Panel
SD	Standard Deviation
SE	Secondary Electron
SEM	Scanning Electron Microscopy
SEM-EDX	Scanning Electron Microscopy coupled with Energy Dispersive X-ray Spectroscopy
Si	Silicon
SNP	Single Nucleotide Polymorphism
SO_4^{2-}	Sulphate
SPSS	Software Package used for Statistical analysis.
SSCP	Single-Strand Conformation Polymorphism
SSLP	Simple Sequence Length Polymorphism
SSR	Microsatellite polymorphism or Simple Sequence Repeat
T	Thymine nucleotide
T	Temperature
TGGE	Temperature Gradient Gel Electrophoresis
ufc	Colony forming unit
UV	Ultraviolet light
VP-SEM	Variable Pressure-Scanning Electron Microscopy
WST-1	[2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium]
XTT	[(2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide]
YEPD	Yeast Extract Peptone Dextrose

Units

%	Percentage
μL	Microliter
µM	Micromolar
Bp	Base pairs
cm	centimeter
g	gram
g/L	gram per liter
h	hour
kV	kilovolt
mg	milligram
mg/g	milligram per gram
min	minute
mM	milimolar
nm	nanometer
ºC	Celsius degree
rpm	Rotation per minute
s	second
U	Enzymatic Activity
V	Volt

Aims and Methodology

In general, the research of the phenomena that induce alterations in mural paintings has neglected the important contribution of the microorganisms for this process. However several mural paintings have many evidences of biological contamination, whose role is imperative to study and understand.

The main goals of this PhD thesis comprise the full characterisation of the biological agents that colonise mural paintings, the identification of the biodeteriogenic agents involved in the biodegradation/biodeterioration processes that induce severe alterations in the paintings, and, the development of the mitigation strategies to eliminate and control the microbial proliferation in these important artworks.

In this way, several mural paintings, set in completely different contexts and environments, were selected and a detailed study was carried out for each of them, in order to gather information about the agents that induce damages in the paintings.

The methodology defined for this work intended:

- To characterise the materials used in the mural paintings, like pigments and mortars, by multianalytical approaches, using non-invasive or μ-invasive and non-destructive techniques like μ-Raman, μ-FTIR, SEM-EDX;
- To find and identify alteration products, in damaged paint areas, by Raman spectrometry and FTIR-ATR;
- To characterise the biological agents present in mural paintings using complementary methodologies including culture-dependent methods and molecular approaches;
- To assess the biological proliferation capacity in mortar microfragments, by SEM analysis;
- To isolate the cultivable microorganisms to perform simulation assays;
- To discriminate biological contamination levels by DGGE;
- To signalise the main biodeteriogenic agents involved in the mural paintings alteration processes;

- To evaluate the presence of metabolic active cells by enzymatic monitorisation (arylsulphatase, dehydrogenase, *β*-glucosidase and phosphatase) and viability assays (MTT cell viability);
- To understand the effect of microbial proliferation in the mural paintings decay;
- To correlate the damages detected in mural paintings with the biological population present;
- To evaluate the antimicrobial effect of selected commercial biocides against the biological population found in the paintings;
- To test possible biocide effects in the paintings by mortar simulation assays;
- To perform *in situ* biocides application;
- To define strategies to eliminate biological contamination and avoid their recolonisation;
- To develop methodologies that could be applied on other cultural assets.

CHAPTER I

State of the Art

1.1. Artworks degradation/deterioration

1.1.1. Mural paintings: general concepts

Mural paintings, also designated wall paintings and murals, are a very old artistic representation, dating back to prehistoric times by the rock paintings. They remaining until nowadays as a well-recognised form of art, widely used in walls and ceilings decoration (Mora *et al.*, 1984; Botticelli, 1992).

The earliest form of mural art was found in caves from the Paleolithic Era like the Lascaux Cave in the Dordogne region of France. This cave contains an impressive display of prehistoric art: the main cavern and several galleries connected to it were decorated with engraved, drawn, and painted figures of animals. The approximately 600 paintings were dated to the late Aurignacian period (15,000 to 13,000 B.C.) and were done with mineral pigments mixed with animal fat in various shades of yellow, red, brown and black (Ciferri, 1999). However, there are other examples of Paleolithic cave art throughout Europe like Cave of Altamira and Cave El Castillo in Northern Spain, and Cave of *Escoural* in Southern Portugal (Portillo and Gonzalez, 2009). This was one of the key moments of the history of rock art, marked by the realistic detail in the animal figures and the combination of engraving and painting (Figure I-1).

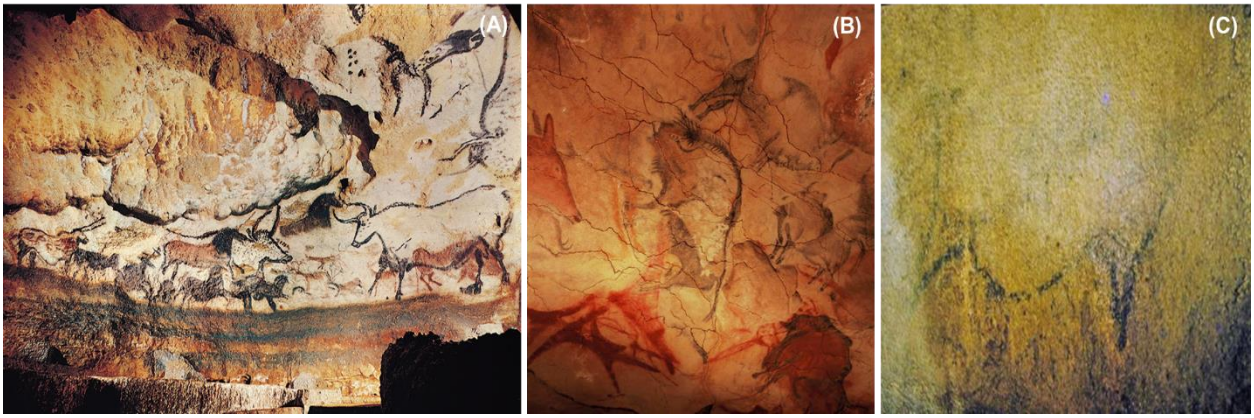


Figure I-1. Prehistoric art expressed in rock paintings present in several Caves of Lascaux - France (A), Altamira - Spain (B) and Escoural - Portugal (C) (Adapted from http://www.arte-coa.pt; http://whc.unesco.org/en/list/310 and http://arqnat.webnode.pt/patrimonio).

Many other ancient murals have survived until today, for example in Egyptian tombs, the Minoan palaces in Crete, Greece and in Pompeii, Italy (Mora *et al.*, 1984).

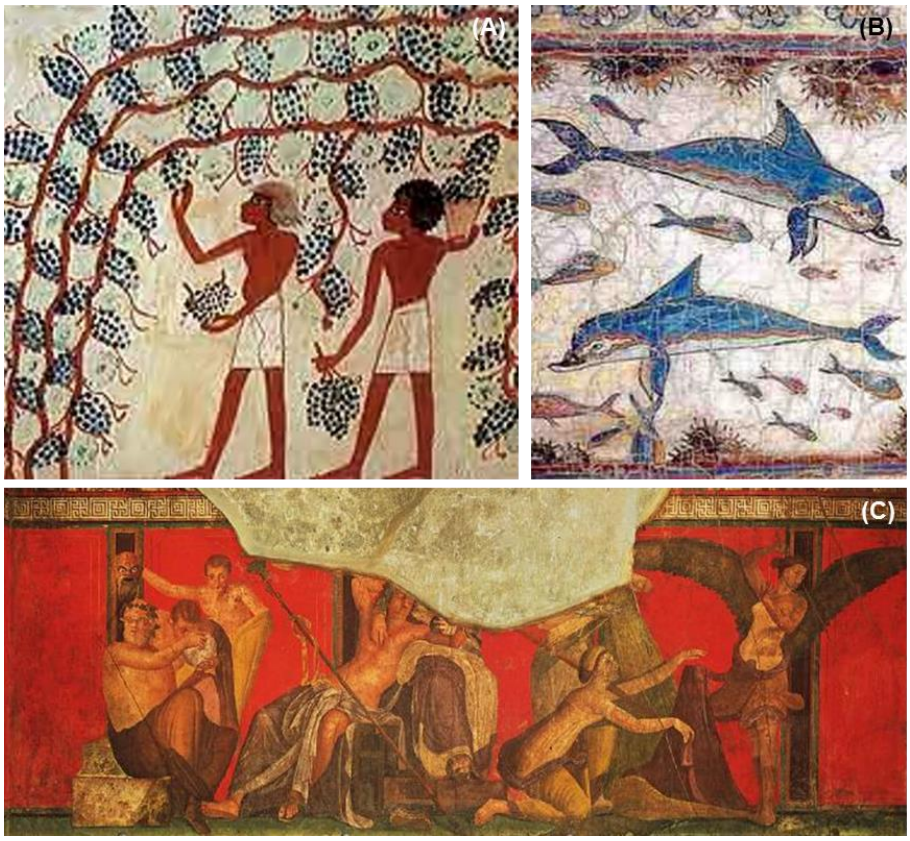


Figure I-2. Ancient mural painting registers of Egyptian tomb painting depicting grape cultivation (A), blue dolphins swimming above a doorway in the Minoan Palace of Knossos, Crete (B) and Villa of the Mysteries, Pompeii (C) (Adapted from http://guity-novin.blogspot.pt; http://www.shutterstock.com and http://www.art-and-archaeology.com).

This artistic expression gained enough significance with Giotto, an Italian painter, in the thirteenth century and in Renaissance period and comes from to nowadays. There are several remarkable mural painting artistes like Michelangelo (Sistine Chapel, Italy), Raphael (Madonna, Italy) and Leonardo da Vinci (The Last Supper, Italy).

Mural paintings refer to a painting that is executed on an architectural support which can be a natural rock, masonry of brick, stone, nogging or pug. These paintings are usually used to decorate walls, vaults, pillars and columns, ornamenting only a small part or all of the architectural surface (Garg *et al.*, 1995).

These artworks, are some of the oldest and most important cultural expressions of Mankind and play an important role for the understanding of Societies and civilisations. These cultural assets have high economic and cultural value and therefore, their degradation is a problem with social and economic impact, and hence hardly need any justification for their preservation (Garg *et al.*, 1995).

The presence of mural paintings in Europe's cultural heritage constitutes a unique richness to Mankind both for its quantity and quality of masterpieces of great intrinsic value (Calicchia and Cannelli, 2005). Portugal has a lot of mural paintings testimonies, distributed from north to south of the country. In Southern Portugal, particularly in the Alentejo region, these artworks achieved great popularity and execution refinement between the late fifteenth and sixteenth century, so it is often known by the golden age of the Portuguese mural painting (de Sousa, 2003; Serrão, 2010). This form of art is quite represented in religious buildings like convents, churches, hermitages and other public and private estates.

1.1.2. Mural paintings: structure and techniques

Mural paintings have a complex matrix constituted by pictorial support and chromatic layer (Figure I-3). Usually, the pictorial support consists of several layers, made of lime and sand of different particle size, that allow the efficient execution of the previous layer. The innermost layer, usually designated as *arricio*, in the field of conservation and restoration of mural painting, is a rougher layer which acts to even out any irregularities in the architectural support and create points of adhesion for the placement of one or more thin layers. The last layer, *intonaco*, intend to create a smooth surface for reception of paint. Thus, the chromatic layer is the visible part of the painting and it is formed by pigments and binders (Botticelli, 1992; Calicchia and Cannelli, 2005).

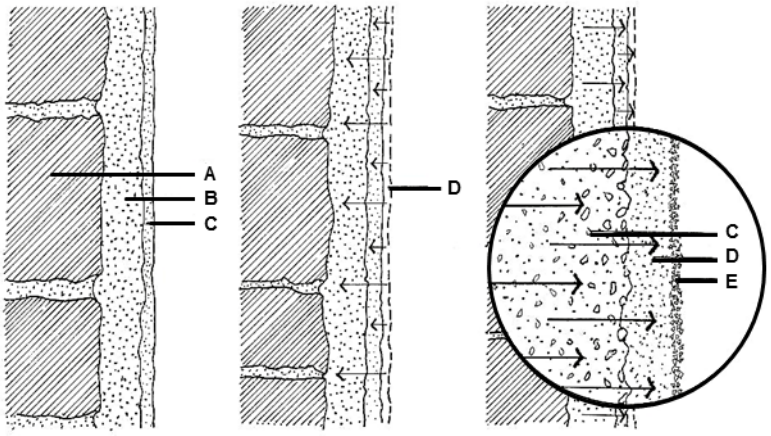


Figure I-3. Schematic representation of a mural painting: A- support, B- *arricio, C- intonaco*, D- Chromatic layer and E- crystals of calcium carbonate in case of *a fresco* technique (Adapted from Boticelli, 1992).

According to the execution technique, mural paintings can be classified as *fresco* and *secco*. In the case of *fresco* technique, the pigments are applied over a freshly wet mortar, in the beginning of the carbonation process (*1*). The pigments can be mixed with water or lime milk, and their fixation occurs during the formation of calcium carbonate matrix resulting from the reaction of calcium hydroxide, contained in the mortar, with carbon dioxide available in the atmosphere.

$$Ca(OH)_2\,(aq) + CO_2\,(g) \rightarrow CaCO_3\,(s) + H_2O\,(g) \qquad (1)$$

A *fresco* technique can be divided in *buon fresco* or *mezzo fresco*, according to the moment that the pigments are applied in the mortar surface. In the case of *mezzo fresco*, the pigment is applied in an advanced carbonation stage of the mortar, while in the *buon fresco* the mortar is completely moist. Thus, this last variant allow paintings with high quality and durability.

In the case of a *secco* technique, the pigments are mixed with a material that promotes their adherence (binders) to the pictorial support and after deposited on a dry mortar surface. This technique can be divided in tempera and oil, according to the binder used to apply the pigment. In the case of tempera the binder used can be an animal glue, vegetal gum or egg, while the oil variant uses siccative oils like linseed oil.

Due to the organic nature of the binder, *secco* paintings are more susceptible to degradation and are in general more fragile and less durable than the *fresco* paintings (Botticelli, 1992).

Given the wide range of organic and inorganic components that are present in these artworks, many different types of microorganisms may grow on these substrates, using them for their development (Ciferri, 1999). The microbial ability to proliferate in the mural paintings, provided in favourable environmental conditions, can promote serious damages. Mural paintings are important elements of Portuguese art, however a large number of these paintings has suffer detriment, fact that require urgent attention and efficient conservation policies (Moropoulou *et al.*, 2003).

Therefore, it becomes imperative to take proper measures for the conservation of mural paintings as well as taking steps for the conservation of the historic buildings (Garg *et al.*, 1995).

1.2. Deterioration of mural paintings

There are several parameters than can promote alterations in mural paintings like environmental factors, geological conditions of the ground, ageing, materials quality and their chemical composition (organic and inorganic nutrient sources), internal mechanical stress and

biological agents. Humidity, temperature, light, CO_2 concentration, atmospheric pressure and pH are physical parameters that can strongly influence mural paintings decay (Garg *et al.*, 1995; Ciferri, 1999; Heyrman and Swings, 2003; Pangallo *et al.*, 2009b ; Nuhoglu *et al.*, 2006; Capodicasa *et al.*, 2010 ; Altenburger *et al.*, 1996 ; Calicchia and Cannelli, 2005; Sanchez-Moral *et al.*, 2005).

Although several biotic and abiotic factors can induce degradation/deterioration in mural paintings, microorganisms are perhaps its main promoters (Rojas *et al.*, 2009).

According to this, living organisms trigger an undesirable process - biodegradation/biodeterioration - of the mural paintings, phenomenon which affects cultural heritage and economically important materials (Allsopp *et al.*, 2004; Rojas *et al.*, 2009; Sterflinger and Piñar, 2013).

The development of microorganisms on mural paintings may cause aesthetic and/or structural damages on these artworks (Figure I-4), such as pigments and mortars discolouration, stains and biofilms formation on the surfaces, efflorescence salts formation, exfoliation of paint layers, formation of paint blisters, cracking and disintegration of paint layers, and, degradation of binders resulting in detachment of the paint layer (Ciferri, 1999; Borrego *et al.*, 2010; Capodicasa *et al.*, 2010; Pepe *et al.*, 2011). On the other hand, some organic coatings can also accelerate mural painting degradation because they alter inappropriately the substrates hydrophilicity. Therefore, humidity changes are more important in mural painting degradation/deterioration than temperature alterations (He *et al.*, 2014).

Particularly relevant are the microbial ability to produce biofilms, where digestive enzymes excreted by microorganisms, with high metabolic activity, are concentrated. The extracellular polymeric substances (EPS) content in a biofilm protects cell enzymes against desiccation and rehydration cycles, thus offering the organisms within the biofilm a distinct advantage over non-embedded cells on external surfaces (Kemmling *et al.*, 2004).

It is believed that aesthetic damages occur earlier than structural damages and can precede serious corruption of the materials, being these damages strongly linked (Sarró *et al.*, 2006; Santos *et al.*, 2009).

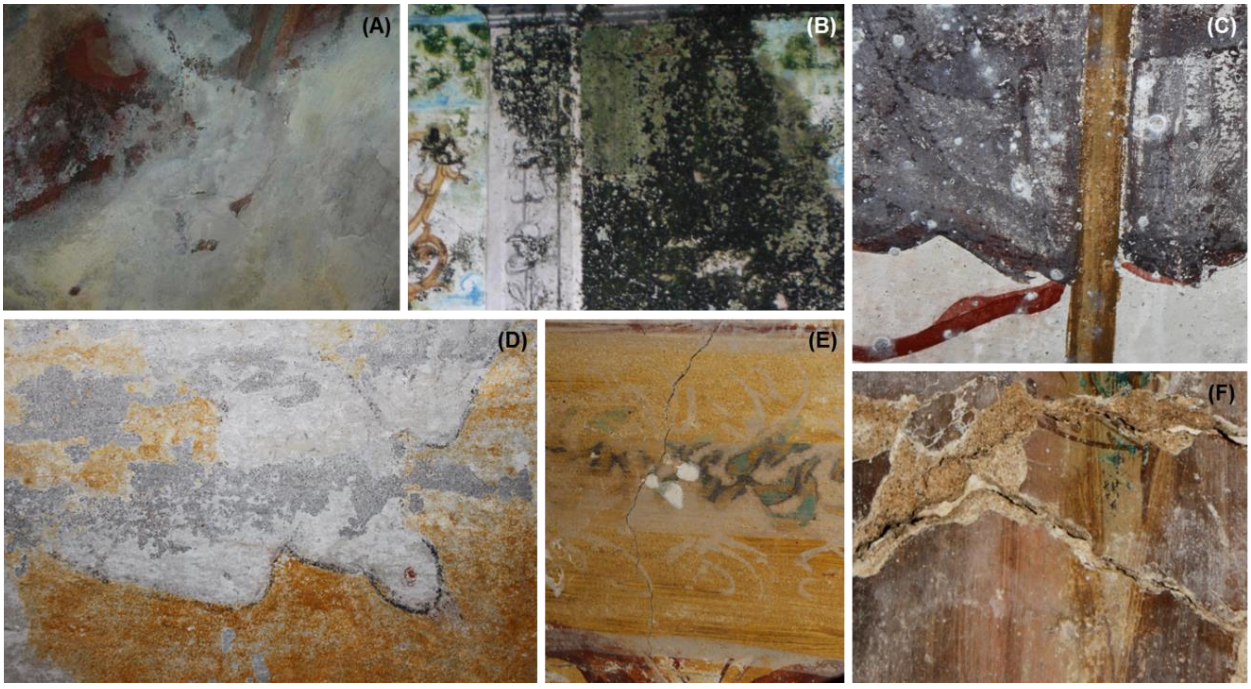


Figure I-4. Main mural painting damages: stains appearance and biofilms formation (A, B), salt efflorescence formation (C), chromatic layer detachment (D), cracks and mortars detachment (E, F).

The microbial flora present in mural paintings, may result from the successive colonisations by different microorganisms, and, each coloniser agent has different ways to compromise the structure and function of the substrates (Nugari *et al.*, 1993b; Borrego *et al.*, 2010). The natural porosity of the paintings and its constitution in organic and inorganic compounds makes their surfaces receptive to microbial spores and vegetative cells transported by airborne particles. These cells can adapt to this environment, grow and proliferate in these surfaces (Saarela *et al.*, 2004; Milanesi *et al.*, 2009). Additionally, airborne particles like dirt, soot and other environmental contaminants contribute to the mural paintings alterations by supplying nutrients for microbial growth (Ciferri, 1999; Kemmling *et al.*, 2004).

In this way, to know the interaction of the microbial population with the physico-chemical properties of the materials, is considered central to understand the long term degradation/deterioration of the mural paintings (Ripka *et al.*, 2006; Herrera and Videla, 2009; Wiktor *et al.*, 2009).

1.2.1. Biological agents involved in artworks decay

Involved in the mural paintings decay are a wide and diversified biological population like bacteria, fungi, algae, lichens and others microorganisms, which act in co-association inducing alterations in these artworks (Capodicasa *et al.*, 2010 ; Jain *et al.*, 2009; Wiktor *et al.*, 2009). They can be quite diverse, being classified according to their nutritional requirements (Zastrow and Straube, 1991; Tolli and King, 2005):

- Autotrophic and heterotrophic organisms assimilate inorganic or organic carbon sources, respectively;
- Phototrophic and chemotrophic organisms get energy from sunlight or oxidation of organic or inorganic compounds, respectively;
- Chemolithotrophic and chemoorganotrophic organisms use inorganic or organic reduced compounds, respectively.

Cyanobacteria are photosynthetic microorganisms that can use CO_2 as a carbon source for growth. Due to their peculiar ability to adapt to extremely low photon flux densities and to a variety of spectral emissions, cyanobacteria are the major organisms responsible for biofilm formation in artworks (Sanchez-Moral *et al.*, 2005).

Chemolithotrophic organisms are found only in prokaryotes and are widely distributed among Bacteria and Archaea and can life in the presence, as well as, in the absence of molecular oxygen. The spectrum of inorganic compounds that can be used as electron donors by chemolithotrophs is rather broad: hydrogen, ammonia, nitrite, sulfide, sulfur, hydrogen and Fe(II) ions.

Among the microorganisms present in mural paintings, lichens play a minor role in their colonisation. Lichens represent the symbionts of fungi (mainly ascomycete) and algae (mainly green algae) or fungi and cyanobacteria (less common). Lichens are comparatively more resistant to extreme temperature and desiccation which allows them to flourish and grow in a wide variety of habitats some of them may be hostile to other forms of lives. They are among the pioneer organisms which inhabit the exposed stone surfaces. They have significant contribution in biogeophysical and biogeochemical deterioration of monumental stone (Dakal and Cameotra, 2012 ; Miller *et al.*, 2012). In addition, algae and bryophytes, often abundant in the plasters and mortars, are considered less important in biodegradation/biodeterioration process. However they support the colonisation and development of allied heterotrophic population of bacteria and fungi (Gómez-Alarcón *et al.*, 1995), which are the main biodeteriogens responsible for aesthetic and structural damages.

Bacteria are suggested by some authors as the first coloniser agent of these artworks, since they have reduced nutritional requirements and provide organic matter to the next colonizers (Garg *et al.*, 1995; Rölleke *et al.*, 1996). These microorganisms are frequently associated to the formation of biofilms, promoting discolouration of the surfaces, due to their development or their metabolic activity that can produce pigmented compounds (Garg *et al.*, 1995; Ciferri, 1999; Gorbushina and Petersen, 2000 ; Milanesi *et al.*, 2006; Guiamet *et al.*, 2011). On the other hand, the growth of biological agents like fungi are identified as a determinant factor in the degradation/deterioration of the murals (Garg *et al.*, 1995; Rölleke *et al.*, 1996).

Fungi are ubiquitously present microorganisms representing the group of chemoheterotrophs, being metabolically more versatile than other biodeteriogens in the microbial kingdom. This versatility allows them to colonise a wide variety of substrates including wood, stone, metal, mortars, paintings and enhances their biodeterioration/biodegradation potential. Their ability to grow on a variety of substrates, tolerating extremes environmental conditions, establishing mutualistic association with cyanobacteria or algae, adopting various structural, morphological and metabolic strategies further enhances their versatility and adaptability (Dakal and Cameotra, 2012a). These microorganisms are particularly dangerous because they show a significant tolerance to adverse environmental conditions. Their hyphae may have high level of proliferation in mortars and their spores, in a dormant state, are commonly present and available for germination. On the other hand, fungal-derived carboxylic acids (e.g., oxalic, citric, succinic, formic, malic, acetic, fumaric, glyoxylic, gluconic, and tartaric acids) can play a significant role in chemical attack (Wiktor *et al.*, 2009; Fomina *et al.*, 2010; Tran *et al.*, 2012). The destructive potential of these microorganisms is shown by mechanical and chemical processes, caused by mycelia penetration inside the mortar, resulting in loss of cohesion and detachment of the paint layer, as well as paint discolouration result of the products of their metabolism secreted in the surface (Altenburger *et al.*, 1996 ; Rölleke *et al.*, 1996 ; Berner *et al.*, 1997; Herrera *et al.*, 2004; Milanesi *et al.*, 2006; Imperi *et al.*, 2007 .; Garg *et al.*, 1995; Ciferri, 1999; Gorbushina and Petersen, 2000 ; Milanesi *et al.*, 2006; Guiamet *et al.*, 2011). According to the literature, fungi of the genera *Penicillium*, *Cladosporium*, *Alternaria*, *Aspergillus*, *Curvularia*, *Dreschlera*, *Chaetomium*, *Fusarium*, *Trichoderma*, *Gliomastix*, *Aureobasidium*, are the most abundant in mural paintings colonisation (Garg *et al.*, 1995; Gorbushina *et al.*, 2004; Sterflinger, 2010).

Besides that, phototrophic microorganisms like cyanobacteria are also associated to mural paintings colonisation, owning an important role in its decay (Altenburger *et al.*, 1996 ; Ariño and Saiz-Jimenez, 1996; Cappitelli *et al.*, 2009; Tran *et al.*, 2012a; Kusumi *et al.*, 2013). These microorganisms have been reported to promote deterioration of the internal wall surfaces and

plasters where there is low light intensity, and, particularly, cyanobacteria are pointed to cause rosy discoloration in indoor environments (Cappitelli *et al.*, 2009).

These microorganisms as well as heterotrophic microorganisms can induce irreversible stainings and chromatic alterations (Rölleke *et al.*, 1998 ; Urzì and Realini, 1998; Gurtner *et al.*, 2000 ; Piñar *et al.*, 2001; Schabereiter-Gurtner *et al.*, 2001 ; Realini *et al.*, 2005; Ripka *et al.*, 2006; Imperi *et al.*, 2007 ; Laiz *et al.*, 2009; Piñar *et al.*, 2009; Jurado *et al.*, 2012; Ortega-Morales *et al.*, 2013; Sterflinger and Piñar, 2013), due to their ability to produce pigmented compounds (Warscheid and Braams, 2000; Polo *et al.*, 2010). An example of these coloured compounds are carotenoids, often responsible by the yellow, orange and red stains that appear in the artworks (Aksu and Eren, 2005; Tinoi *et al.*, 2005; Abdel-Haliem *et al.*, 2013; Olivares *et al.*, 2013).

On the other hand, mural paintings are often affected by black stains that can be caused mainly by the secretion of metabolites or the pigmentation of fungi, especially melanins. Fungi like *Cladosporium*, *Acremonium* or *Gliomastix* have been implicated in the biodegradation/biodeterioration of mural paintings (Nugari *et al.*, 1993a; Orial and Mertz, 2006; Kiyuna *et al.*, 2011).

Thus, some of the fundamental challenges for the mural paintings biodegradation/biodeterioration interpretation are:

- To characterise the biological population present in the mural paintings;
- To understand the role of each microorganism in the biodegradation/biodeterioration process;
- To identify the main biodeteriogenic agents;
- To understand the relationship between microorganisms and their interactions with each other and with their environment - population dynamics.

1.3. Biological agents characterisation

1.3.1. Culture dependent methods

Although the involvement of microorganisms in the degradation process is well known, the specific role of the different groups and species that compose the microbial communities is not yet well understood, because methodologies tend to identify only easily cultivable and omit slow growing and uncultivable microorganisms. The identification of the microbial diversity present in cultural heritage is a crucial step to develop and apply correct conservation and mitigation methodologies and to prevent further contaminations (Ramírez *et al.*, 2005).

The traditional way to identify the microbial diversity is based on the cultivation of microorganisms in specific nutrient media, but, only a small portion, typically far less than 1% of organisms can be cultivated by standard techniques and the cultivable microorganisms underrepresent the microbial diversity present in the Earth (González and Saiz-Jiménez, 2005). A wide variety of culture media, both solid and liquid, can be used for this purpose based on the type of microorganisms that proliferate in the mural paintings. Despite the limitations inherent of this approach, culture based techniques and development of new culture media is still encouraged due to the advantage of having pure cultures isolated to carry out physiological and metabolic studies (Dakal and Arora, 2012).However, to understand the phenomena that promotes the degradation of mural paintings it is crucial the deeper knowledge of the microbial population that colonise these artworks. In this way, techniques based on nucleic acids allow the differentiation of microorganisms within complex microbial communities or the identification of isolated microorganisms (Portillo and Gonzalez, 2009).

According to this, DNA sequencing approaches are very useful to phylogenetic identification, and have been applied in several areas, being useful in artworks to analyse the microbial diversity (Rölleke *et al.*, 1996 ; Rölleke *et al.*, 1998 ; Saiz-Jimenez and Laiz, 2000; Schabereiter-Gurtner *et al.*, 2001b; Saarela *et al.*, 2004; Carmona *et al.*, 2006; Cappitelli *et al.*, 2009; Olivares *et al.*, 2013).

Thus sequencing small subunits (SSU) ribosomal DNA genes like 16S and 18S, universally present in all prokaryotes and eukaryotes, respectively, provide an efficient mean to identify microorganisms from cultural assets. These ribosomal sequences possess variable and highly conserved regions, which are used as phylogenetic markers to identify and distinguish between microorganisms on all phylogenetic levels (Hill *et al.*, 2000; Kennedy and Clipson, 2003; Dakal and Arora, 2012).

On the other hand, sequencing of the internal transcribed spacer (ITS) region, located between 18S and 5.3S rDNA, is also a diagnostic tool for identifying fungi. This region is very variable in sequence composition and also vary in length between species, and thus can be used to profile the number of ribotypes present in a community (Kennedy and Clipson, 2003; Anderson and Cairney, 2004; Dakal and Arora, 2012).

In the case of yeast characterisation, sequencing the D1/D2 domain of 26S/28S rDNA region has been used to identify these microorganisms from different sources. This approach is rapid and precise compared with the physiological method for the yeast identification, and has also been applied to study the phylogeny of different yeast groups and species-level differentiation

(Lachance *et al.*, 2003; Couto *et al.*, 2005; Dagar *et al.*, 2011; Kiyuna *et al.*, 2012; Lv *et al.*, 2013; Hesham *et al.*, 2014; Selbmann *et al.*, 2014).

The DNA sequence analysis can be accomplished using BLAST server of NCBI (www.ncbi.nlm.nih.gov) using Blastn program which is specifically designed for comparing query nucleotide sequence with nucleotide sequences in database (Burgess *et al.*, 2010).

Other nucleic acid approaches can be applied to detect uncultivable microorganisms and to identify microbial isolates with more efficiency, since the DNA is common to all organisms and may give further information. On the other hand, molecular techniques that circumvent the need for isolation and cultivation are highly desirable for in-depth characterisation of microbial communities present in mural paintings.

1.3.2. Molecular approaches for microbial characterisation

The ongoing advances in genomics and sequencing technologies are allowing a *new era* of microbial community analyses using culture-independent approaches which complement the information obtained by culture-dependent methods (Figure I-5). A multiplicity of molecular methods based on the analysis of nucleic acids, proteins, and lipids have been developed to describe and characterise the phylogenetic and functional diversity of microorganisms, and, can be highly effective for mural paintings invasive species monitoring. Thus, molecular approaches such as genetic fingerprinting, metagenomics, metaproteomics, metatranscriptomics and proteogenomics are crucial for the full identification of the microbial diversity present in mural paintings and understanding their interactions with biotic and abiotic factors (Rastogi and Sani, 2011).

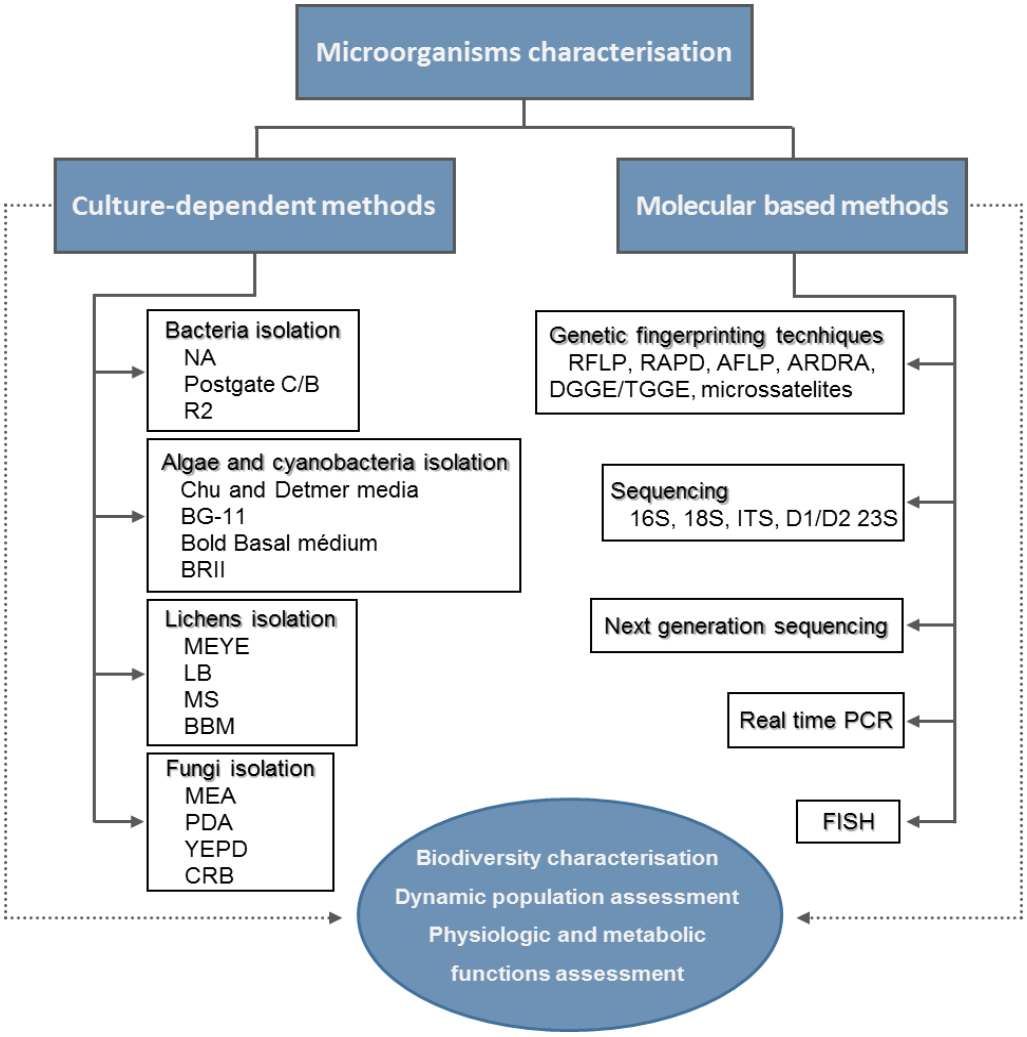


Figure I-5. Multianalytical approaches to characterise biological agents present in mural paintings.

In general, molecular strategies include polymerase chain reaction (PCR), where total DNA/RNA extracted is used as a template for the characterisation of microorganisms. Generally, the PCR product produced, reflects a mixture of microbial gene signatures from all organisms present in a sample and can generate a profile of microbial communities based on the direct analysis of PCR products by fingerprinting techniques, using several molecular markers (Muyzer *et al.*, 1993; Rastogi and Sani, 2011). Molecular markers are DNA sequences which show polymorphisms (heritable DNA sequence differences) between individuals genetically related. These approaches are widely applied for studies of population genetics, mapping and similarity analysis and even genetic distance. Therefore, according to these molecular markers, there are several techniques based on genetic fingerprinting, that can be used to microbial diversity

characterisation, such as: Restriction Fragment Length Polymorphism (RFLP), Random Amplified Polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP), Microsatellite polymorphism or Simple Sequence Repeat (SSR), Single-Strand Conformation Polymorphism (SSCP), Simple Sequence Length Polymorphism (SSLP), Denaturing/Temperature Gradient Gel Electrophoresis (DGGE/TGGE) and others. These genetic fingerprinting techniques, based on direct analysis of PCR products, generate a profile of microbial communities, are rapid and allow simultaneous analyses of multiple samples. Fingerprinting approaches have been devised to demonstrate an effect on microbial communities or differences between microbial communities but do not provide direct taxonomic identification, being sometimes necessary sequencing techniques (Muyzer *et al.*, 1993; Rastogi and Sani, 2011).

Between several fingerprinting techniques, Restriction Fragment Length Polymorphism (RFLP) is one example of them, based on a difference in homologous DNA sequences that can be detected by the presence of fragments of different lengths after digestion with specific restriction endonucleases. RFLP, as a molecular marker, is specific to a single clone/restriction enzyme combination. RFLP analysis exploits polymorphisms in restriction enzyme recognition sites on PCR amplicons to generate DNA fragments of varying sizes. Sequence variation is visualised in the form of peaks on an electropherogram (Lott *et al.*, 2014). Although now largely obsolete due to the rise of inexpensive DNA sequencing technologies, RFLP analysis was the first DNA profiling technique inexpensive enough to see widespread application. RFLP analysis was an important tool of genome mapping and localisation of genes in areas like biology, medicine and food science (Lin and Hwang, 2007; Pourahmad and Richards, 2013; Sarin *et al.*, 2013).

Random Amplified Polymorphic DNA (RAPD) is a powerful fingerprinting technique that involves the amplification of random segments of genomic DNA by short arbitrary primer of about 10 nucleotides that binds to random sections of the genome. The amplification of these sequences results in different banding patterns between strains (Lynch and Milligan, 1994; Burgess *et al.*, 2010). The PCR amplicons generated are separated on agarose or polyacrylamide gel depending on the genetic complexity of the microbial communities, and, a phylogenetic tree is drawn by the UPGMA cluster program, to make the correlations between samples. This technique is highly dependent on the experimental conditions (e.g., annealing temperature, $MgCl_2$ concentration), the quality and quantity of template DNA and primers used. Thus, several primers and reaction conditions need to be evaluated to compare the relatedness between microbial communities and obtain the most discriminating patterns between species or strains. The advantages of RAPD profiling are that it requires no sequence information, and, it is quicker and

easier in comparison with other profiling methods, such as denaturing gradient gel electrophoresis (DGGE), detecting differences between closely related species, needing only small quantities of DNA for assays (Burgess *et al.*, 2010; Ben Salem *et al.*, 2014).

This fingerprinting technique is used to study the microbial diversity or variability and their ecological distribution. RAPD is a very convenient and cost effective method employed for bacterial identification and variability estimation. The PCR based method of gene typing based on genomic polymorphism is a recent approach which is widely used for the assessment of inter- and intraspecific genetic variation and uses a single short random oligonucleotide primer. The RAPD technology is well suited to DNA fingerprinting although it suffered from a certain lack of reproducibility due to mismatch annealing (Saxena *et al.*, 2014).

In the case of fingerprinting techniques application in cultural assets like mural paintings, stone and others, RAPD methodology seems to be the most used to characterise and distinguish isolate microorganisms and communities (Zanardini *et al.*, 1997; Gorbushina *et al.*, 2004; Ripka *et al.*, 2006; Suihko *et al.*, 2007).

Amplified Fragment Length Polymorphism is other DNA fingerprinting technique that detects genomic restriction fragments and resembles in that respect the RFLP technique, with the major difference that PCR amplification instead of Southern hybridisation is used for detection of restriction fragments (Vos *et al.*, 1995). This technique is based on the selective PCR amplification of restriction fragments from total digestion of genomic DNA, by two restriction enzymes. It involves two amplification steps: a low-level or pre-selective amplification, followed by a more selective amplification, which generates a set of fragments that can be used as the discriminatory marker set for a particular sample. The amplified fragments are separated by gel electrophoresis and visualised through autoradiography or fluorescence methodologies, or via automated capillary sequencing instruments (Hookey *et al.*, 1999; Lazzi *et al.*, 2009).

AFLP can simultaneously screen many different DNA regions distributed randomly throughout the genome and generates many genome wide polymorphic markers with no prior sequence information, and showed more discriminatory power than RAPD, RFLP and microsatellites, due to its higher reproducibility, resolution, and sensitivity. This technique has become widely used for the identification of genetic variation in strains or closely related species of plants, fungi, animals and bacteria (Lazzi *et al.*, 2009; Thakur *et al.*, 2014).

Between the several existing electrophoretic techniques, denaturing gradient gel electrophoresis (DGGE) and/or temperature gradient gel electrophoresis (TGGE) have been used to characterise the microbial diversity in different ecosystems. DGGE has been used to determine

and identify the genetic diversity of the microbial communities present in mural paintings, representing a powerful tool for monitoring the biological population in these artworks and also other cultural assets (Rölleke *et al.*, 1996 ; Gurtner *et al.*, 2000 ; Möhlenhoff *et al.*, 2001).

These techniques separate amplified rDNA (ribosomal DNA) fragments of similar length (200-700 bp) but with different sequences, according to their melting properties. Whereas DGGE uses denaturing chemicals such as formamide and urea, a temperature gradient is applied in TGGE (Muyzer *et al.*, 1993; Rölleke *et al.*, 1996 ; Rantsiou *et al.*, 2005; Justé *et al.*, 2008; Malik *et al.*, 2008).

DGGE technique has the advantage of directly profiling microbial populations present in specific ecosystems by separating PCR products originated from universal primers, on the basis of the melting domain of the DNA molecules (Muyzer *et al.*, 1993; Rantsiou *et al.*, 2005; Justé *et al.*, 2008).

The detection of microorganisms is mainly based on the small subunit ribosomal DNA genes, 16S rDNA for prokaryotes and 18S rDNA for eukaryotes. Ribosomal DNA is the most commonly employed target for PCR amplification prior to DGGE because they are present in every organisms and they contain variable and highly conserved regions which allow to distinguish between organisms on all phylogenetic levels (Heyrman and Swings, 2003; Ercolini, 2004; González and Saiz-Jiménez, 2005).

Double-strand DNA fragments (Figure I-6) are subjected to an increasing denaturing environment as they encounter increasing concentrations of the denaturing agents and partially melt in discrete regions called ‘‘melting domains”, and, depends on the hydrogen bonds formed between the GC and AT base pairings and the attractions between neighbouring bases of the same strand. GC pairs are much more stable to denaturation than AT pairs. This technique uses a chemical gradient of urea and formamide created within an acrylamide gel. Usually, the PCR products applied in a DGGE gel are obtained by PCR amplification using a GC-rich tail at the 5’-end of one primer, generally composed by about 40 bases like 5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G and it will be continued by the priming sequence complementary to the target DNA to be amplified. This GC rich tail is highly resistant to chemical denaturation (Ercolini, 2004; Gonzalez and Saiz-Jimenez, 2004).

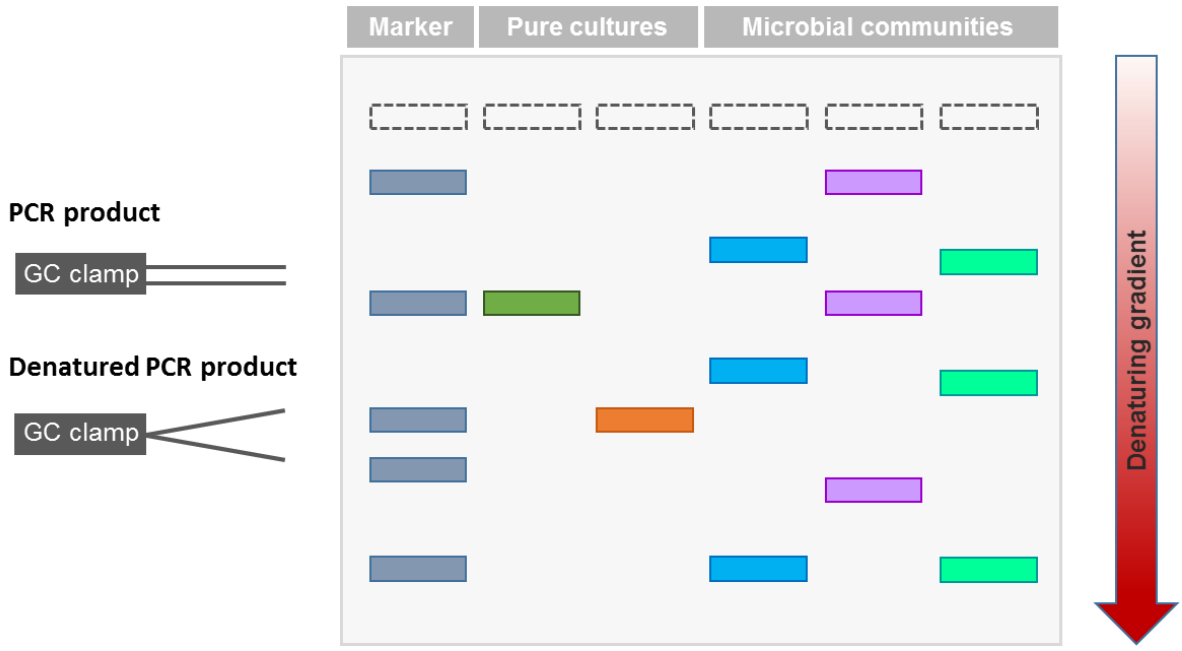


Figure I-6. Illustrative scheme of Denaturing Gradient Gel Electrophoresis process. This technique uses a gradient of urea and formamide in a polyacrylamid gel to separate the PCR products. Through this gradient the double stranded PCR products melt depending on their GC content (Based on Gorbushina *et al.*, 2004; Ripka *et al.*, 2006).

The technique benefits from the facility to analyse and compare numerous samples on a single gel and allows a rapid and simultaneous comparison between samples, which is useful to get information about the diversity and distribution of the population by the different sampling places. The accuracy of the comparison, however, is heavily dependent upon the inclusion of suitable internal standards and assumes that the resolution and quality of the gels have been standardised. This parameter is particularly crucial where comparison between several different gels is required as a result of large sample numbers. Thus, the reproducibility between gels has been highlighted as one of the main pitfalls of DGGE. In addition to the previously described, it is possible to obtain more information about the microbial diversity by gel-based community profiling techniques, excising and sequencing individual DGGE bands, thereby obtaining taxonomic information for interesting members of the community via database searches and/or phylogenetic analysis (Anderson and Cairney, 2004).

DNA sequencing is a basic and essential tool in molecular biology and applied biosciences, allowing analyses ranging from single nucleotide polymorphism (SNP) identification to whole genome sequencing. This approach, to characterise several DGGE bands are high time consuming and sometimes the difference between nucleotide sequences are insufficient to produce separate bands, conducting to mix DNA and consequently to an inappropriate DNA for

sequencing and identification. Thus it is necessary the application of high-throughput techniques that allow a full characterisation of the microbial population present on mural paintings (Gharizadeh *et al.*, 2006).

In addition to fingerprinting techniques, there are other molecular approaches that can be exploited to improve the understanding of the biological agents involved in the alteration phenomena of mural paintings.

Real-time PCR or quantitative PCR (q-PCR) has been used in microbial investigations to measure the abundance and expression of taxonomic and functional gene markers. Unlike traditional PCR, which relies on end-point detection of amplified genes, this technique uses either intercalating fluorescent dyes such as SYBR Green or fluorescent probes (TaqMan) to measure the accumulation of amplicons in real time during each cycle of the PCR. Software records the increase in amplicon concentration during the early exponential phase of amplification which enables the quantification of genes (or transcripts) when they are proportional to the starting template concentration. When real-time PCR is coupled with a preceding reverse transcription (RT) reaction, it can be used to quantify gene expression (Rastogi and Sani, 2011). This technique is highly sensitive, accurate and allows the simultaneous analysis on the same samples, monitoring functional genes involved in metabolic or catabolic pathways, microbial ecology distribution systems. It can also be applied to study changes in expression of particular genes in response to environmental conditions alteration, disinfection treatment, wastewater treatment systems and cultural assets (Kim *et al.*, 2013; Martin-Sanchez *et al.*, 2013; Douterelo *et al.*, 2014).

Fluorescence *In Situ* Hybridisation (FISH) is a molecular diagnostic technique and has been used for localisation of specific nucleic acid sequence in natural context, enabling *in situ* phylogenetic identification and enumeration of individual microbial cells by whole cell hybridisation with oligonucleotide probes (Dakal and Arora, 2012). The oligonucleotide probes used in FISH are generally between 15 and 30 nucleotides long and covalently linked at the 5’ end to a single fluorescent dye molecule that allows detection of probe bound to cellular rRNA by epifluorescence microscopy. Common fluorophors include fluorescein, tetramethylrhodamine, Texas red and, increasingly, carbocyanine dyes like Cy3 or Cy5. The intensity of fluorescent signals is correlated to cellular rDNA contents and growth rates, which provide insight into the metabolic state of the cells (Amann *et al.*, 2001; Polo *et al.*, 2010).

FISH technique is very powerful, rapid and straightforward and has the advantage to detect microorganisms across all phylogenetic levels. FISH probes can be generated without prior isolation of the microorganism (Hill *et al.*, 2000).

This methodology was used to follow the dynamics of bacterial populations in agricultural soils, aquatic systems, wastewater, and more recently in artworks (Amann *et al.*, 2001; Baskar *et al.*, 2006), and is a promising approach to identify fungal contamination diversity on mortar samples (González *et al.*, 2014; Vieira *et al.*, 2014).

1.3.3. Next generation DNA sequencing

Recent developments in new sequencing chemistries, bioinformatics and automated instruments have revolutionised the knowledge of microbial diversity. Nowadays, there are five Next Generation Sequencing (NGS) platforms, including the Roche/454 FLX, the Illumina/Solexa Genome Analyzer and the Applied Biosystems (ABI) SOLiD Analyzer are currently dominating the market. The other two platforms, the Polonator G.007 and the Helicos HeliScope, have just recently been introduced and are not widely used (England and Pettersson, 2005; Mardis, 2008; Shendure and Ji, 2008; Zhang *et al.*, 2011).

Pyrosequencing technology is a novel DNA sequencing method based on the sequencing-by-synthesis principle. This technology was developed at the Royal Institute of Technology (KTH – university in Stockholm, Sweden), and is the first alternative to the conventional Sanger method for *de novo* DNA sequencing. This bioluminometric real-time DNA sequencing technique employs a cascade of four enzymatic reactions producing sequence peak signals. It has the potential advantages of accuracy, flexibility, parallel processing and can be easily automated (Ahmadian *et al.*, 2006 ; Gharizadeh *et al.*, 2006; Gong *et al.*, 2010 ; Fakruddin *et al.*, 2012).

The next generation sequencing technology, pyrosequencing, allows high-throughput sequencing and has revolutionised the study of microbial diversity. This methodology is currently used in multidisciplinary fields in academic, clinical and industrial settings, particularly to identify mammal species, to study microbial diversity in soils, freshwater, human guts, wastewater treatment facilities and others (Karlsson and Holmlund, 2007; Roesch *et al.*, 2007; Jones *et al.*, 2009; Roh *et al.*, 2009; Nam *et al.*, 2011; Ye and Zhang, 2011).

In addition to the broad range of applications listed, this technology has also been used in Single Nucleotide Polymorphism (SNP) genotyping, *de novo* mutation detection, gene identification and microbial genotyping (Ahmadian *et al.*, 2006 ; Gharizadeh *et al.*, 2006; Fakruddin *et al.*, 2012).

Pyrosequencing technology is a non-electrophoretic real-time ssDNA sequencing method based on the detection of released pyrophosphate during nucleotide incorporation in the DNA-strand (Figure I-7). The DNA synthesis is catalysed by four kinetically well-balanced enzymes: DNA polymerase (E.C. 2.7.7.7), ATP Sulfurylase (E.C. 2.7.7.4), Luciferase (E.C. 1.13.12.7) and Apyrase (E.C. 3.6.1.5) (Ronaghi, 2001; Ahmadian *et al.*, 2006 ; Trama *et al.*, 2007; Petrosino *et al.*, 2009; Fakruddin *et al.*, 2012; Leite *et al.*, 2012).

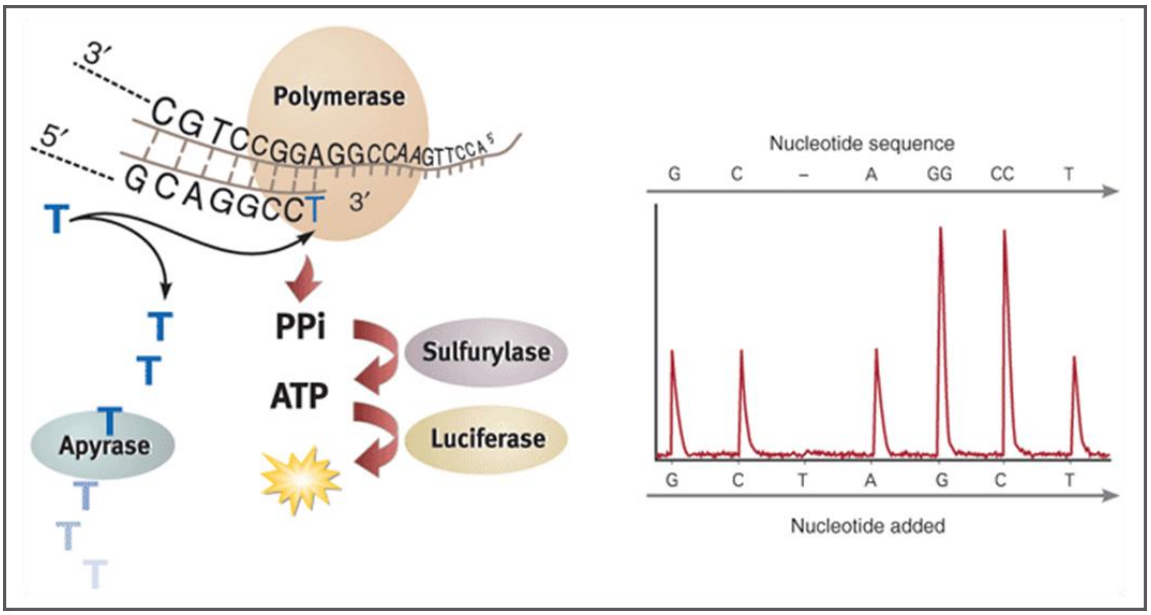


Figure I-7. Schematic representation of 454 Pyrosequencing technology (Adapted from England and Pettersson, 2005).

The first reaction involved in pyrosequencing is the DNA polymerisation and occurs in the presence of DNA polymerase and complementary nucleotide (A, C, G or T) which are incorporated into the single-stranded DNA (ssDNA) sample leads to generation of pyrophosphate (PPi) in a quantity equimolar to the number of incorporated nucleotides (Gong *et al.*, 2010).

$$(DNA)_n + dNTP \rightarrow (DNA)_{n+1} + PP_i \text{ (Polymerase)}$$

The inorganic pyrophosphate (PPi) released, works as substrate for ATP Sulfurylase, which produces ATP.

$$PP_i + APS \rightarrow ATP + SO_4^{2-} \text{ (ATP Sulfurylase)}$$

Thereafter, ATP is converted by luciferase for producing bioluminescence which is proportional to the amount of DNA and the number of the incorporated nucleotides.

$$\text{Luciferase + D-luciferin + ATP} \rightarrow \text{Luciferase-luciferin- AMP + } PP_i$$

$$\text{Luciferase-luciferin-AMP + } O_2 \rightarrow \text{Luciferase + oxyluceferin + AMP + } CO_2 \text{ + light}$$

The unincorporated nucleotides and the generated ATP are degraded by Apyrase allowing iterative addition of next nucleotide dispensation.

$$ATP \rightarrow AMP + 2P_i \text{ (Apyrase)}$$

$$dNTP \rightarrow dNMP + 2P_i \text{ (Apyrase)}$$

This degradation between base additions is crucial for synchronized DNA synthesis asserting that the light signal detected when adding a certain nucleotide only arises from incorporation of that specific nucleotide (Ronaghi, 2001; Ronaghi and Elahi, 2002; Ahmadian *et al.*, 2006 ; Petrosino *et al.*, 2009; Gong *et al.*, 2010a; Siqueira *et al.*, 2012).

The generated light is observed as a peak signal in the pyrogram (corresponding to electropherogram in dideoxy sequencing) proportional to the number of nucleotides incorporated (a triple dGTP incorporation generates a triple higher peak) (Fakruddin *et al.*, 2012).

This methodology was applied for the first time on mural paintings studies in 2014 (Rosado *et al.*, 2014d) and revolutionised the knowledge of the microorganisms that colonise these artworks.

1.4. Biochemical markers

Apart from the characterisation of the microbial population that colonise mural paintings, it is crucial to understand the role of each microorganism in the biodegradation/biodeterioration process, in order to identify the main biodeteriogenic agents.

Biodeteriogenic organisms have the ability to use a substrate to sustain their growth and reproduction, producing alterations (Sequeira *et al.*, 2012).

The microbial population present in mural paintings consists of a very broad range of organisms in different physiological states: active, dead or in a dormant state. The active microorganisms are involved in the ongoing utilisation of substrates and associated biochemical transformations. The living microorganisms in a dormant state does not contribute to ongoing processes currently but can contribute under altered circumstances. Dead microbial biomass act as an additional pool of available substrate but do not contribute actively to any biochemical

process. However, only active microorganisms are involved in the ongoing processes and consequently, all processes should be related to the mass of active (Blagodatskaya and Kuzyakov, 2013).

In this way, the signalisation of the active biological population is crucial for the identification of the biodeteriogenic agents involved in the mural paintings destruction.

1.4.1. Cell viability assessment

One of the earliest methods for assessing cell viability was trypan blue dye exclusion assay, which is still widely used today. It is based on the principle that viable cells have an intact cell membrane which can therefore exclude the trypan blue dye. Dead cells take up trypan blue, and appear blue, as their membrane is no longer able to control the passage of macromolecules. The assay requires the cells to be in a single cell suspension and they are then visualised and counted under a microscope using a Neubauer chamber of a defined volume (Stoddart, 2011).

There are yet other dyes that give information about the presence of active or inactive cells. They can bind to the cell components such as nucleic acids (acridine orange, SYBR Green I, 4,6-diamidino-2-phenylindole), proteins (fluorescein iso-thiocyanate) or polysaccharides of cell walls (phenol aniline blue, 5-4,6-dichlorotriazinyl aminofluoroscein) and can cross intact cell membranes. Another group of dyes binding to the nucleic acids (propidium iodide and ethidium bromide) are unable to penetrate membranes and cannot stain living cells. These dyes are commonly used to identify dead membrane-destructed cells (Blagodatskaya and Kuzyakov, 2013).

A variety of tetrazolium compounds have been used to detect viable cells. The most commonly used compounds include: MTT, XTT, MTS and WST-1, however MTT is the best known metabolic dye. These compounds fall into two basic categories: 1) MTT which is positively charged and readily penetrates viable eukaryotic cells and 2) those such as MTS, XTT, and WST-1 which are negatively charged and do not readily penetrate cells. The latter class (MTS, XTT, WST-1) is typically used with an intermediate electron acceptor that can transfer electrons from the cytoplasm or plasma membrane to facilitate the reduction of the tetrazolium into the colored formazan product (Roehm *et al.*, 1991; Roslev and King, 1993; Goodwin *et al.*, 1995; Knight and Dancis, 2006).

These assays were usually applied to evaluate cytotoxicity or in cell proliferation assays, which are widely used in immunology, toxicology and cellular biology (Barltrop and Owen, 1991; Cory *et al.*, 1991).

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay relies on the ability of living cells to reduce this tetrazolium salt into an insoluble blue/purple formazan crystals, which after solubilisation can be quantified spectrofotometrically. The formazan generated is proportional to the living cells present in the sample (Mosmann, 1983; Freimoser *et al.*, 1999; Mota *et al.*, 2012).

XTT [(2, 3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide] is used to assess cell viability as a function of redox potential. Actively respiring cells convert the water-soluble XTT to a water-soluble, orange coloured formazan product. Unlike MTT, XTT does not require solubilisation prior to quantitation, thereby reducing the assay time in many viability assay protocols. Moreover, the sensitivity of the XTT reduction assay is reported to be similar to or better than that of the MTT reduction assay (Knight and Dancis, 2006).

Other chromogenic assays that involve the biological reduction by viable cells of the tetrazolium compound is MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium]. The MTS assay reagent is composed of MTS and the electron coupling agent phenazine methosulfate (PMS). The formazan product of MTS reduction is soluble in tissue culture medium. This reaction only takes place when mitochondrial reductase enzymes are active, and therefore the conversion can be directly related to the viability of cells in culture (Malich *et al.*, 1997; Soman *et al.*, 2009; Willems *et al.*, 2011).

A tetrazolim salt WST-1 [2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium] produces a highly water soluble formazan upon metabolically active cells, allowing a direct and user-friendly colorimetric measurement of cell viability and proliferation (Lin *et al.*, 2012).

1.4.2. Enzymatic systems

Microorganisms play a central role in the decomposition and mineralisation of the materials due to their metabolic activity. However, their activity depends on the physical properties of the materials where the microorganisms are, the organic matter content and the mechanism of action of each agent (Jastrzębska and Kucharski, 2007).

The enzymes produced by microorganisms, convert large molecules into low molecular moieties, which then can be assimilated by other organisms, and are generally regarded as a rate-limiting step in the decomposition and nutrient cycling. Their monitorisation has been used in soil, water and wastewater quality assessment (Bergstrom *et al.*, 2000; Pozo *et al.*, 2003; Klose

and Ajwa, 2004; Jastrzębska and Kucharski, 2007; Floch *et al.*, 2009; García-Ruiz *et al.*, 2009; Kang *et al.*, 2009; Antunes *et al.*, 2011; Balestri *et al.*, 2013).

Enzymes like arylsulphatase, dehydrogenase, *β*-glucosidase and phosphatase can constitute important biomarkers to assess the physiological features of the microbial communities and to evaluate their biodegradative and biodeteriorative potential.

β-Glucosidase, phosphatase and arylsulphatase enzymes, hydrolyse and catalyse specific reactions involved in the biogeochemical transformations of carbon (C), phosphorus (P) and sulphur (S). The enzymes *β*-glucosidase (EC 3.2.1.21) catalyse the hydrolysis of cellobiose, and thus plays a major role in the initial phases of the decomposition of organic C compounds. Arylsulfatases (EC 3.1.6.1) are exoenzymes involved in the sulphur cycling that hydrolyze sulfate esters with an aromatic moiety (phenol esters of sulfuric acid). Phosphatases (EC 3.1.3.2) catalyse the hydrolysis of a variety of organic phosphomonoesters and are therefore important in organic P compounds mineralisation. These enzymes regenerate inorganic nutrients from organic materials and have been reported as the rate-limiting step in the nutrient cycling process. On the other hand, organic phosphorus (P) must be mineralized into inorganic orthophosphate (PO_4^{3-}) ions to be assimilated by many organisms. Only enzymes produced by plants and/or microorganisms are able to hydrolyse organic P into phosphates. Dehydrogenases (EC 1.1.) are intracellular enzymes used as an indicator of microbial respiration rate and gives information about the active microbial community in a particular environment and can be considered an accurate measure of the microbial oxidative activity (Taylor *et al.*, 2002; Pozo *et al.*, 2003; Klose and Ajwa, 2004; Kang *et al.*, 2009; Stege *et al.*, 2009).

The enzymatic monitorisation provides numerous information about the metabolism of the microbial communities, however these approaches can be limited by several parameters like pH, temperature, enzyme and substrate concentration, hindering the understanding of the real metabolic activity.

Thus, in order to avoid these limitations, alternative methodologies must be taken into account, once that, understanding how the microorganisms act in the mural paintings decay and if they are metabolically active or not, are central goals on the biodegradation/biodeterioration artworks process. One of the alternatives to overcome this difficulties is based on the nucleic acid analysis. Through DNA it is possible to get information about the presence of biological agents, once DNA is universally present in both active and inactive microorganisms. However, RNA studies provide more valuable information than DNA in revealing active microbial communities versus dormant microbial cells. This is due to the fact that rRNA and mRNA are indicators of functionally active microbial cells (Rastogi and Sani, 2011; Blagodatskaya and Kuzyakov, 2013). Thus, community

profiling based on direct RNA extraction reflects the metabolically active microorganisms (Anderson and Parkin, 2007), which can be further correlated with the damages observed in the surfaces.

1.4.3. Biomarkers profiling by MALDI-TOF

Matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF) mass spectrometry is known as an extremely sensitive analytical tool for characterising different types of biological compounds including proteins, peptides and lipids, but also microorganism identification as well as DNA sequence analysis (Kirpekar *et al.*, 1998; Fenselau and Demirev, 2001; Gut, 2004; Seng *et al.*, 2009; Wieser *et al.*, 2012; Cho *et al.*, 2013).

In recent years procedures have often been developed that use mass spectrometry for the direct determination of protein in a complex mixture of biological origin. In particular, the application of MALDI mass spectrometry permits to obtain biomarker profiles directly from unfractionated microorganisms like viruses, bacteria and fungal cells and spores. This approach enables to detect, characterise and identify peptides and proteins from intact microorganisms and is applied in biotechnology, cell biology and pharmaceutical research. For example, protein expression profiles from bacterial and eukaryotic cells and cell-free extracts could be rapidly obtained by MALDI-TOF-MS analysis. In the case of mass spectrometry of DNA, the process is more complex than protein analysis due to the formation of sodium and potassium adducts which complicate mass spectra interpretation. Thus, the introduction of 3-hydroxy-picolinic acid as a matrix for DNA together with extensive washing procedures made oligonucleotide analysis possible (Marvin *et al.*, 2003).

In practice, a microbial sample is mixed with a matrix on a conductive metal plate. The mixture can be deposited on the metal support or alternatively the microbial sample is deposited and dried out on the support before the addition of the matrix. After the crystallisation of the matrix and the compound, the target on the metal plate is introduced in the mass spectrometer where it is bombarded with brief laser pulses from usually a nitrogen laser. The matrix absorbs energy from the laser leading the desorption of the analytes that are then vaporised and ionised in the gas phase. This matrix assisted desorption and ionisation of the analytes leads to the formation of predominantly singly charged sample ions. The desorbed and ionised molecules are first accelerated through an electrostatic field and are then ejected through a metal flight tube that is subjected to a vacuum until they reach a detector, with smaller ions traveling faster than larger ions. The TOF required to reach the detector is dependent on the mass (m) and charge (z) of the

bioanalyte and is proportional to the square root of m/z. Thus, bioanalytes with different m/z that composed a complex sample are separated according to their TOF and create a mass spectrum that is characterised by both the m/z and the intensity of the ions, which is the number of ions of a particular m/z that struck the detector (Croxatto *et al.*, 2012).

All of these methodologies are useful to identify the microorganisms present in the mural paintings and to assess their role in the alterations induced in the mural paintings. However other approaches more easily applicable by non-specialists are necessary to be developed, in order to be applied in Conservation-Intervention practice to increase the protection and preservation of our heritage assets.

1.5. Mitigation strategies

The microbial flora present in artworks, like mural paintings, is wide diversified and result from the successive colonisations by different microorganisms. Its biological attack occurs at favourable temperature and relative humidity conditions for the development of microorganisms, and, each coloniser agent has different ways to compromise the aspect, structure and stability of paintings and consequently the building where these are present. Therefore, it becomes imperative to take proper measures for the conservation of mural paintings as well as taking steps for the conservation of the historic buildings (Nugari *et al.*, 1993b; Garg *et al.*, 1995; Borrego *et al.*, 2010)

Microbial growth and propagation on material surfaces can be controlled by physical and chemical manipulations of the material and by creation of artificial environments. As a control measure, lowering humidity is a very effective way to slow down the growth of microorganisms on surfaces in an enclosed environment and prevention against potential contamination will prolong the life time of the objects. However this procedure is not possible for all cultural heritage artworks, being more suitable under museum conditions, where art pieces should be carefully protected environmentally and the numbers of visitors should also be controlled to maintain a relatively constant temperature and humidity, in order to decrease contamination factors (Gu, 2003).

In the case of built artworks, the removal process of the microbial population must be carefully evaluated in order to promote an efficient elimination process, avoiding material destruction and ensuring that the biological recolinisation does not occur for a long period of time.

Thus, to control the biodegradation/biodeterioration process, different approaches can be used, as such as: indirect control by altering environmental conditions, mechanical removal of

biodeteriogens, chemical treatment with biocides and by physical eradication methods (Scheerer *et al.*, 2009).

1.5.1. Physical treatments

The application of irradiation treatment for microbial elimination and cultural heritage artefacts protection has been used in several studies.

Gamma-irradiation can have several advantages for the conservation of objects of cultural heritage. It is highly penetrating and therefore very efficient in killing microbial communities colonizing these objects. Furthermore, this technique is of use to conservators as it is not producing hazardous traces for paintings, it does not cause the formation of secondary radioactivity nor the formation of toxic residues and it is cost attractive. The required dose of gamma irradiation depends on the contamination level, the microbial diversity and its capacity for irradiation resistance. Nevertheless, gamma irradiation is not suitable for large paintings and it does not have a long-lasting effect. Beyond this limitation, a major problem in using gamma irradiation to eliminate colonising microorganisms is the possible deterioration of the object to preserve. The colour stability might be affected as chemical and physical properties of pigments may be changed due to gamma irradiation (Katušin-Ražem *et al.*, 2009; Scheerer *et al.*, 2009; Abdel-Haliem *et al.*, 2013).

The use of UV-C irradiation is an alternative to chemical products because this process does not generate pollution phenomena and the physical support remains unaltered. UV-C irradiation is harmful to living organisms due to its short wavelength, which confers highly energetic photons and germicidal properties upon these organisms, compromising the viability and metabolic activity of the microorganisms (Borderie *et al.*, 2014).

Titanium dioxide is a photo-catalytic nanoparticle with antibacterial and antifungal abilities due to the production of reactive redox species (hydroxyl radicals, superoxide anions and hydrogen peroxide) which induce damages in the cell membrane and can inactivate a wide range of organisms like bacteria, viruses, fungi and algae. Titanium dioxide was proposed for preventing biodeterioration of mortars in cultural heritage buildings. In Palácio Nacional da Pena (Sintra, Portugal) treatments showed the biocidal and preventing biodeterioration properties of titanium dioxide against lichens and other phototropic microorganisms. However, despite these good indications it is necessary to take into account risks to humans as well as for the paint materials,

in the case of mural paintings, because these particles are not as well studied neither their effect (De Filpo *et al.*, 2013).

1.5.2. Biocides treatment

Biocides are commonly applied in repairing, cleaning and maintenance of artworks. Their application aims to prevent and/or control microbial growth. In this way, biocides can be applied before conservation-intervention process to eliminate microorganisms already present, and, after the intervention as preventive effect to slow down the re-colonisation of restored surfaces (Blazquez *et al.*, 2000; Warscheid and Braams, 2000; Ascaso *et al.*, 2002; Gu, 2003; Domenech-Carbo *et al.*, 2006; Urzì and De Leo, 2007; Moreau *et al.*, 2008; Fonseca *et al.*, 2010; Gaylarde *et al.*, 2011; de los Ríos *et al.*, 2012; Maxim *et al.*, 2012; Pinna *et al.*, 2012a; Speranza *et al.*, 2012; De Filpo *et al.*, 2013).

After a conservation-intervention process the spectrum of compounds is increased and the microbial proliferation can be promoted (Ciferri, 1999; Pinna *et al.*, 2012b). Thus, to try to delay their recurrence a biocide treatment should be performed to ensure the durability of the artwork.

Biocides are chemical compounds that have ability to control biological growth/act against biological agents. The requirements for a good biocide are:

- High effectiveness against biodeteriogens;
- Absence of interference with the constituent materials;
- Low toxicity to human health;
- Low risk of environmental pollution.

The commercial biocides available are mainly alcohols, aldehydes, organic acids, carbon acid esters, phenols and their derivatives, halogenated compounds, metals and metal-organic substances, among others. Compounds like quaternary ammonium salts, metals and metal organic substances and heterocyclic organic products, have been widely applied for the control of microbial growth on artworks. Among the products currently used, quaternary ammonium salts are a group of substances widely applied in artworks treatment due to its broad-spectrum action and low toxicity. The antimicrobial effect of quaternary ammonium compounds is probably based on the inactivation of proteins and enzymes and the detrimental impact on the microbial cell membrane. Their effectiveness is dependent on their chemical structure, such as the presence of an aromatic ring structure and the respective length of the four radicals. These compounds affect

a broad microbial spectrum ranging from bacteria, fungi to algae and lichens (Warscheid and Braams, 2000; Sequeira *et al.*, 2012).

Table I-1. Biocides used in mural paintings treatment.

Biocide	Classe/ Active principle	Action form	Ref.
Igran 500FW	Triazines/Terbutryn	Photosynthesis inhibition; affects electron transport	(Rosado *et al.*, 2014a)
New Des	Sulphonamines/Streptamine H	Inhibitor of cell division of prokaryotic cells	(Blazquez *et al.*, 2000; Domenech-Carbo *et al.*, 2006; Gazzano *et al.*, 2013)
Preventol®PN	Chlorophenols/ Pentaclorophenolate	Oxidation affects the oxidative phosphorylation	(Blazquez *et al.*, 2000; Maxim *et al.*, 2012)
Preventol®R80	Ammonium quaternary compounds/ Benzalkonium chloride	Affect active transport and destabilizes the membrane integrity	(Blazquez *et al.*, 2000; Ascaso *et al.*, 2002; Nugari *et al.*, 2009)
Wikamol Murosol	Organometallics/ Tributyltin oxide	Inhibitor of Metabolism	(Rosado *et al.*, 2014a)

Despite the well-established biocides efficiency, some studies have suggested that the combined application of hydrophobic compounds and biocides is more effective against microbial recolonisation than single biocide application. The application can be done in a single step when the water-repellent and the biocide are mixed together, or in two steps when the biocide is applied before or after the water-repellent (Urzì and De Leo, 2007; Pinna *et al.*, 2012).

1.5.3. Natural alternatives

Due to the limitations related with the use of chemical compounds, natural products represent a huge potential source of compounds with antimicrobial properties, which can be an useful and advantageous alternative for the chemical products.

Natural substances with antimicrobial action have been identified from a very wide range of sources, including plants, microorganisms and animals. In this way, several strains of *Bacillus subtilis* and *Bacillus amyloliquefaciens* have been referred to produce lipopeptides. In response

to nutritional stress, a variety of processes are activated by *Bacillus* strains, including sporulation, synthesis of extracellular degradative enzymes and antibiotic production (Klich *et al.*, 1991; Dieckmann *et al.*, 2001; Caldeira *et al.*, 2006, 2007; Caldeira *et al.*, 2008). Many strains are known to suppress fungal growth *in vitro* due to the production of antifungal antibiotics (Hiradate *et al.*, 2002; Yu *et al.*, 2002) especially the nonribosomally synthesized cyclic lipopetides surfactin, iturin and fengycin. Bioactive peptides show a great potential for biotechnological applications. These compounds, made of amino acids and a fatty acid, are easily biodegradable. The synthesis of lipopeptide compounds are common in nature because the mechanism behind its production is directly related to defence to stress situations, like sporulation, and because these compounds can bring benefits to the individual. They also act as surfactants, being molecules with a low molecular weight capable of changing the physical and chemical properties of interphases. In the nature, these lipopeptides increase the surface area from non-soluble hydrophobic growth substrates and the solubility of hydrophobic substances improving their biologic availability, and participate in the adherence and detachment of microorganisms from surfaces (Stein, 2005; Thasana *et al.*, 2010; Caldeira *et al.*, 2011a; Velho *et al.*, 2011).

Recent studies have shown that there is a strong relation between the molecular structure and their antifungal properties; in general more carbon atoms in the fatty acid chain enhances the antifungal and hemolytic activity, as it seems to increase interactions with biological membranes (Akpa *et al.*, 2001; Etchegaray *et al.*, 2008). These amphiphilic cyclic biosurfactants have many advantages over other biocides: low toxicity, high biodegradability and environmentally friendly characteristics (Caldeira *et al.*, 2011b). Preliminary studies with lipoptides resulting from *Bacillus* sp. metabolism revealed inhibitory effect against biodeteriogenic fungal strains isolated from mural paintings (Silva *et al.*, 2014), suggesting them as potential products to be applied in remediation and preventive strategies to protect microbial attack of artworks.

In this context, lichen secondary metabolites (LSM) have also been suggested as potential natural antimicrobial compound, because their chemical simplicity makes their synthesis potentially easy in the laboratory. LSM are a group of more than 800 compounds, which include aliphatic, cycloaliphatic, aromatic and terpenic components, synthesised by lichen-forming fungi. Many of these compounds are well known for having allelopathic effects on bryophytes and vascular plants. Antibiotic, antiviral and anti-proliferative functions have been also recognised, suggesting their potential use for therapeutic applications. The antimicrobial activity of LSM has been assessed against a wide set of bacteria and filamentous fungi, mainly of medical interest. Some of these LSM compounds like usnic acid, norstictic acid and parietin were tested on stone materials, showing that lichen secondary metabolites as allelopathic agents against rock dwelling

microorganisms and as potential natural sources for their control on stone materials in restoration and conservation program (Gazzano *et al.*, 2013).

Another alternative is based on the antibiotics production by several microorganisms. There are several studies that report this application. For example, the antibiotic 6 Penthyl α Pyrone phenol, produced by *Trichoderma harzianum*, was applied as a successful technique for elimination of *Aspergillus niger* and *Aspergillus flavus* from mural paintings. This compound is non-toxic, non-expensive, practical, durable, and does not reveal deteriorative effect on the colours of the paintings (Helmi *et al.*, 2011).

Thus, understanding the biodegradation/biodeterioration processes requires a well-defined intervention plan (Figure I-8) that focuses aspects from the characterisation of the materials used, biological agents, as well as remediation strategies to prolong the longevity of the artworks.

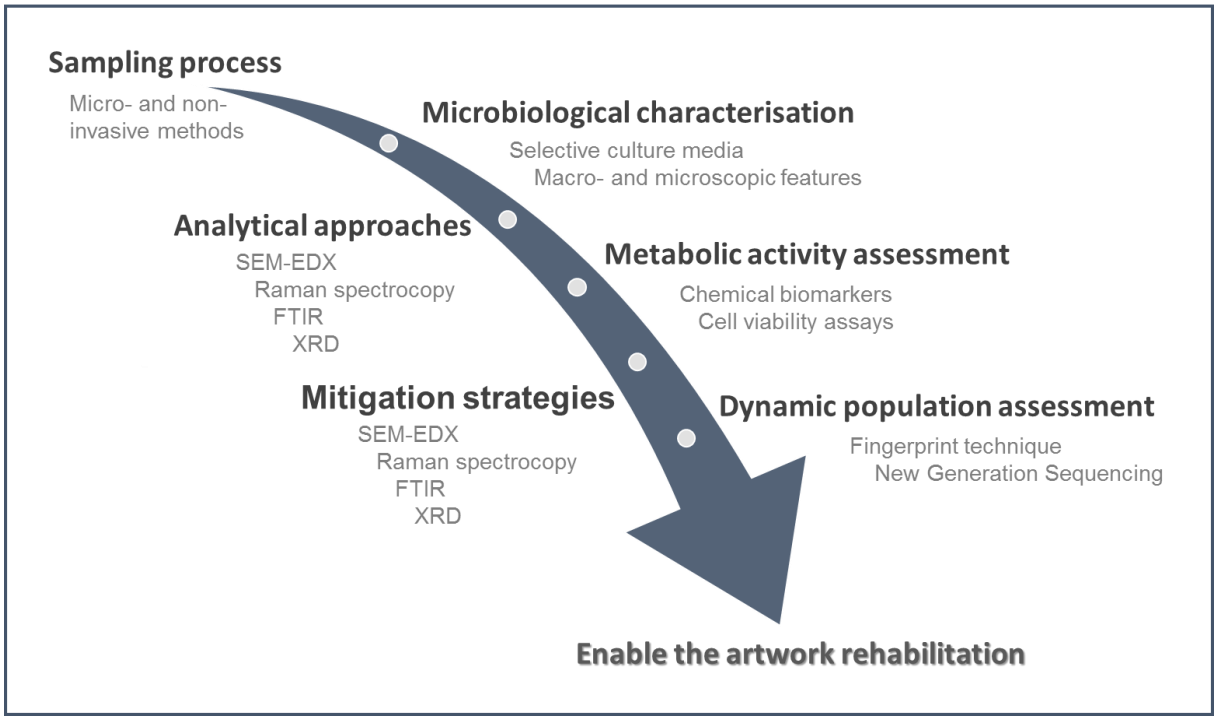


Figure I-8. Strategic plan to provide a complete diagnostic of the main problems that affect the integrity of the paintings, encompassing micro-analytical approaches, culture dependent methods, molecular approaches, biochemical markers and mitigation strategies.

According to this, to study the alteration phenomena that promote damages in mural paintings, it is essential to know the materials applied, being fundamental the application of micro-analytical methods, which must be non-invasive or micro-invasive and non-destructive. There are several procedures with the advantage to be applied *in situ* or need only microsamples for analysis.

Studies on mural paintings materials have involved a large number of different instrumental analytical techniques (Stuart, 2007) including optical and scanning electron microscopy (Ortega-Avilés *et al.*, 2001; Sánchez del Río *et al.*, 2004; Barilaro *et al.*, 2005; Baraldi *et al.*, 2006), infrared spectroscopy (Barilaro *et al.*, 2005; Salvadó *et al.*, 2005; Baraldi *et al.*, 2006; Hernanz *et al.*, 2006), Raman spectroscopy (Edwards *et al.*, 1999; Smith and Barbet, 1999; Wang *et al.*, 2004; Hernanz *et al.*, 2006), X-ray fluorescence (XRF) spectroscopy (Aloupi *et al.*, 2000; Ortega-Avilés *et al.*, 2001; Gil *et al.*, 2008), X-ray diffraction (Barilaro *et al.*, 2005; Salvadó *et al.*, 2005; Baraldi *et al.*, 2006; Hernanz *et al.*, 2006) and seldom synchrotron advanced techniques (Ortega-Avilés *et al.*, 2001; Pagès-Camagna *et al.*, 2006). Particularly promising is the development of non-destructive techniques such as *in situ* XRF (Ferrero *et al.*, 2002; Uda, 2004; Gil *et al.*, 2008) and *in situ* Raman (Perardi *et al.*, 2000; Vandenabeele *et al.*, 2000; Pérez-Alonso *et al.*, 2006), which has recently enabled the *in situ* study to avoid sampling the mural paintings.

These techniques provide information about the material composition, biological contamination presence and characterisation, and, identification of alteration products, useful data to complement the biochemical and microbiological population dynamics.

Table I-2 compare different complementary methodologies, used in this work, emphasizing the advantages and disadvantages of multianalytical approaches, combining culture dependent methods, DNA approaches and microanalytic techniques.

Table I-2. Multianalytical approaches to characterise mural paintings alterations, combining culture dependent methods, molecular approaches, analytical methods and biochemical markers.

Methods	Advantages	Disadvantages
Culture dependent methods	▪ Provide high cells density, useful to *in vitro* assays	▪ Sequencing is needed for identification ▪ Some microorganisms are not cultivable ▪ Incomplete screening
DGGE	▪ Use metagenomic DNA ▪ Information about the dynamics of the populations ▪ Useful to compare microbial contamination between different places/samples	▪ Sequencing of bands ▪ Incomplete screening ▪ Do not allow obtain cells
Pyrosequencing	▪ Powerful novel technique in biodegradation studies of artworks ▪ Full characterisation of the microbial population ▪ Large number of samples can be analysed in a short time	▪ Expensive ▪ Do not allow obtain cells
Raman microspectrometry	▪ No sample preparation is required ▪ Non-destructive method ▪ High sensitivity ▪ Rapid screening for detect microbial presence	▪ Sample heating by the laser radiation can destroy the sample ▪ Incomplete screening ▪ Do not allow obtain cells
FTIR-ATR	▪ No sample preparation is required ▪ High reproducibility ▪ Easy-to-use, fast, and versatile technique ▪ Solids, pastes, gels, liquids and powders can be analysed ▪ Rapid screening for detect microbial presence	▪ Samples could be destroyed by the pressure ▪ Incomplete screening ▪ Do not allow obtain cells
SEM and SEM-EDX	▪ Image with high magnification and resolution ▪ Elemental composition of the materials allowing their localisation in the samples ▪ Detect proliferation of the microorganisms in the samples	▪ Coating samples with gold or carbon destroy them ▪ Do not allow obtain cells
Dehydrogenase	▪ Detect cell viability of the microbial population ▪ Cell viability could be correlated with contamination levels ▪ Biochemical marker function	▪ Destructive ▪ Time-consuming ▪ Do not allow obtain cells

(Rosado *et al.*, 2014b)

The rationale behind this PhD research project has been the development of integrated studies that combine the characterisation of mural painting materials and the assessment of the extrinsic causes and mechanisms of degradation, using novel techniques and implementing new analytical protocols for mural paintings biodegradation assessment. The research was developed in close collaboration with conservator-restorers and heritage researcher with the aim to develop tools and tackle some of their needs and questions. Several historical mural paintings were selected as case studies under the framework of this PhD, which represent different research scenarios and conditions.

This PhD thesis is organised into six chapters; an introductory chapter, four chapters dedicated to the results and discussion, being most of them published in scientific papers, and a final chapter presenting the main conclusions obtained during this research.

Chapter I describes general aspects related to mural paintings constitution and cultural importance, giving an overview about the phenomena that affect mural paintings, exploiting the significant importance of microorganisms in the decay of this artworks, presenting several methodologies for their identification and different approaches to control their development, based on an extensive bibliographical research.

In Chapter II are presented multianalytical approaches to identify the alteration causes of mural paintings decay, using non- and micro-invasive and non-destructive methodologies.

Chapter III presents a combined application of culture dependent methods and molecular approaches to characterise the microbial population thriving in the paintings, using novel and innovative approaches.

Chapter IV is focused on the monitorisation of biological systems, to signalise the main biodeteriogenic agents involved in in the decay of mural paintings.

Chapter V describes the development of mitigation strategies to be applied in mural paintings, to eliminate and control microbial proliferation, in order to promote the rehabilitation and enrichment of these artworks.

Final considerations and future perspectives are presented in Chapter VI, emphasising the advantages of the strategies outlined.

CHAPTER II

The role of microorganisms in the mural paintings pathologies

Some results of this chapter were published in the following scientific papers:

Rosado T, Gil M, Mirão J, Candeias A and Caldeira AT (2013) Oxalate biofilm formation in mural paintings due to microorganisms - a comprehensive study, *International Biodeterioration & Biodegradation* 85:1-7.

Rosado T, Reis A, Candeias A, Mirão J, Vandenabeele P and Caldeira AT (2014) Pink! Why not? On the unusual colour of Évora Cathedral, *International Biodeterioration & Biodegradation* 94:121-127.

Rosado T, Mirão J, Candeias A and Caldeira AT (2014) Characterizing Microbial Diversity and Damage in Mural Paintings, *Microscopy and Microanalysis*, 1-6 (doi:10.1017/S1431927614013439).

1. Overview

Three different cases alteration of mural paintings and mortars, with pronounced aesthetic and structural damages, were analysed in order to identify the causes that alter the artworks that will be presented.

This investigation revealed a strong relationship between the microbiological proliferation and the damaged areas, evidencing the important role of the microorganisms in the degradation/deterioration process.

The formation of oxalates, lead pigments oxidation and carotenoids development noticeably contribute to murals and/or mortars alteration, and are correlated in this study with the presence of biodeteriorative microorganisms. These alterations, attributed to metabolic activity of microbial cells, promote polychromy degradation of specific pigments, efflorescence's appearance and biofilms formation, culminating in some cases, in structural damages, affecting mortars integrity.

The study of the mechanisms underlying the microbiological attack of mural paintings has been explored to understand as much as possible the proliferative ability and biodeteriorative capacity of microorganisms.

The biodeterioration/biodegradation study is an important issue for the conservation of cultural heritage that needs urgent answers to their rehabilitation. In this way, the role of microorganisms in surfaces alteration will be exploited.

2. Introduction

The biodeterioration/biodegradation of historic monuments occurs as a consequence of chromatic alterations, stains appearance, biofilms formation, secretion and deposition of organic and inorganic compounds promoting salt encrustation and efflorescence formation, physical intrusion/penetration of microorganisms inducing cracks and detachment of some fragments (Rojas *et al.*, 2009; Dakal and Cameotra, 2012). These alterations promote serious aesthetic and structural problems that need to be signalised and characterised to fully understand the degradation/deterioration process (Capodicasa *et al.*, 2010; Pepe *et al.*, 2011).

Microorganisms play a geoactive role in the biosphere because they can initiate, support and accelerate some geochemical and geophysical reactions which lead to biodeterioration of artworks. Among the numerous microorganisms involved in this process, filamentous fungi, yeasts, bacteria, algae and lichens constitute the commonly microbiological agents present in artworks, whose development is supported by favourable temperature, relative humidity conditions and nutrients availability (Nugari *et al.*, 1993b; Garg *et al.*, 1995; Ciferri, 1999; Heyrman and Swings, 2003; Pangallo *et al.*, 2009b; Borrego *et al.*, 2010). Thus, to give an overview about the microflora involved in the biodeterioration/biodegradation processes, it is necessary to use different approaches in order to characterise the coloniser population, to assess their physiological/biological potential and to identify the alterations caused in the materials.

Some authors suggest bacteria as the first agents in the colonisation of mural paintings, because they have reduced nutritional needs, providing organic matter to the next colonisers. Bacterial growth is frequently associated to the formation of biofilms, promoting discolouration of the pigments. On the other hand, the growth of biological agents such as fungi is identified as a determinant factor in the degradation of the murals (Garg *et al.*, 1995; Rölleke *et al.*, 1996). Their proliferation is enhanced in situations of high humidity and temperature (Garg *et al.*, 1995; Gorbushina *et al.*, 2004; Sterflinger, 2010). Their development in mural paintings can induce discolouration and deterioration of the surfaces, leading to stains appearance that alter the colour of the paint layer, and, on the other hand, hyphae penetration may lead to fragments detachment (Garg *et al.*, 1995; Ciferri, 1999; Gorbushina and Petersen, 2000 ; Milanesi *et al.*, 2006; Guiamet *et al.*, 2011).

Among the several chromatic alterations involved in the degradation/deterioration of artworks, pigments oxidation is one of the problems that affects drastically painted areas.

Pigments like red lead and lead white have been widely employed in paintings since Antiquity. Admixtures of lead white and red pigments like red lead, vermillion and red ochre for example,

were employed on paintings to produce flesh tones/carnations. However, some pigments like lead white ($2PbCO_3 \cdot PbOH_2$) and red lead (Pb_3O_4) can suffer alterations, associated to darkening and/or whitening processes (Petushkova and Lyalikova, 1986; Aze *et al.*, 2006; Aze *et al.*, 2008; Kotulanová *et al.*, 2009a). The transformation of these pigments can be due to natural aging, light interaction or promoted by several environmental parameters where humidity play an important role in the activation of chemical processes, as well as in the support of microbial development, which may generate colour alterations of lead based pigments (Giovannoni *et al.*, 1990).

In the case of the whitening process, compounds like hydrocerussite ($2PbCO_3.Pb(OH)_2$) cerussite ($PbCO_3$) and anglesite ($PbSO_4$) can appear in artworks as pigment degradation products, whereas *plattnerite* (PbO_2) and galena (PbS), a black/brown product are found in darkening areas (Smith *et al.*, 2001; Aze *et al.*, 2008; Kotulanová *et al.*, 2009a).

Black galena production can be caused by the reaction of lead based compounds with sulphur-containing compounds and gases. Another mechanism for the darkening of lead-based pigments is their oxidation to the black-brown mineral *plattnerite* (Kotulanová *et al.*, 2009a). Nevertheless, in mural paintings, the darkening phenomenon has been mainly attributed to the *plattnerite* formation (Smith *et al.*, 2001; Smith and Clark, 2002). This degradation product can result of the red lead or lead white oxidation, wherein the alteration of lead oxidation state of Pb^{2+} to Pb^{4+} promotes the chromatic alteration of these pigments (Aze *et al.*, 2006). P*lattnerite* formation was also attributed to the metabolic activity of microorganisms (Petushkova and Lyalikova, 1986; Giovannoni *et al.*, 1990; Qingping *et al.*, 1999; Smith *et al.*, 2001; Aze *et al.*, 2008).

Another phenomenon that promotes mural paintings decay is associated to oxalates formation. There are two possible sources, chemical and biological, for the formation of oxalates onto the surface of the painting. According to the second hypothesis, microorganisms such as bacteria, fungi, algae and lichens have been identified as the main responsible for their formation (Çaliskan, 2000; Cariati *et al.*, 2000; Edwards *et al.*, 2000; Rampazzi, 2004). In fact, the metabolism of these microorganisms excrete oxalic acid ($H_2C_2O_4$), which can react with the calcite ($CaCO_3$) present in the painting giving rise to calcium oxalate (CaC_2O_4) formation, in different states of hydration like *whewellite* ($CaC_2O_4.H_2O$) and *weddellite* ($CaC_2O_4.2H_2O$), very insoluble compounds, leading to the formation of efflorescence and consequent deterioration of the paintings (Švarcová *et al.*, 2009; Guggiari *et al.*, 2011). Calcium oxalates formation can also occur as a defence mechanism of the microorganisms in situation with excess of calcium, to prevent the toxicity for the cell (Pinna, 1993).

In addition to the degradation/deterioration problems already described, mortars discolouration is also an alteration that affect the integrity of the artworks. These alterations are mainly

associated to phototrophic microorganisms such as cyanobacteria and algae, owning an important role in the mortars decay (Altenburger *et al.*, 1996 ; Ariño and Saiz-Jimenez, 1996; Cappitelli *et al.*, 2009; Tran *et al.*, 2012 ; Kusumi *et al.*, 2013). These microorganisms have been reported to promote deterioration of the internal wall surfaces and plasters where there is low light intensity, and, particularly, cyanobacteria are pointed to cause rosy discoloration in indoor environments (Cappitelli *et al.*, 2009). Nevertheless, these microorganisms and others can induce irreversible stainings and chromatic alterations (Rölleke *et al.*, 1998 ; Urzì and Realini, 1998; Gurtner *et al.*, 2000 ; Piñar *et al.*, 2001; Schabereiter-Gurtner *et al.*, 2001b; Realini *et al.*, 2005; Ripka *et al.*, 2006; Imperi *et al.*, 2007 ; Laiz *et al.*, 2009; Piñar *et al.*, 2009; Jurado *et al.*, 2012; Ortega-Morales *et al.*, 2013; Sterflinger and Piñar, 2013), due to their ability to produce pigmented compounds (Warscheid and Braams, 2000; Polo *et al.*, 2010). An example of these coloured compounds are carotenoids, that are tetraterpenoids - highly unsaturated isoprene derivatives - chiefly synthesised by filamentous fungi and yeasts but also by some species of bacteria, algae and lichens. Carotenoids are the most widely distributed class of dyes in nature, displaying yellow, orange, and red colour (Aksu and Eren, 2005; Tinoi *et al.*, 2005). The main function of the carotenoids is harvesting the energy of light, protection of living organisms against oxidative damage by quenching photosensitizers, interacting with singlet oxygen and scavenging peroxy radicals, thus preventing the accumulation of harmful oxygen species, and in stabilisation of certain pigment-protein complexes (Davis, 1991; Sandmann *et al.*, 1999).

To understand some alteration phenomena that induce serious damages in artworks, putting in question their durability, three different cases were selected for study. Each case is inserted in a different context: one church with regular religious ceremonies celebration, the *Santa Clara* Church, a convent (*Nossa Senhora da Saudação* Convent) with low luminosity, high humidity conditions, closed to visitors, both with mural paintings, and the Évora Cathedral, one of the most emblematic monuments of Évora, with a lot of visitants along the year.

The criteria for selection of these artworks were: alteration status, degradation levels and chromatic alterations. Three different problems were studied:

a) Darkening of carnation areas - *Santa Clara* Church
b) Chromatic alterations of green areas - *Nossa Senhora da Saudação* Convent
c) Mortars discolouration (pink stains) - Évora Cathedral

2.1. *Santa Clara* Church

Santa Clara church was built at the end of 16th century in Sabugueiro (Arraiolos, Southern Portugal), with decorative campaigns that extended during the 17th and 18th centuries, classified as Monument of Public Interest in 2001. It is one of the Mannerist exemplars of the region, evidenced by the classic simplicity of the late-sixteenth-century. The inside has a single nave covered with sixteenth century mural paintings, composed by 18 panels. The several paintings present in the church are essentially representations of themes of Marian life and worship of the Lusitanian, as well as: Annunciation, Crib, Adoration of the Kings and representation of several Saints (A.A.V.V., 1978).

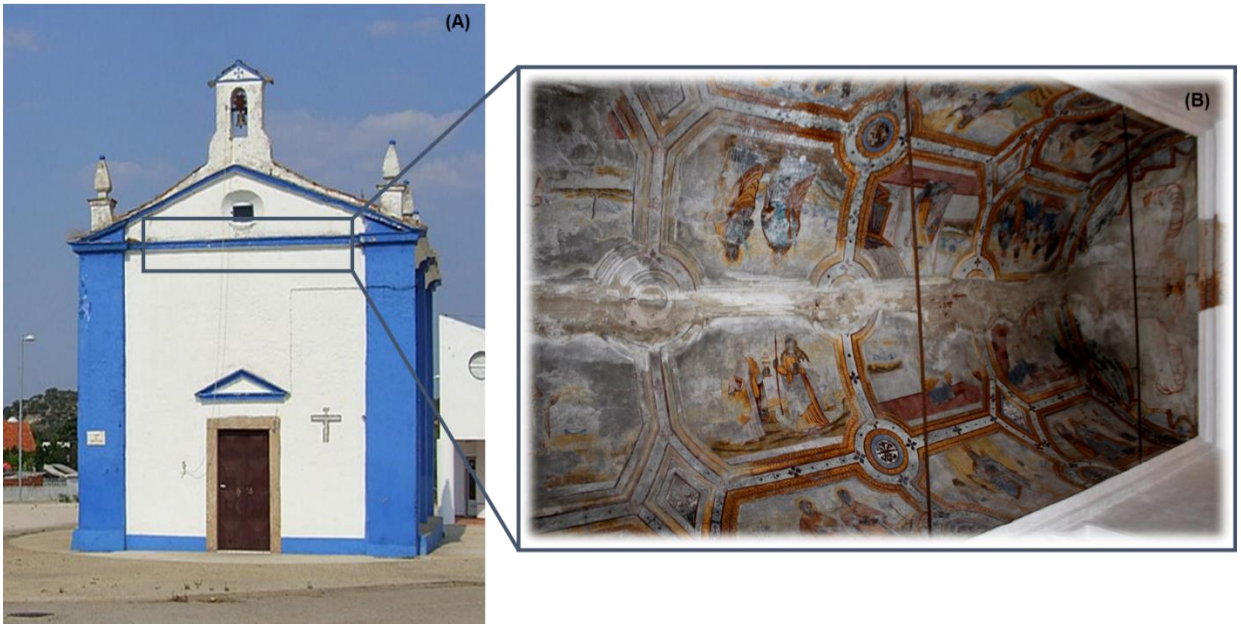


Figure II-1. *Santa Clara* church in Sabugueiro, *Portugal* (A) and general view of the ceiling mural paintings (B).

2.2. *Nossa Senhora da Saudação* Convent

The Low Choir of the extinct Dominican Convent of *Nossa Senhora da Saudação*, founded during D. Manuel I reign inside the medieval castle of Montemor-o-Novo (Alentejo, Southern Portugal) have a set of mural paintings, with good artistic quality. These paintings are among the most important mural paintings cycles from the first quarter of the 17th century in Évora Archiepiscopate, and are attributed to *José de Escovar*, an easel and mural painter with Spanish ancestry, one of the most laborious and active painters working in Évora. Covering the vault and the walls there are 17 painted scenes which includes catechetical passages from Jesus Christ

and other Saints iconography. These paintings have survived with structural damages to the 1755 earthquake that have completely destroyed the town of Lisbon and have escaped in the 20th century to vandalism during the years that the convent was abandoned (Gil *et al.*, 2014). Hidden by darkness for at least sixty years, these murals show severe paint losses in some of the scenes mainly due to salts formation and also chromatic alterations that affect green areas.

Figure II-2. General view of *Nossa Senhora da Saudação* Convent, Montemor-o-Novo, Portugal (A), cloister (B) and Low Choir of the Convent (C).

2.3. Évora Cathedral

Évora Cathedral or *Santa Maria* Church is one of the most emblematic monuments in Évora, Southern Portugal, a monumental town classified by UNESCO as World Heritage. This monument is the biggest Portuguese Cathedral and has a Romanic-Gothic style or Gothic with Cistercian and Medicant influences. Its construction dates back to the 13th century and was inspired by the model of Lisbon's Cathedral and other foreign cathedrals.

This monument has suffered several conservation-restoration interventions through the ages, without, however, any type of previous knowledge about the type of mortars and materials used. Recent works (Adriano *et al.*, 2009; Silva *et al.*, 2010) focused on the material characterisation of the renders, have shown that the inner walls of the Cathedral are composed of dolomitic aerial lime mortars with siliceous aggregates similar in composition to the granodiorites of Évora's region with crushed ceramics as additives which can be dated back to a 16th century documented rehabilitation intervention. These works, however, were unable to detect any pigment and hence to explain the pink colour that covers the majority of the inner walls surface.

Figure II-3. General view of the façade (A), lateral (B) and indoor of Évora Cathedral, Évora, Portugal.

Thus, according to the different alteration problems reported, it was considered imperative to perform a full study in order to identify the phenomena that promote these damages in the mural paintings and mortars, combining analytical methodologies for the material characterisation of the surface layers and microbiological approaches to evaluate the contamination levels and correlate them with the chromatic alteration detected. This information together with the alteration products characterisation has proven to be a good indicative to identify the biodeteriogenic agents responsible for the decay of these artworks.

3. Experimental Section

3.1. Sampling process

The sampling process was carried out using micro- and non-invasive methods (Annexe C), following the requirements for conservation purposes, minimising the structural and aesthetical impact of the paintings, collecting the minimum amount of sample required for the different assays and sufficient to ensure the representativeness of the areas in analysis. Three different artworks visibly altered were analysed: *Santa Clara* Church, *Nossa Senhora da Saudação* Convent and Évora Cathedral.

Microsamples were collected in areas with different alteration signs, using sterile cotton swabs placed in suspension of transport MRD medium (Maximum Recovery Diluent, Merck)/NaCl 0.85% solution for microbiological experiments, and with sterile scalpels and microtubes for mortar microfragments (100 mg) analyses, whose scheme collection for each case is represented in the figure II-4, figure II-13 and figure II-18. Samples were conserved at 4ºC until utilisation.

3.2. Evaluation of microbial contamination in mortars

In order to assess the degree of deterioration of the support and the type of colonising microorganisms, samples were used as such or coated with Au-Pd (Balzers Union SCD 030) during 30 s, and observed in a HITACHI S-3700N variable pressure scanning electron microscope (VP-SEM) with accelerating voltage of 18-20 kV. Microanalysis of the selected samples were performed using the same microscope coupled with a Bruker XFlash 5010 energy dispersive X-ray spectrometer to allow microstructural characterisation of the mortars and elemental composition (point analysis and 2D mapping). EDX analyses were performed at 20 kV.

3.3. Isolation and characterisation of microbial population

Samples collected with sterile cotton swabs were mechanically shaken for 1h and inoculated (100 µL), under aseptic conditions, in different culture media (Annexe A), specific to each microorganism like: NA (Nutrient Agar) for bacteria, MEA (Malt Extract Agar) and CRB (Cook Rose Bengal) for filamentous fungi, and, YEPD (Yeast Extract Peptone Dextrose Agar), for yeasts. The cultures were incubated at 30ºC for 24-48 h 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.

3.4. 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). The preparations made for fungal isolates were stained with methylene blue, observed with a 20x 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. Identification was performed by sequencing 16S rDNA or ITS region for bacterial or fungi isolates, by outsourcing service. The genomic DNA extraction was carried out by using a kit (NucleoSpin DNA Extraction kit - Macherey-Nagel, Düren, Germany). For bacteria, 16S rDNA was amplified using the primers 5'-ACG GGT GAG TAA CAC GTG-3' and 5'-GCT CCG TCA GAC TTT CGT-3' or 5'-AGA GTT TGA TCC TGG CTC AG-3' and 5'- GAC GGG CGG TGT GTA CAA-3'.

For fungi, the region containing partial portions of the small subunit (18S), both internal transcribed spacers (ITS) and the 5.8S of the rDNA repeat unit was amplified using the oligonucleotides primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS4 (5'TCCTCCGCTTATTGATATGC-3').

PCR reactions were carried out on initial denaturing at 95ºC for 3 min followed by 30 cycles at 92ºC for 30 s, 55ºC for 30 s, and 72ºC for 1 min. The reaction was completed with 10 min extension at 72ºC. PCR products were analysed by agarose gel (1%) electrophoresis, purified with the NucleoSpin Extract II Kit (Macherey-Nagel) and sequenced by capillary electrophoresis using the ABI PRISM 3730 xl sequencer (Applied Biosystems) with the Kit BDT v1.1 (Applied Biosystems).

The nucleotide sequences were aligned with those retrieved from the GenBank (NCBI) databases for the homology analysis using the BLASTN 2.2.25+ program.

3.5. Alteration status assessment

To investigate the alteration products formed in the deteriorated paint areas, microanalyses were performed on the mortar microfragments.

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 sample. Ordinary acquisition time was of the order of 10-20 s with 5 cm^{-1} of spectral resolution. The back-scattered light is collected by the objective (10x or 50x), and then captured by a CCD (Charge Coupled Device) detector.

Some samples, those having compounds with a low Raman scattering, were also analysed by FTIR spectroscopy using a (Bruker ALPHA) equipped with the attenuated total reflection (ATR - QuickSnap) set up coupled with crystal diamond. 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.

3.6. *In vitro* simulations assays

3.6.1. Green areas chromatic alteration by microbial communities of *Nossa Senhora da Saudação* Convent

To study the influence of metabolic activity of microorganisms on the green areas alteration a combinatory strategy was used:

a) Development of laboratorial cultures with high density of cells corresponding to mixed cultures of active cultivable microorganisms present in degraded green areas of the paintings;
b) Development of laboratorial cultures with high density of cells corresponding to pure cultures, using isolated microorganisms;
c) Simulation of the influence of these cultures on real sterilised microsamples.

Mixed cultures were performed using cotton swabs/microfragments collected in degraded green areas of the Low Choir paintings of the Convent of *Nossa Senhora da Saudação*. These

samples were used to inoculate 50 mL cultures (Malt Extract and Nutrient Broth liquid culture medium) for fungal and bacterial population development. Cultures were incubated at 28ºC (for fungi) or 30ºC (for bacteria) in an orbital shaker at 150 rpm (Heidolph unimax 1010), during 7 days. Pure cultures were performed under the same conditions, using the different isolated strains (*Bacillus* sp., *Cladosporium* sp.*, Penicillium* sp., *Nectria* sp.) to inoculate the liquid cultures.

These cultures were analysed by Raman microscopy to detect the presence of oxalates in the metabolic pool. Then, 10 µL of these cultures were applied on sterilised microfragments of mortar (around 20 mg) and incubated during two weeks at 30ºC. After this period the potential for biofilm production and the proliferation on the mortar was evaluated by SEM.

3.6.2. Mortars alteration of the Évora Cathedral

The predominant isolated microorganism colonies, *Rhodotorula* sp. yeast, exhibited a strong pink/dark orange colour that was further investigated to establish the effect of their growth on the mortars, by different sets of experiments:

- insertion of original historical mortar on sterilised liquid culture media under controlled conditions for microbial population analysis;
- liquid cultures of isolated *Rhodotorula* sp. yeast for production of metabolic compounds.

Mortar microfragments (0.1 g) were inoculated in 50 mL culture media (Malt Extract and Nutrient Broth liquid culture medium) for fungal and bacterial population development. Cultures were incubated at 28ºC (for fungi) or 30ºC (for bacteria) in an orbital shaker at 150 rpm (Heidolph unimax 1010), during 14 days. In the case of *Rhodotorula* liquid cultures, fresh yeast slant was washed with 2 mL of NaCl 0.85% solution and the suspension was inoculated in the same conditions mentioned above.

These cultures were analysed by Raman microscopy and FTIR-ATR (see section 3.5. Alteration status assessment) to detect the presence of carotenoids in the metabolic pool.

Standard samples were prepared by mixing weighed amounts of commercial *β*-carotene (Sigma-Aldrich, 95%) with pulverised sterile mortar from Évora Cathedral (16, 58, 122 and 212 mg *β*-carotene g^{-1} mortar). These mixtures were analysed by Raman spectroscopy (see section 3.5.Alteration status assessment) to evaluate the Raman accuracy on the detection of carotenoids in complex samples like mortars. The analyses were complemented with FTIR-ATR measurements.

4. Results and Discussion

The identification of the agents that promote degradation/deterioration of the mural paintings involves a detailed study, using different techniques, requiring sometimes the prior isolation of the microorganisms that proliferate in these artworks. Thus, the strategy adopted to identify the pathology, that promotes alteration in the artwork, defines the type of analysis to be performed. Therefore, the collection of samples is an extremely important step, where the careful observation of the paintings allows us to get an idea about the areas possibly affected and the type of changes that are occurring.

The main problems detected in the mural paintings studied are related with colour alterations due to pigment changes or surfaces modifications, salt efflorescences formation, cracks appearance in the walls, and, in some cases detachment of fragments. In this way, the identification of the alteration products that promote the modification of the surfaces and the signalisation of the main parameters involved in this event, it is crucial to understand and diagnose the problem, in order to allow the conservation and preservation of artworks.

4.1. Darkening of carnation areas - *Santa Clara* Church

The *Santa Clara* church has a set of mural paintings covering the vault, where it is possible to observe a wide range of religious scenes paintings, but unfortunately, they have suffered degradation/deterioration due to darkening of some figures represented, and salt efflorescences appearance. These alterations affect mainly carnations areas like faces, arms, hands and feet, where it was possible to observe black spots that cover some of the mentioned areas, or, in some cases affect all the carnation areas. In addition to the blackening process that affect the carnation areas, it was also detected the presence of salt efflorescences. Nevertheless, salt efflorescences were also detected in other areas, particularly in the blue areas of the painting.

The eighteen panels represented in this church report several biblical episodes, and for this work, only two panels were selected, the Annunciation (Panel 1) and the Apparition of the Virgin to *D. Fuas Roupinho* (Panel 2). These panels were selected due to the presence of evident alteration signs. Throughout the text, each one of these panels will be designated by SCP1 (*Santa Clara* Panel 1) and SCP2 (*Santa Clara* Panel 2), following up the respective number of the sampling area (Figure II-4). The sampling points are represented in Figure II-4.

Figure II-4. *Santa Clara* church panels with representative scheme of sampling process. (A) Panel 1 (SCP1) – Annunciation, (B) Panel 2 (SCP2) – Apparition of the Virgin to *D. Fuas Roupinho*.

Therefore, it is crucial to identify the phenomena that induced these alterations in the mural paintings, and, if it is associated to microorganisms presence, in order to give useful information for a possible intervention-restoration process to avoid the dissemination of the problem.

In order to find answers to this problem, multianalytical protocol was developed.

The first approach focuses on the mortars observation and material characterisation, important component to the knowledge of the original constituents of the paintings, followed by the identification of alteration products and investigation about the presence of biological contamination. Thus, samples with different alteration status were selected for these analyses:

a) Chromatic layers in the flesh tones that suffer darkening process
b) Areas with efflorescence salts

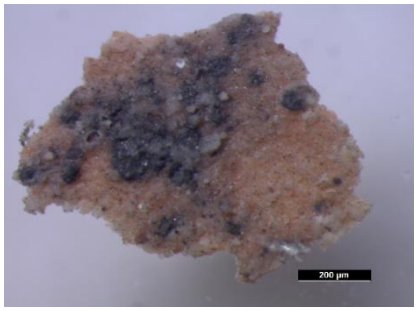
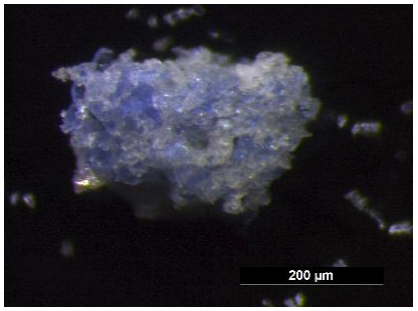
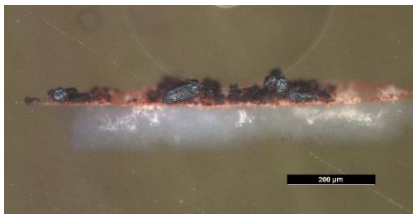
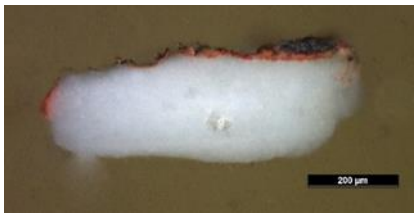
4.1.1. Mortar microfragments analysis

Several mortars microfragments from altered areas (carnations and blue zones) were used as such and mounted into a stratigraphic section for optical microscopy observation (Table II-1).

The microscopic analysis performed on samples from flesh tones areas (SCP1_5 and SCP2_13) allows the observation of white and red pigment particles. This result indicates that the carnations hue and colour were obtained by the mixture of two different pigments (compounds identification in the section 4.1.2. Alteration products identification). On the other hand, these samples from flesh tones/carnations, that present darkening and also salt efflorescences, show, in addition to the white and red pigments already observed, small black particles in the upper layer of the paint surface. These particles seem to be associated with the alterations induced in the paintings, whereby analyses in these specific areas were performed.

In the case of blue areas (SCP2_10), the pigment particles seem to be stable, without visible changes, however efflorescence salts are observed in these zones, as it was also detected in the carnation areas (Annexe C1).

Table II-1. Optical microscopy observations of mortar microfragments and respective cross section from different altered areas of the painting.

	Sample identification		
	SCP1_5	**SCP2_13**	**SCP2_10**
Sample location			
Mortar microfragment			
Cross section			

After optical microscopy observation of the most altered areas, it was necessary to characterise the chemical composition of the samples, in order to knowledge the materials used and understand the alterations suffered.

Thus, through chemical analyses by SEM-EDX, the elemental composition of the pigments was obtained. Microchemistry analysis on the chromatic layers of the mortar microfragment SCP1_5 sample (Figure II-5, A-D) allowed the identification of lead (Pb), as the main element in the paint surface, which may indicate the presence of lead based compounds, fact that was confirmed by Raman analysis (section 4.1.2. Alteration products identification). It was also detected iron (Fe), calcium (Ca) and magnesium (Mg) in this sample, as it is shown in the Figure II-5.

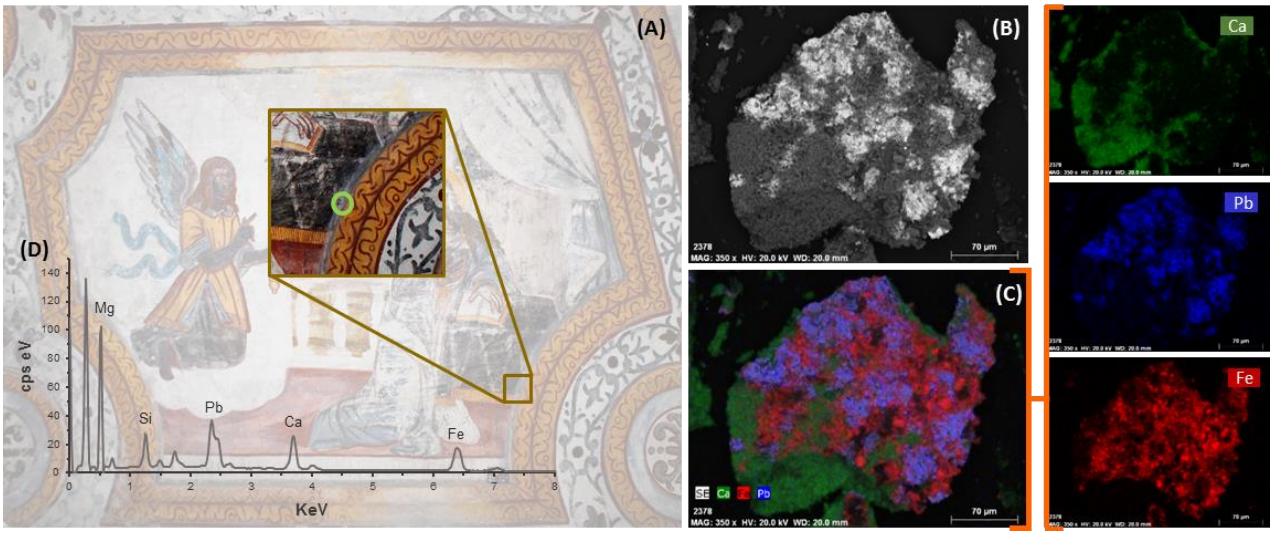


Figure II-5. Analysis of mortar microfragment SCP1_5 from Panel 1 of the Santa Clara church (A), by SEM observation in back-scattered mode (B) and EDX 2D elemental maps (C) with individual element distribution of calcium (Ca), lead (Pb) and iron (Fe) within mortar, and, EDX spectrum (D).

Other sample from a darkened area (SCP2_7) was analysed, as cross section, in order to obtain information about the chromatic layers, but also the mortar composition (Figure II-6, A-D). The elemental composition is similar to the previous one, when calcium was mainly located in the mortar layer, together with magnesium, while lead is only present in the chromatic layer, suggesting the presence of a lead compounds, as had already been observed.

The calcium (Ca) and magnesium (Mg) detected in the mortar layers, can be indicative of a dolomitic lime mortar.

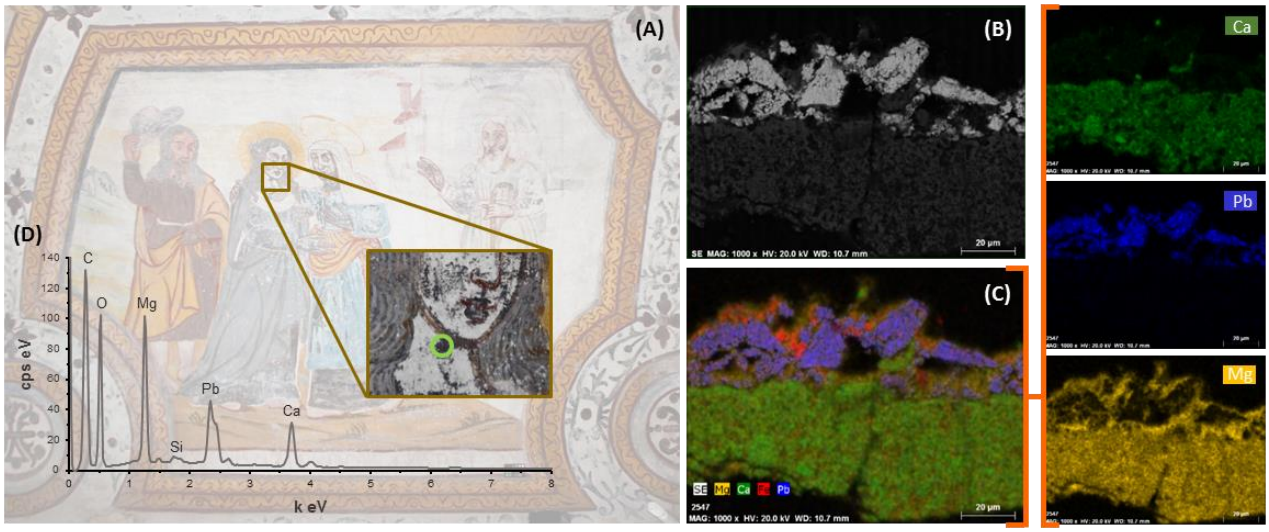


Figure II-6. Analysis of mortar microfragment SCP2_7 from Panel 2 of the *Santa Clara* church (A), by SEM observation in back-scattered mode (B) and EDX 2D elemental maps (C) with individual element distribution of calcium (Ca), lead (Pb) and magnesium (Fe) within mortar, and, EDX spectrum (D).

The presence of lead in the chromatic layers of these paintings is common in all the carnation areas analysed. This colouration, according to the results, was obtained by a mixture of red and white pigments, which can be lead based compounds. Therefore, standard lead compounds like lead white (Figure II-7 A) and red lead (Figure II-7 B) were analysed by SEM, and, the results evidence similarities in the morphological aspect of the standard grain particles with the mortar microfragment samples (Figure II-7 D-F), being possible to observe the presence of these two pigments in the paint areas (Figure II-7 E,F).

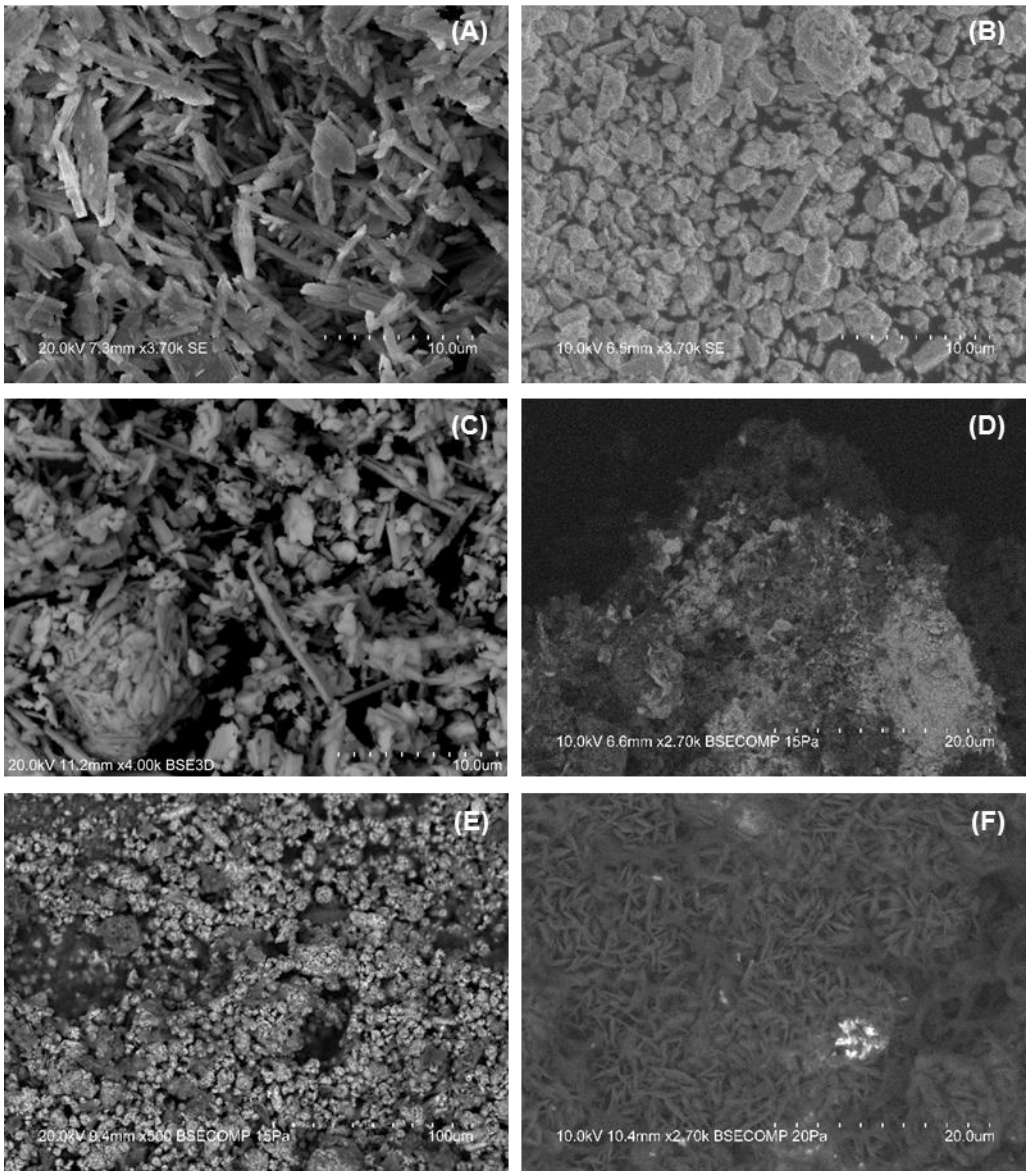


Figure II-7. SEM micrograph of lead based compounds standards like lead white (A), red lead (B) and a mixture of these two compounds (C), and, mortar microfragments from altered areas: SCP1_5 (D), SCP1_8 (E) and SCP2_7 (F).

Apart from the chromatic alterations, samples from salt efflorescence areas (SCP1_11) were also analysed, revealing in their constitution calcium (Ca), magnesium (Mg), silicon (Si) and aluminium (Al), elements that are part of the mortar layer (Figure II-8). These salt efflorescences were detected in the surface of the pictorial support, fact that can be indicative of the dissolution and surface recrystallisation of the mortars binder.

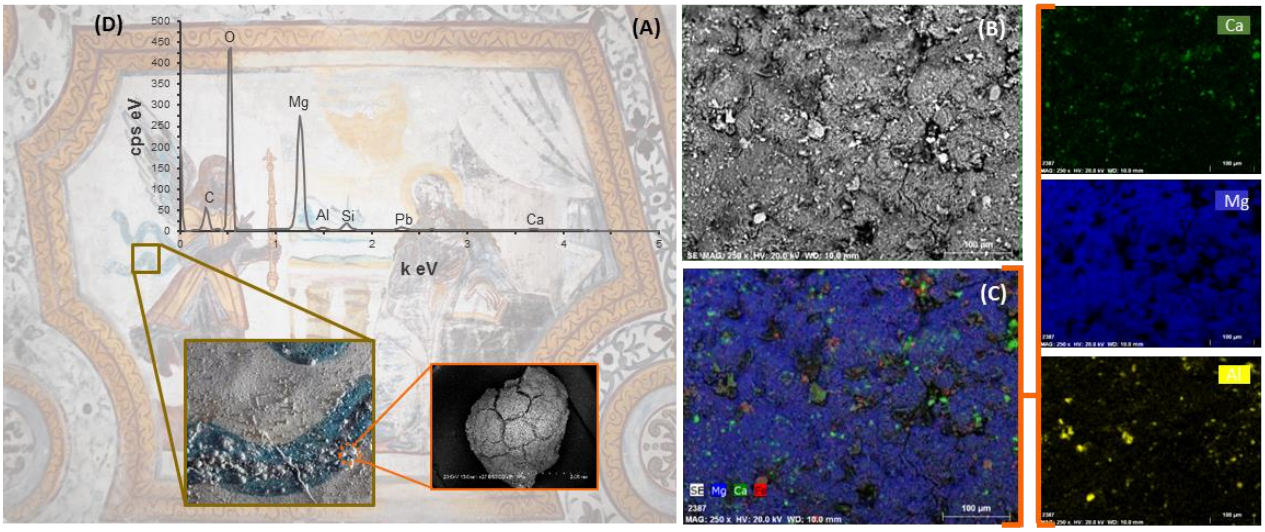


Figure II-8. Analysis of mortar microfragment SCP1_11 from Panel 1 of the *Santa Clara* church (A), by SEM observation in back-scattered mode (B) and EDX 2D elemental maps (C) with individual element distribution of calcium (Ca), magnesium (Mg), silicon (Si) and aluminium (Al) within salt efflorescence, and, EDX spectrum (D).

Salt efflorescences are frequently found in artworks with porous nature like mural paintings and stone monuments, promoting their degradation/deterioration. These porous materials are suitable for salt solution migration to the surface, where crystallisation can occur and consequent alteration of the paint layers. In this way, due to pigments sensitivity to moisture, alkalinity and air pollution, some of them, such as azurite, smalt, cinnabar, lead white and red lead can suffer this process, causing alteration on the wall paintings (Dei *et al.*, 1998; Kotulanová *et al.*, 2009b).

In accordance with the foregoing, these efflorescence salts were also detected in areas with carnations blackening, as it is possible observe in Figure II-9 by the microchemical analysis performed. Thus, in the mortar microfragment, SCP2_8 from Panel 2, it is possible to observe calcium and magnesium particles (Figure II-9B) in the surface of the chromatic layer, which is mainly composed by lead. The presence of these elements in the paint surface are probably from the mortar, which promote the efflorescence appearance, altering the paintings. Besides that, it is possible to observe several voids/empty spaces between the pigment particles (Figure II-9C), suggesting the degradation of the binder used in the painting. This fact can be promoted by microorganisms that use the organic material for their development.

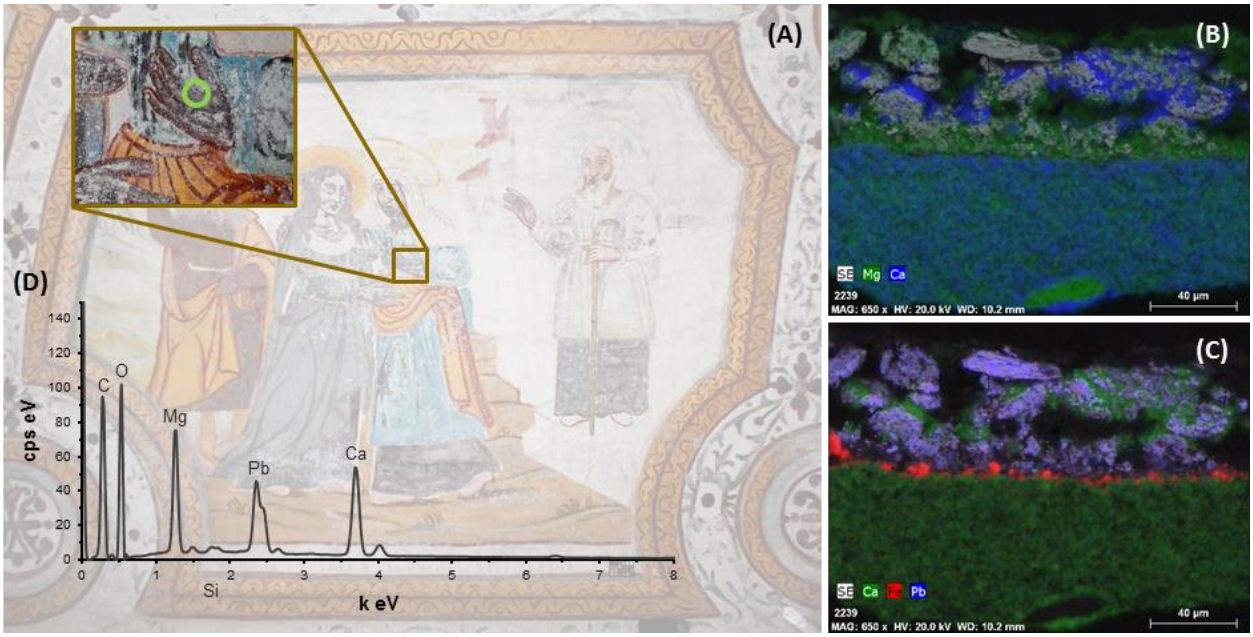


Figure II-9. Analysis of mortar microfragment SCP2_8 from Panel 2 of the *Santa Clara* church (A), by SEM observation in back-scattered mode (B, C) and, EDX spectrum (D).

4.1.2. Alteration products identification

In order to identify possible alteration products, a Raman comparative study of carnation areas with and without alterations due to black spots was performed.

Through Raman analysis of the microfragments it was possible to identify, in areas without alteration, the presence of bands characteristics of lead compounds like lead white (288, 356, 369, 435, 607, 961, 1047, 1088 and 1126 cm^{-1}) and red lead (117, 149, 227, 316, 392, 485 and 546 cm^{-1}), by the comparison of the standard pigments analysed (Figure II-10, A-B), whose combined use allows the flesh tones tonality, as previously reported.

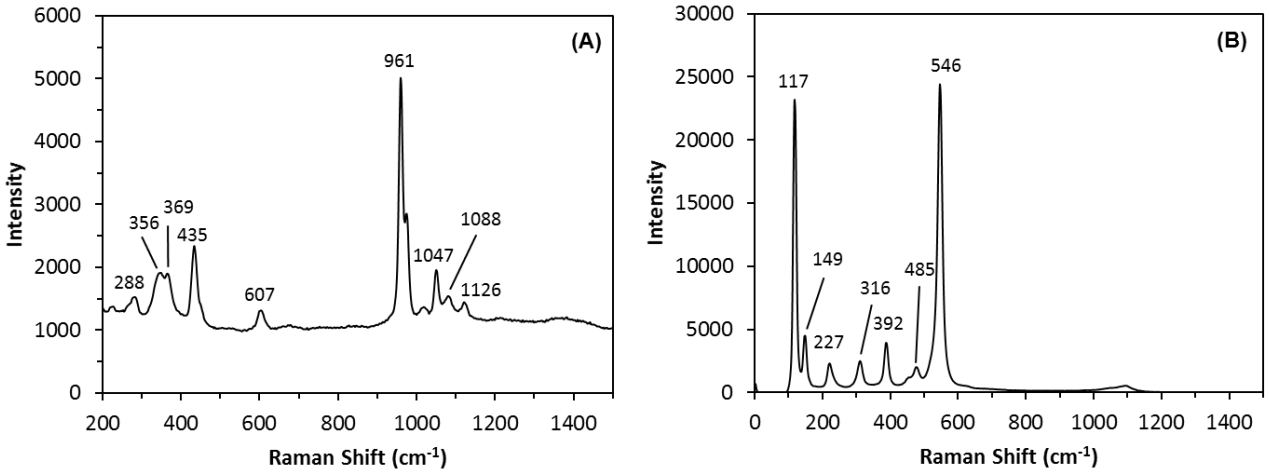


Figure II-10. Raman spectra of lead white (A) and red lead (B) standards.

In microfragments from chromatic layers in the flesh tones/carnations that suffer darkening process were detected characteristic bands of *plattnerite* (PbO_2) by the presence of 405, 523 and 676 cm^{-1} bands (Figure II-11A-C). The formation of this compound could be associated to lead based pigments oxidation, like lead white and red lead. The presence of these compounds in mortars was confirmed due to the detection of some Raman signature of these pigments (Figure II-11).

In addition, in the black spots that affect a broad area of the paintings, specifically in the carnation zones, were identified characteristic signals of *plattnerite*. This alteration product was detected in all the microfragments analysed, evidencing that the presence of this compound is responsible for the darkening process in the flesh tones of the painting. In fact, PbO_2 resulting from the oxidation of lead pigments, wherein the alteration of lead oxidation state of Pb^{2+} to Pb^{4+} is already related to promote chromatic alteration of these pigments (Qingping *et al.*, 1999; Aze *et al.*, 2006; Kotulanová *et al.*, 2009b). In *Santa Clara* Church the oxidation of lead based compounds affect a broad area of the paintings, causing a pronounced darkening on the carnations in the majority of the figures represented.

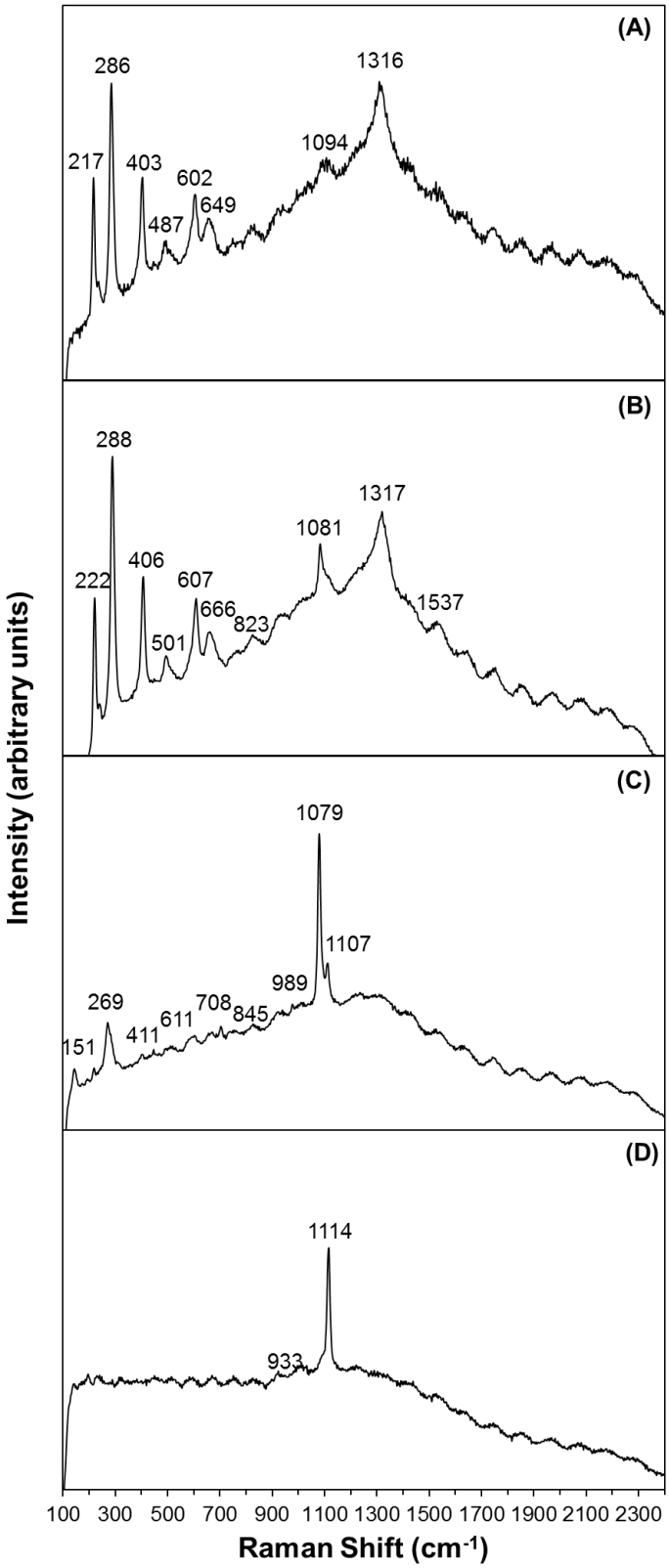


Figure II-11. Raman analyses of several altered areas of *Santa Clara* Church affected by darkening process SCP1_9 (A), SCP2_13 (B), SCP2_1 (C) and with salt efflorescences formation SCP1_11 (D).

In addition, Raman analysis of the darkened carnation show also characteristic bands of organic material like carbohydrates and proteins (1319 and 1554 cm^{-1}, C-H, C-C and C=O stretching vibrations) (Rampazzi, 2004; Jehlicka *et al.*, 2007; Jehlicka *et al.*, 2010; Edwards *et al.*, 2011), which is compatible with microbial contaminations (Figure II-11A, B).

Areas with salts efflorescence formation were also analysed by Raman spectroscopy (Figure II-11D), and the results suggest the presence of *anhydrite* (calcium sulphate - $CaSO_4$). This salt may have been formed due to the available calcium present in the mortars through the reaction of calcium carbonate with atmospheric sulphur dioxide (SO_2) (Prasad *et al.*, 2001).

4.1.3. Microbiological contamination assessment

Conversion of lead based compounds into black mineral *plattnerite* is normally attributed to the exposure to an alkaline environment or warm situations. According to some authors, fungi are able to dissolve Pb_3O_4, causing the accumulation of Pb^{2+} ions and leading the subsequent recrystallization of Pb^{4+} ions into *plattnerite* (Fomina *et al*., 2005*)*.

To evaluate the microbial proliferation, mortar microfragments were analysed by scanning electron microscopy. The micrographs obtained, revealed the presence of microbial contamination in all the areas analysed (Figure II-12) in opposition to areas without alteration signs, where the presence of microorganisms was nearly absent. In the case of angel arm darkening (SCP1_10), a micellar structure of filamentous fungi was observed, covering all the microfragment surface (Figure II-12 SCP1_10, A-D). These microorganisms were also observed in the black spots of the angel face (SCP2_1), however in a lower extension (Figure II-12, SCP2_1, A-D) than in the SCP1_10 sample. The angel hand (SCP2_13) was also affected by the darkening process and in this area it is possible to observe filamentous fungi proliferation (Figure II-12, SCP2_13, C) but also yeast cells covering all the sample surface (Figure II-12, SCP2_13, D). Microbial contamination was also identified in the areas with salts efflorescence formation (SCP1_11), corresponding to an high fungal proliferation (Figure II-12, SCP1-11, C-D).

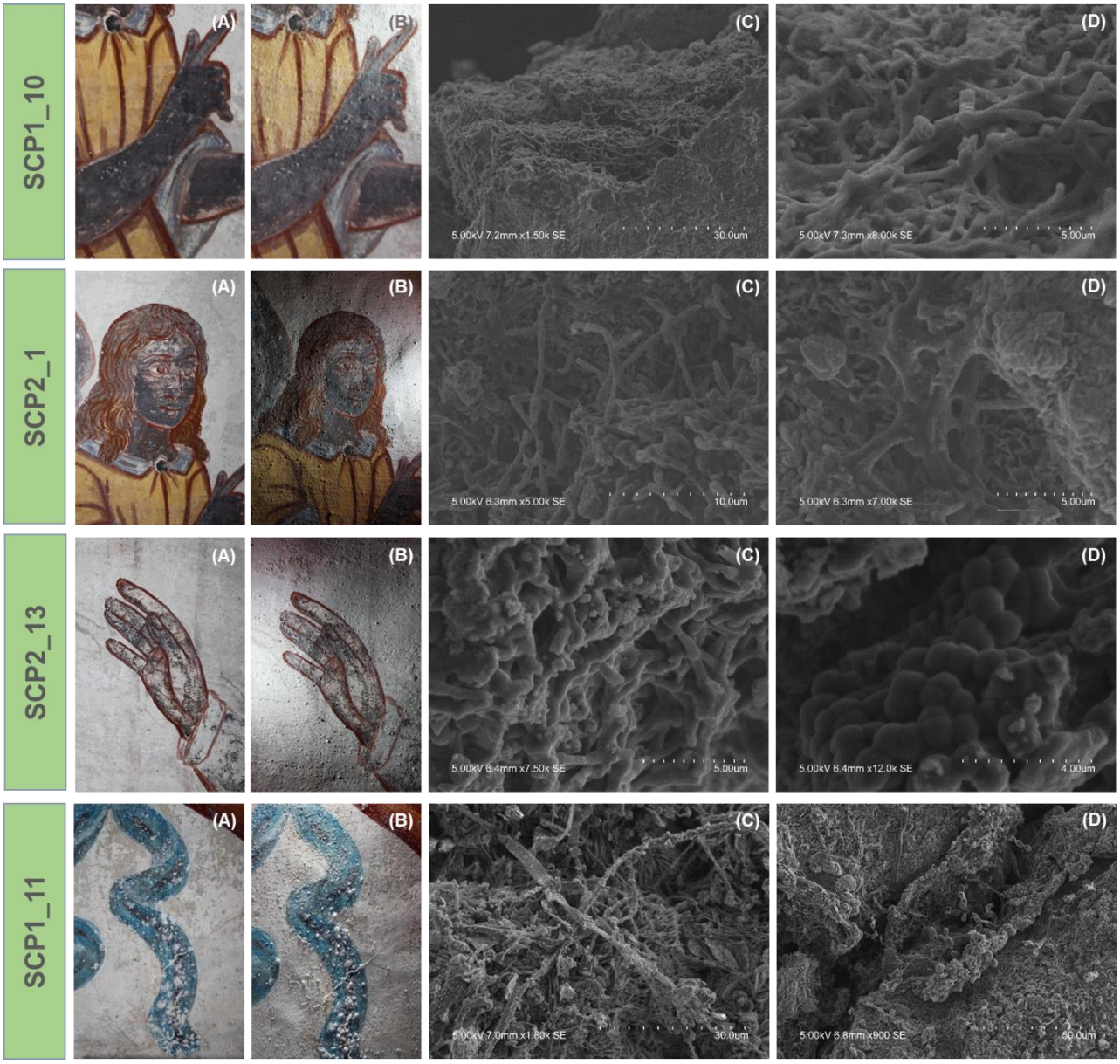


Figure II-12. Analysis of several altered areas from Santa Clara Church, focusing on darkening areas and salt efflorescence formation, evidencing details of the areas with alteration by: photographs captured under frontal (A) and raking (B) light, and, SEM micrographs in secondary electron mode (C, D).

Thus, this biological contamination seems to contribute for the alterations observed in the *Santa Clara* Church, whose high contamination level were found in cracked areas, pigments oxidation and materials crystallisation.

In this way, to complement these results, culture-dependent methods were applied in order to characterise the cultivable microbial population that colonise these paintings.

The results show that the cultivable microorganisms thriving in the *Santa Clara* mural paintings are essentially filamentous fungi of the genera *Aspergillus*, *Cladosporium* and *Penicillium*, but were also isolated unclassified fungi, and several unidentified bacterial strains.

The areas that suffer chromatic alterations, due to darkening process, show high contamination levels, particularly by fungi of the genus *Penicillium*. The microorganisms identified in this case study are widely found in other mural paintings, whose incidence was detected in areas with significant alterations (Rosado *et al.*, 2013a). These microorganisms seem to be related with the chromatic alteration detected, probably induced by their metabolic activity and excretion products (Qingping *et al.*, 1999; Aze *et al.*, 2008). At this moment it is not possible to identify the main parameter that promote this degradation process, however results obtained in this study, clearly show that microbial contamination give an important contribution to lead based compounds oxidation.

To try to get a deeper insight on this process, simulation assays are in progress, in order to understand the role of the microorganisms on the pigments oxidation. For this purpose and to mimic the paintings of *Santa Clara* Church, several mortar specimens, painted with lead white and red lead, were inoculated with the different microorganisms isolated and natural aging is in course.

4.2. Green pigments alteration - *Nossa Senhora da Saudação* Convent

One of the chromatic alterations that affect mural paintings is the degradation of copper green pigments. In the Low Choir of the *Nossa Senhora da Saudação* Convent, the painting areas with these pigments were altered by the appearance of a brown veil, which cover the surfaces promoting in several cases the alteration of a broad area of the mural paintings.

The Low Choir of the *Nossa Senhora da Saudação* Convent has a mural paintings with a good artistic quality, dating from the seventeenth century that needs an urgent attention to preserve them. In this way, these paintings were studied and the main problems that causes the chromatic alteration were identified.

In Figure II-13 it is possible to observe the sampling scheme used for this study.

Figure II-13. Sampling location on green, brown and golden areas of the panels 2, 4, 5, 8, 13, and from Left and Right wall (□ - samples collected with sterile cotton swabs; △ - microfragments collected with sterile scalpels and microtubes) present in the Low Choir of the Convent of *Nossa Senhora da Saudação* Church, Montemor-o-Novo, Portugal).

Several samples were collected in green, brown and golden areas, with visible alterations signs and different approaches were performed in order to characterise these alterations.

4.2.1. Microbial community identification

Mortar microfragments from altered painted areas were analysed by SEM, allowing the observation and identification of the presence of microbial communities thriving in the paintings. This technique provides an image of high magnification and resolution, and, hence infer about the existence of contamination, by direct observation. In the collected mortar microfragments were observed (Figure II-14) filamentous fungi and hyphae proliferation within the structure of the mortar, which may explain the presence of some cracks in the paint. The image obtained by SEM allows also the observation of fungal hyphae and micellar structures of filamentous fungi penetrating the microstructure of the mortars, therefore promoting the proliferation of these microorganisms in depth.

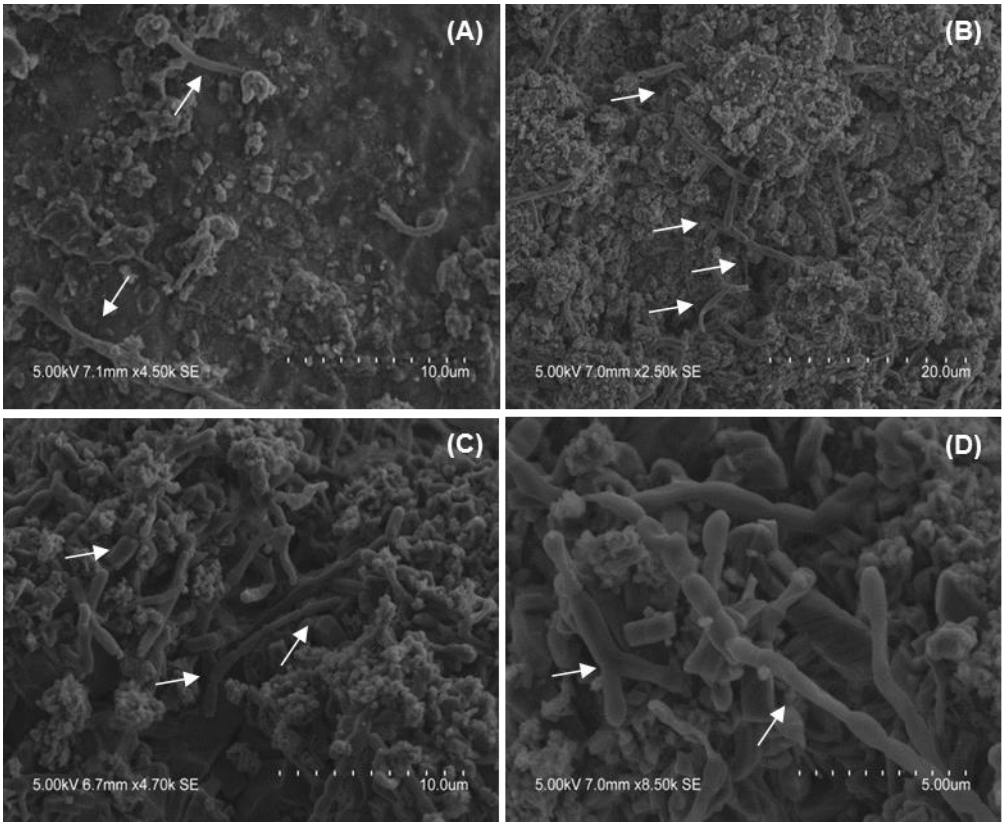


Figure II-14. SEM micrograph of mortar microfragments. The arrows indicate the presence of filamentous fungi and hyphae proliferation by the surface of the mortar (A, B) and the penetration of these microorganisms in depth (C, D).

To evaluate the microbial population present in the paintings, an *in vitro* growth assay was envisaged, using different culture media. The characterisation of the isolated microorganisms was performed according to macroscopic (colour, size and morphology) and microscopic features (type of reproductive structures and colour of colonies in the case of bacteria) and in some cases by DNA sequencing. Although this approach does not enable a full characterisation of the microbial community, since some microorganisms do not have the capacity to grow under *in vitro* conditions, however, it allows to obtain isolated microorganisms, required for other assays, namely for simulation of biodegradation process with high density of cells.

The results showed that the microorganisms growing on these paintings are bacteria, filamentous fungi and yeasts. With this study it was possible to isolate seventeen strains of fungi and, based on molecular approaches attribute genera *Cladosporium* (six isolates), *Penicillium* (one isolate) and *Nectria* (one isolate). On the other hand, the bacterial population isolated is mainly composed by bacilli Gram-positive, being possible to assign the identification of three different *Bacillus* sp. strains.

Left Wall and Panel 2 (Figure II-13) correspond to the zones of the painting where the isolation procedure allowed a greater fungal diversity. Panel 13 (Figure II-13) showed a high bacterial number of isolated strains. Microbial colonisation may be one of the factors contributing to chromatic alterations observed in the painting, namely surface veils and colour alteration of original green areas. Several studies indicate that microorganisms are the main responsible for the biofilms formation on the paintings, promoting alterations in the pigments (Nugari *et al.*, 2009; Zammit *et al.*, 2011).

4.2.2. Green areas alteration

4.2.2.1. Evidence of alterations by oxalates formation

Pigment analysis of green areas, by Raman spectroscopy, have allowed to identify the main green pigment used as malachite, as is shown in the figure II-15 and according to standard analysis.

Mortar microfragments from altered green areas were also analysed by Raman spectroscopy allowing the detection of oxalate compounds.

The Raman spectra present in the figure II-15 show the presence of calcium oxalates in the analysed samples. Different forms of calcium oxalates can be observed. These oxalates can

occur as *whewellite* ($CaC_2O_4.H_2O$, calcium oxalate monohydrate) and *weddellite* ($CaC_2O_4.2H_2O$, calcium oxalate dihydrate), the latter being less stable (Cariati *et al.*, 2000; Hernanz *et al.*, 2007).

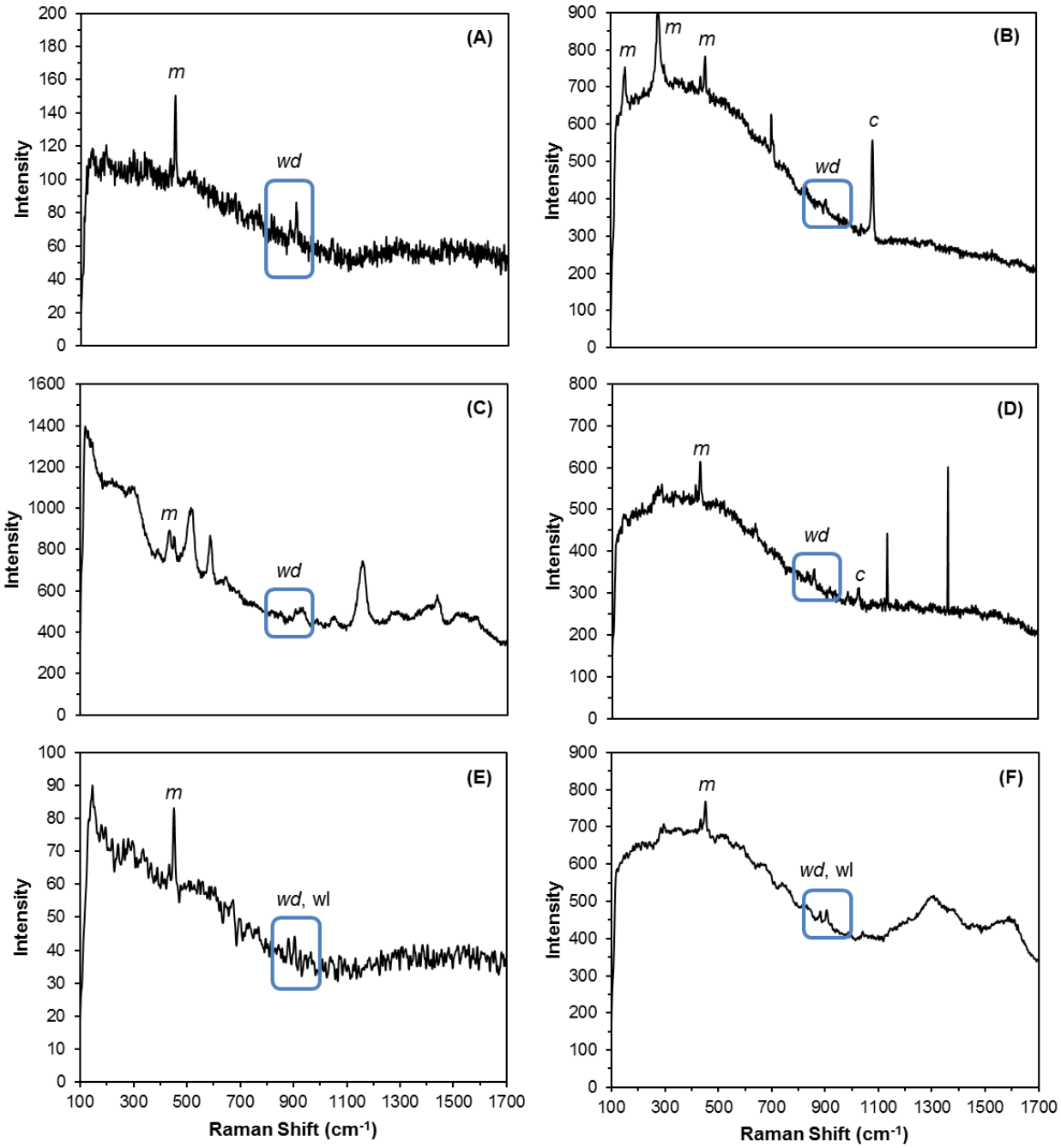


Figure II-15. Raman spectra of mortar microfragments (A-F) collected in the Low Choir of the Convent of *Nossa Senhora da Saudação* Church. The oxalates bands are evidenced in the spectra. Peaks of malachite pigment (*m*), oxalate compounds like *weddellite* (*wd*) and *whewellite* (*wl*), and calcite (*c*) are evidenced in the spectra.

Raman spectra of the several samples analysed (Figure II-15, A-F) allows the detection of a band around 900 cm^{-1}, characteristic of calcium oxalate compounds. The presence of the 455 cm^{-1}, 909 cm^{-1} (Figure II-15, A-F) and 1440 cm^{-1} (Figure II-15,D) bands are characteristic of *weddellite*, however, in Figure II-15 C it is also present a band at 945 cm^{-1}, characteristic of *whewellite* (Pérez-Alonso *et al.*, 2004; Villar *et al.*, 2004). In all the analysed samples it is observed a peak at 1080 cm^{-1}, characteristic of calcite ($CaCO_3$) (Daniilia *et al.*, 2008), the mortars binder. This compound may be an available source of calcium, which react with oxalic acid produced by metabolic activity of bacteria and fungi, forming the calcium oxalates compounds founded in these paintings.

The results obtained in the green zones allowed to detect the presence of calcium oxalates, associated to high levels of microbial contamination, factors that seem to be responsible for the alteration on the green pigments (malachite) that compose the mural paintings present in the Low Choir of the *Nossa Senhora da Saudação* Convent. Thus, the results suggest that the chromatic changes in the paint layers can be due to the presence of calcium oxalates. Several studies have reported the occurrence of *weddellite* in degraded areas of mural paintings (Pérez-Alonso *et al.*, 2004; Nevin *et al.*, 2008; Sarmiento *et al.*, 2008) due to the metabolic activity of the microorganisms, which secrete oxalic acid that reacts with calcium compounds present on the surface (Sarmiento *et al.*, 2008).

4.2.2.2. Analysis of oxalates in simulated assays

The influence of microorganisms metabolic activity on the green areas alteration was studied using:

- microsamples removed from degraded areas of the painting which retain the presence of total microbial communities, and,
- simulation assays performed with real sterilised mortar microsamples, inoculated with high cells density of pure and mixed cultures, isolated from the painting,

that were performed as mentioned in the section 3.6 of the Experimental Section.

These cultures were analysed by Raman spectroscopy to detect the presence of oxalates in the metabolic pool. The results indicate the presence of oxalates in all bacterial cultures. Figure II-16 shows an example of a Raman spectrum of the metabolic pool, from mixed bacterial cultures from Panel 13, where it was also found oxalate compounds. It was possible to detect the presence of *whewellit*e (885 cm^{-1}, 1464 cm^{-1} and 1490 cm^{-1}) and *weddellite* (455 cm^{-1}, 908 cm^{-1} and 1464 cm^{-1}).

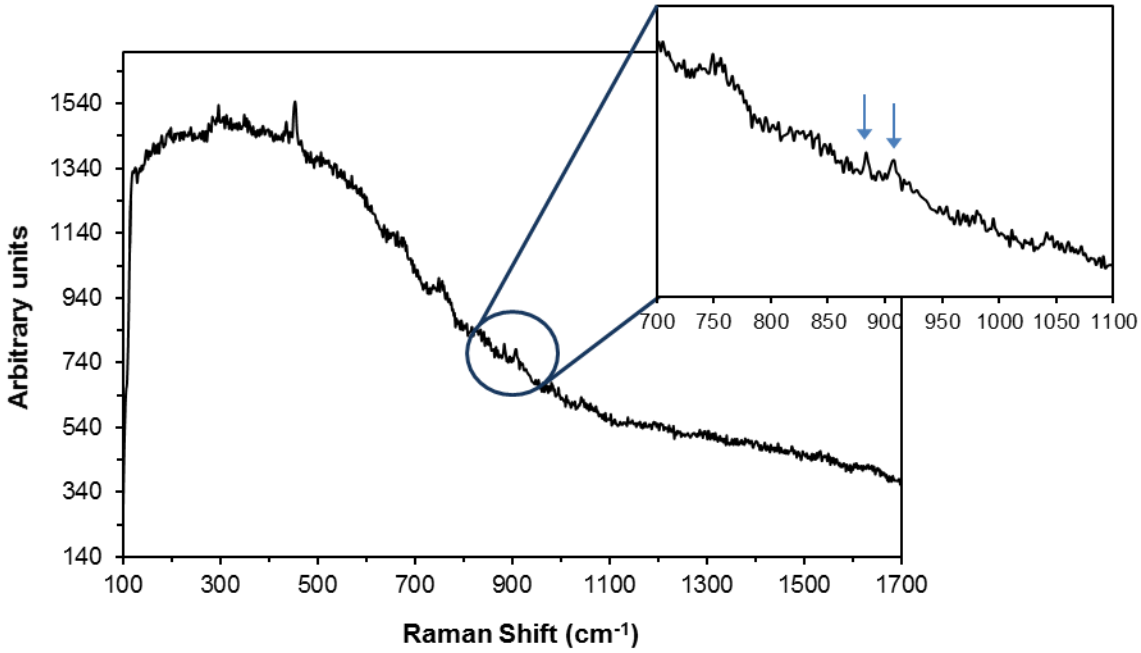


Figure II-16. Raman spectrum of bacteria mixed cultures, coming from Panel 13, to simulate oxalates production.

This is consistent with the results obtained in the mortar microfragments analyses, where these oxalates were firstly detected.

To complement the results, simulation assays were performed. Sterile mortar microfragments were inoculated with the bacterial pool from Panel 13, as mentioned above, incubated and analysed by scanning electron microscopy.

Figure II-17B shows a SEM micrograph of a mortar microfragment submitted to a mixed culture of bacteria taken from Panel 13, where it is evident a biofilm formation involving all the surface of the mortar, indicating the potential proliferation of the bacteria. The results clearly show the influence of the microorganisms, namely bacterial communities, in the colour alteration, due the formation oxalate biofilms. Simulated assays in mortar microfragments, with mixed culture of fungi (Figure II-17D) obtained from the Left Wall were also performed in order to clarify the fungal impact in the mural painting biodeterioration.

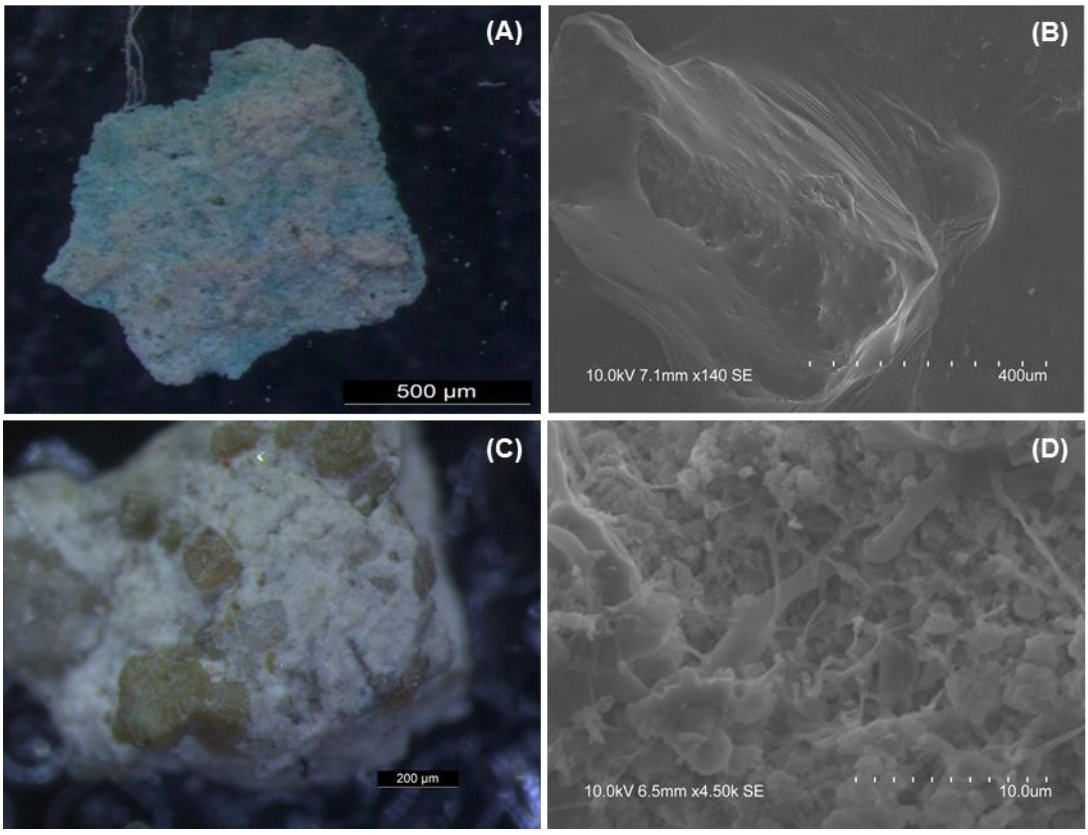


Figure II-17. Magnifying glass observation of a mortar microfragment (A, C) and SEM micrograph of biofilm formation by bacteria taken from Panel 13 on the sterilised mortar microfragment (B), and, hyphae proliferation of fungi taken from Left wall (D), after *in-vitro* growth.

These assays allow to infer that these microorganisms have the capacity to proliferate within the mortar microfragments. Furthermore, observation of real mortar samples, show that the proliferation of these microorganisms seem to be correlated with cracks observed and the detachments of some areas of the painting, not only on the Left Wall but also on all panels of the Low Choir of the *Nossa Senhora da Saudação* Convent. Thus, fungal proliferation appears associated to mortar structural damages, whereas bacteria development is a determining factor in the chromatic alterations.

All the panels analysed have biological contamination, either by bacteria or filamentous fungi proliferation, which seems to be associated with chromatic alterations, development of biofilms (particularly due to bacterial contamination), cracking (chiefly promoting by fungal contamination) and detachment of some areas of the painting.

4.3. Mortar discolouration - Évora Cathedral

Évora Cathedral is one of the most emblematic monuments of Évora that has suffered some chromatic alterations in the inner walls, acquiring pink/dark orange stains, covering a broad area of the mortars. Several studies were performed in order to characterise the mortars composition (Adriano *et al.*, 2009; Silva *et al.*, 2010), however the analyses were unable to detect any pigment and hence to explain the pink colour that covers the majority of the inner walls surface. Thus, in order to identify the phenomena that promote the pink/orange stains appearance in the mortars of the Évora Cathedral inner walls, a multidisciplinary approach was adopted.

The first step of this study was the samples collection, whose sampling locations are represented in the Figure II-18.

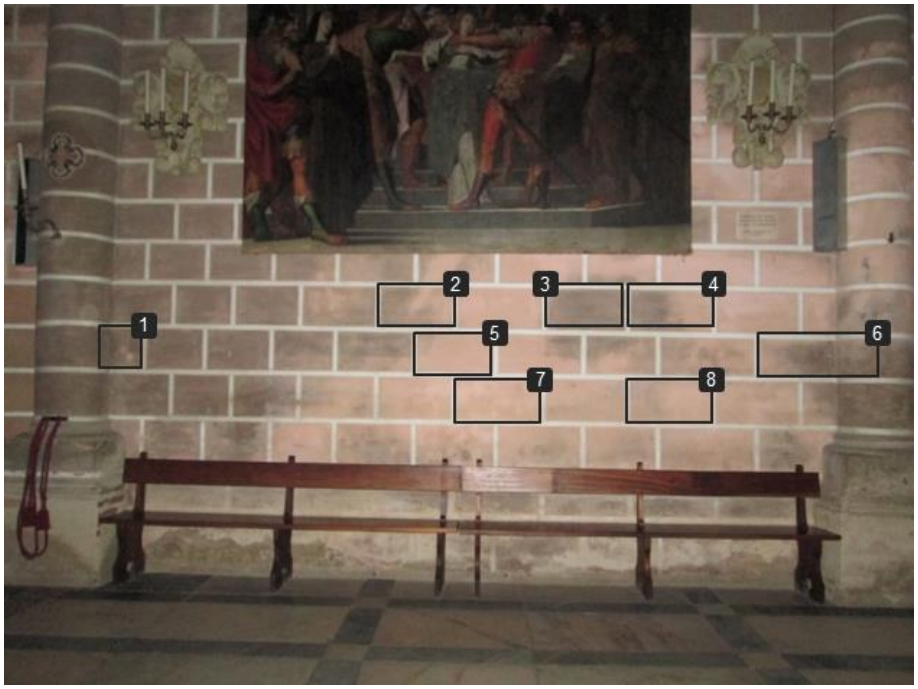


Figure II-18. Sampling location in the inner wall of the Évora Cathedral with pronounced signs of alteration: pink/orange spots covering the surface.

In this process, several samples were collected in order to allow the characterisation of the materials used, investigate the presence microbiological agents and understand the alterations that promote mortars discolouration.

4.3.1. Material characterisation

The chemical analyses performed in mortars from inner walls of Évora Cathedral do not show pigment or an inorganic chromophore in their composition. Elemental point spectra and two-dimensional (2D) elemental mapping by SEM-EDX (Figure II-19) showed the association of aluminium (Al), calcium (Ca), potassium (K), iron (Fe) and silicon (Si) in the mortars composition, suggesting a dolomitic aerial lime mortars with siliceous aggregates. Each chemical element micrograph (Figure II-19 Al, Ca and Si) shows the distribution of each of them in the mortar surface. Additionally, the support microstructure shows porosity and particle size (Figure II-19A,B) characteristic of this type of materials.

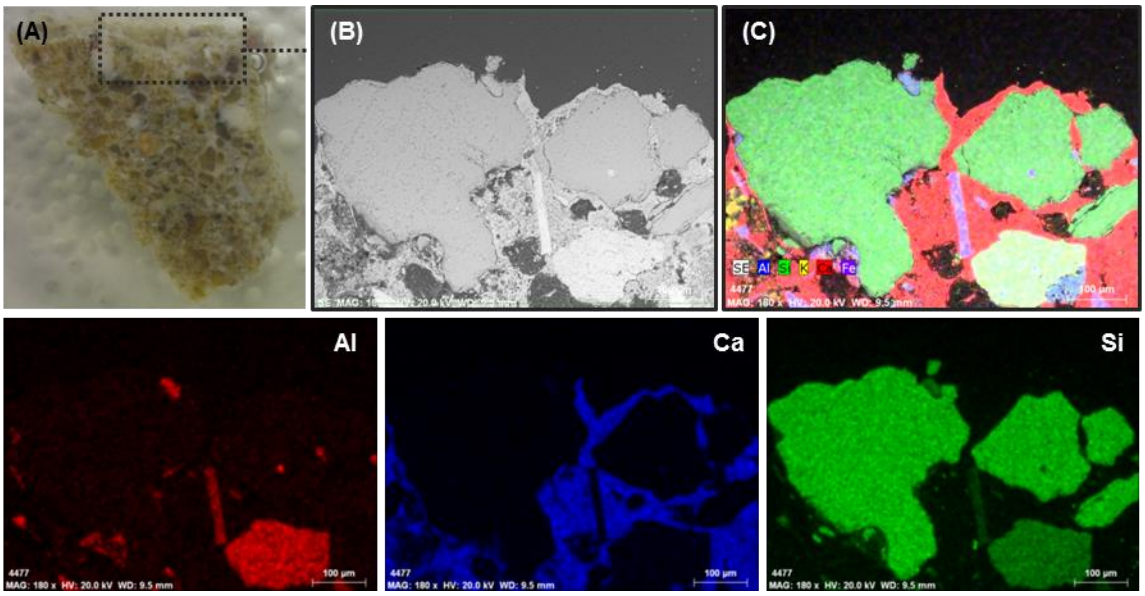


Figure II-19. Analysis of cross section by stereozoom observation (A), SEM micrograph in back-scattered mode (B), EDX 2D map (C) and elemental map distribution of aluminium (Al), calcium (Ca) and silicon (Si) in mortar microfragment from Évora Cathedral inner walls.

These results confirm that the pink colour observed in the inner walls of Évora Cathedral is not due to inorganic pigment presence, as has already been suspected and indicated in previous work (Adriano *et al.*, 2009; Silva *et al.*, 2010). Therefore, to understand the reasons that led to the appearance of these stains the present work envisaged a study of the possible biological nature of this phenomenon.

Pigment formation may be a consequence of microorganisms metabolic activity. Many fungi produce organic pigments of different colours (green, grey, blue, purple, pink, violet, orange, and others), belonging to different classes of compounds like anthraquinones, xanthones or

carotenes, and are characteristic of different species, but the colour of the stain depends not only upon the chemical composition of the pigment but also on the other factors like the composition of painting constituents, presence of other microbial species or environmental conditions. Thus, pigments release on the material support or the presence of microorganisms containing pigments, can cause the appearance of different coloured stains or patches on mural paintings (Garg *et al.*, 1995).

4.3.2. Microbial diversity characterisation

The microfragments collected in the pink stained inner walls of the Cathedral, analysed by SEM, presented strong signs of microbiological contamination by yeast, bacteria and filamentous fungi (Figure II-20A-C). Yeast contamination forms a biofilm on the surface of the mortar totally covering the fragment analysed (Figure II-20A). In the case of fungal contamination it is possible to observe the proliferation of micellar structures upon/over the mortar surface (Figure II-20C).

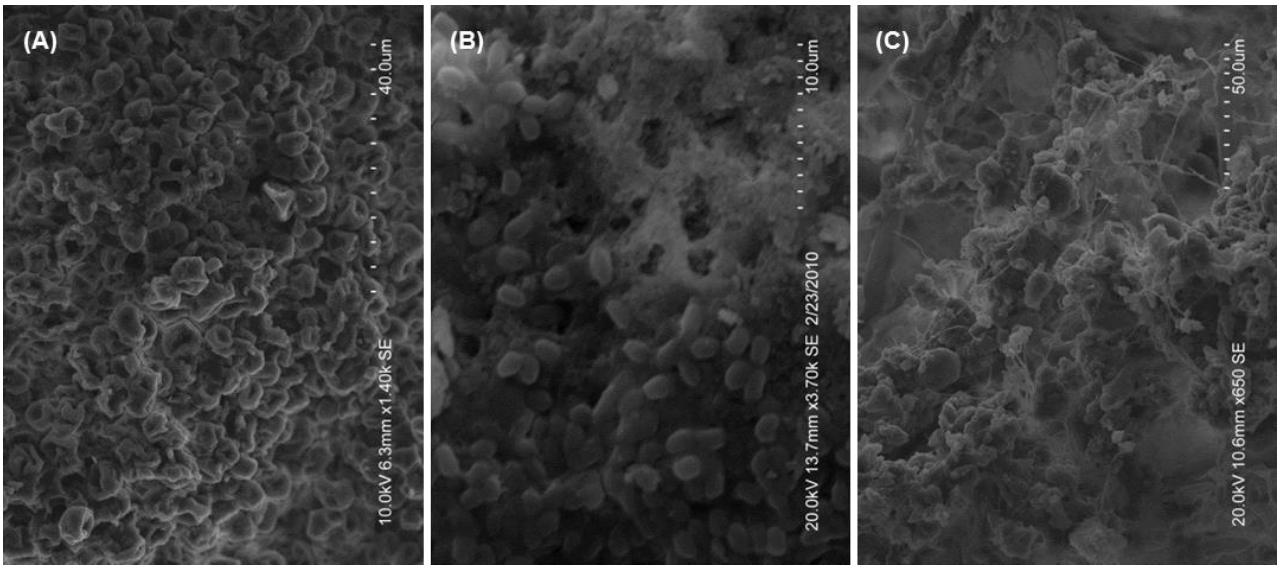


Figure II-20. SEM micrographs of mortar microsamples. Cluster of yeast (A) on the wall surface, bacteria (B) and filamentous fungi (C) proliferating in the inner wall of the Évora Cathedral.

Once detected biological contamination in the samples, solid culture-dependent methods were applied, being possible to isolate several bacterial strains (e.g. Gram-positive cocci/bacilli), 3 yeast strains in particular one of the genera *Rhodotorula* and filamentous fungi, 5 strains of the genera *Penicillium*, one strain of the genera *Cladosporium,* mycelium and also sterile mycelia.

Particularly relevant was the fact that, the predominant isolated microorganism, *Rhodotorula* sp. yeast, exhibited a strong pink/dark orange colour. Given these results, liquid medium cultures were carried out with microfragments from pink zones. These assays allowed us to obtain high cell densities revealing a potential proliferative capacity of microorganisms present in the walls of the Évora Cathedral (Figure II-21A). In addition, a pink colour is visible in the cultures which appears to be indicative of the presence of microorganisms able to produce coloured compounds (Figure II-21B).

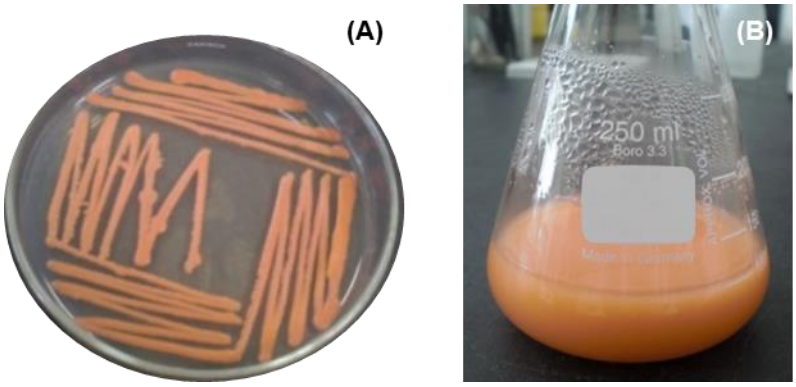


Figure II-21. Solid (A) and liquid (B) cultures of *Rhodotorula* yeast isolated from the inner wall of the Évora Cathedral with pink stains.

These results suggest that the presence of the *Rhodotorula* yeast may be one of the causes of the pink stains on the walls of the Cathedral, an interesting result, once till now, this microorganisms was not yet considered in this kind of deterioration. In the literature the chief microorganisms associated to this alteration are phototropic bacteria and algae (Ariño and Saiz-Jimenez, 1996; Urzì and Realini, 1998; Cappitelli *et al.*, 2009; Tran *et al.*, 2012 ; Kusumi *et al.*, 2013; Ortega-Morales *et al.*, 2013), however the results obtained in this work suggest *Rhodotorula* as a biodeterioration agent, nevertheless it is important to be aware that all biodeterioration processes are probably the result of complex microbial interactions.

4.3.3. Analytical approaches to identify products alteration

Besides the biological approach that allowed the knowledge of the Cathedral colonisers, an analytical study was also performed to characterise the chromatic and microstructural alterations observed in the walls.

Raman spectroscopy analyses allowed the detection of carotenoid bands in the isolated yeast, *Rhodototula* sp., and in the microsamples collected. Figure II-22 presents Raman spectra of β-carotene standard (Figure II-22A), with three intense bands at 1008, 1154 and 1514 cm^{-1}, and, of *Rhodotorula* yeast and Évora Cathedral mortar sample (Figure II-22B) where 1154 and 1514 cm^{-1} bands (Baranska *et al.*, 2006; Lin *et al.*, 2007; Vítek *et al.*, 2009) are visible.

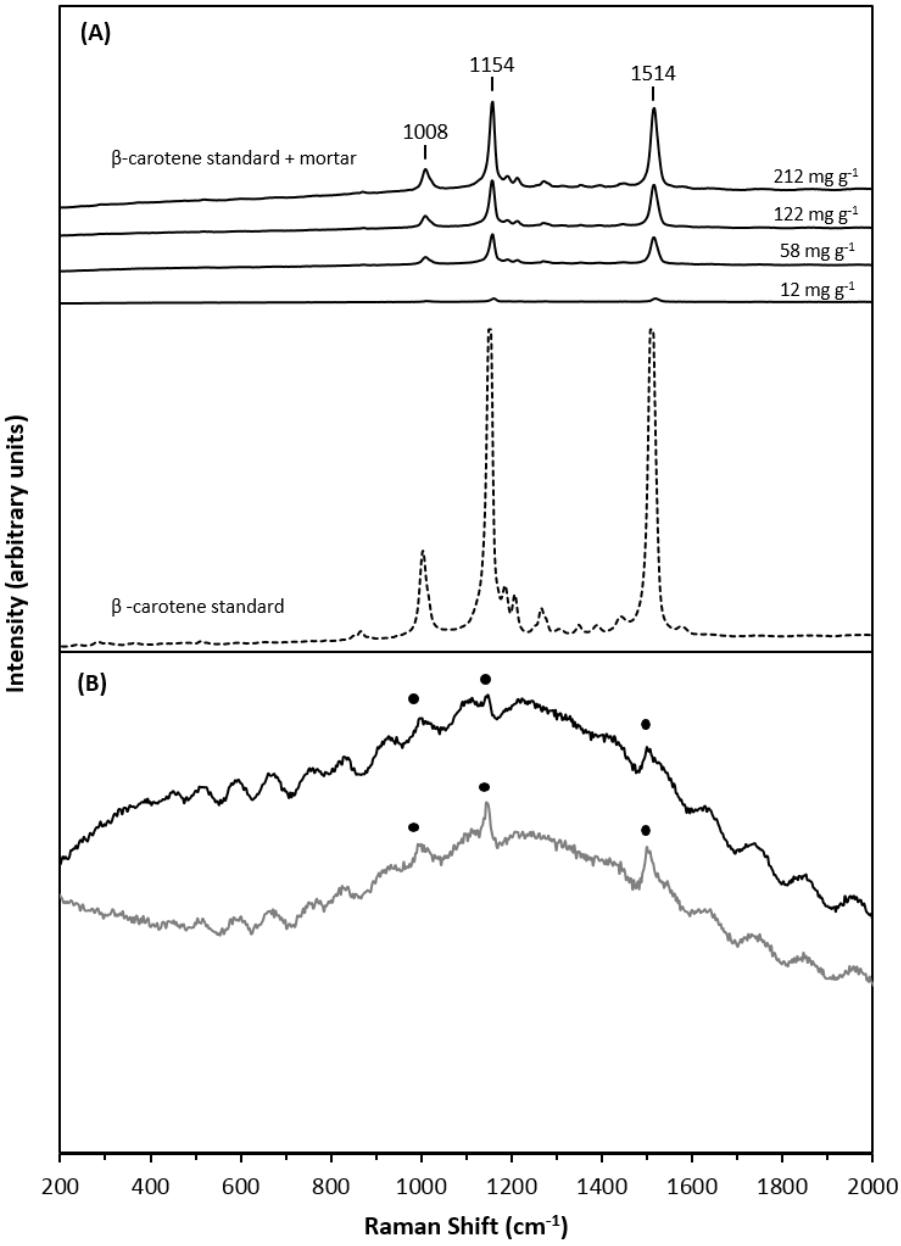


Figure II-22. Raman spectra of (A) β-carotene standard and mixtures of β-carotene/*Évora* Cathedral sterilised mortar at different concentrations (12, 58, 122 and 212 mg of β-caroten/g of sterilised mortar); (B) microsamples collected in the inner wall of the *Évora* Cathedral with pink stains (dark grey) and *Rhodotorula* yeast (light grey) isolated from the same place. Carotenoids bands are evidenced in the spectra.

Raman spectra of carotenoid compounds are typically dominated by two Raman bands at 1150 cm^{-1} and at 1500 cm^{-1}, attributed to the in phase ν(C-C) and ν(C=C) stretching vibrations, respectively (Merlin, 1985; Agalidis *et al.*, 1999). Due to resonance enhancement, these Raman bands are very intense when irradiated with a green 532 nm laser. Raman spectroscopy was used to study polyenes and carotenoids in different biological matrices, such as microorganisms, fruits and feathers (Veronelli *et al.*, 1995; Silva *et al.*, 2008; Abdel-Haliem *et al.*, 2013; Jehlička *et al.*, 2013; Jehlička and Oren, 2013; Kusumi *et al.*, 2013). When recording Raman spectra of pure molecules, the exact band position can be related to the length of the polyene chain (Brambilla *et al.*, 2012). However, it has been well demonstrated by Oliveira et al. (de Oliveira *et al.*, 2009) that the band positions may shift depending on the molecular environment: interactions with other molecules in the matrix or with other carotenoids present may cause small shifts. Moreover, polar and non-polar interactions affect the conformation of the polyene chain, hence wavenumber shifts are observed. Therefore, it is difficult to provide an exact identification of the carotenoid molecules that are present inside the microorganism (Jehlička and Oren, 2013).

Raman microspectroscopy revealed to be a non-destructive tool for the identification of carotenoids and was successfully applied for their detection in real and biological samples, without any preliminary preparation. The results obtained are indicative the presence of this compound in the inner walls of the Cathedral, and therefore the main chromophore responsible for the alterations detected. In fact Raman analyses reliably identifies carotenoids on paintings but cannot pin-point the producing species, because the same pigment is produced by more than one specie. Thus, the combination of culture-dependent methods and Raman microspectroscopy allows the association of the microbial community isolated with the pigment identification.

Carotenoids provide the strongest bands in the spectrum but, although not well-resolved and very weak, calcium oxalate compounds (bands at 915 and 1460 cm^{-1}) also seem to be present (Urzì and Realini, 1998; Edwards *et al.*, 2003; Villar *et al.*, 2004; Ortega-Morales *et al.*, 2005; Villar *et al.*, 2005; Nevin *et al.*, 2008; Rosado *et al.*, 2013a). The production of calcium oxalate compounds can be indicative for the biodeteriorative ability of the microorganisms in accumulating calcium ions from the substratum or from the environment.

Raman spectroscopy has proven to be a powerful tool for the characterisation of several biomarkers which are produced by microbial colonies in extreme habitats as part of their survival strategy (Imperi *et al.*, 2007b; Vítek *et al.*, 2009). This technique was successfully applied in this study and can to provide the causal link between yeast production of carotenoid pigments and colour alteration of mortars.

On the other hand, mixtures of *β*-carotene with Évora Cathedral mortar, in different concentrations (12, 58, 122 and 212 mg g^{-1}), were analysed by Raman spectroscopy in order to validate the application of this methodology to *β*-carotene identification within mortar grains. The results obtained revealed that Raman spectroscopy is useful for the detection of carotenoids compounds, even in low concentrations (Figure II-22A). The analytical potential of this technique for the identification of *β*-carotene in complex samples was confirmed, as has been previously presented by other authors (Goodwin *et al.*, 2006; Imperi *et al.*, 2007b; Vítek *et al.*, 2009; Kusumi *et al.*, 2013).

Additionally to the Raman analyses, we have conducted FTIR-ATR measurements with the intention to complement the experiments. FTIR-ATR spectra (Figure II-23) revealed characteristic bands of carotenoids in the isolated yeast and in the samples from pink stained sites.

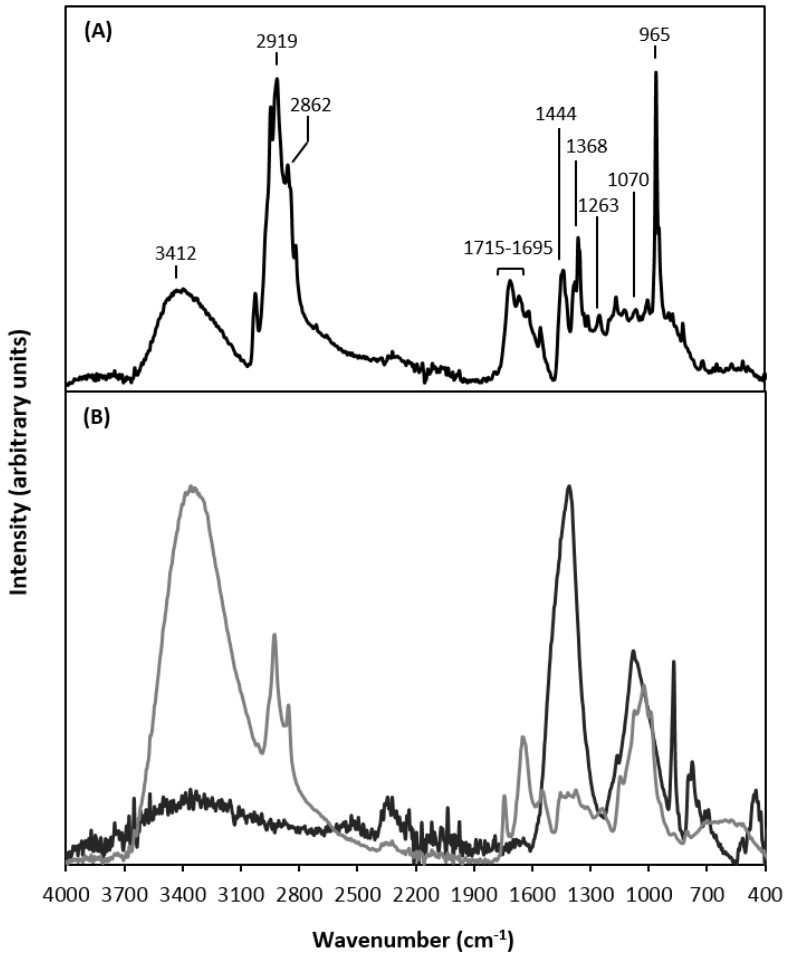


Figure II-23. FTIR-ATR spectra of *β*-carotene standard (A), microsamples collected in the inner wall of the *Évora* Cathedral (B) with pink stains (dark grey) and *Rhodotorula* yeast (light grey) isolated from the same place (B). Carotenoids bands are evidenced in the spectra.

The *β*-carotene spectrum (Figure II-23A) exhibited a spectral region between 3500-3000 cm^{-1}, characteristic of the O–H stretching vibrations, and 1715-1695 cm^{-1} corresponding to a carbonyl group ν(C=O), bands at 2919 and 2862 cm^{-1} for asymmetric and symmetric stretching vibrations of the CH_2 and CH_3, 1444 cm^{-1} for CH_2 scissoring, 1368 cm^{-1} for splitting due to dimethyl group, 1263 cm^{-1} corresponding to ν(C–O) of ester or acid groups, 1070 cm^{-1} is assigned to the C–O stretching band and 965 cm^{-1} for trans conjugated alkene-CH=CH- out-of-plane deformation mode (Tarantilis *et al.*, 1998; Ammawath and Man, 2010; Adamkiewicz *et al.*, 2013). This last band is a good indicator of the presence of carotenoids and was detected in *Rhodotorula* sp. and in the Évora Cathedral samples (Figure II-23B).

These findings are consistent with the results obtained by the previous technique. In this way, carotenoids seem to be responsible for the pigmentation acquired by inner wall of the Cathedral, due to *Rhodotorula* proliferation, behaviour detected in Figure II-20A, which can induce pink stains. Thus, biological activity contributed to the colour alteration of the mortar, and its interaction with the support is crucial to understand the long term deterioration.

The results have shown that carotenoid compounds are correlated with the degradation/deterioration of the inner walls of the Évora Cathedral, due to development and metabolic activity of living organisms, particularly *Rhodotorula* yeast. These compounds are responsible for pink/orange spots that cover the wall surface and alter the original aspect.

5. Conclusions

In this study we developed an analytical strategy that enables to understand several deterioration processes that occur in mortars and mural paintings. Complementary non-destructive microanalytical techniques were applied to characterise the materials used and the alteration products formed.

Among the results obtained it was possible to understand that:

In the case of *Santa Clara* church, the process that caused chromatic alteration on the mural paintings was a result of the lead based compounds oxidation by the formation of *plattnerite*, which promotes the darkening of these areas. Salt efflorescences formation promotes alterations in the painting, due to the recrystallisation of the mortars components on the surface, inducing the destruction of the support. The high microbial contamination levels and the particular presence of *Penicillium* and *Cladosporium*, seem to contribute to the black spots development and efflorescence formation.

The alterations detected in the green areas of the Low Choir of *Nossa Senhora da Saudação* Convent allowed to establish a strong correlation with the presence of microbial contamination. The cracks and detachment of some areas of the paintings was supported by fungal proliferation and propagation of micellar structures in depth, affecting the mortar integrity. Bacterial development appears to be responsible for biofilm formation in the paint surface, promoting chromatic alterations of the green areas, particularly due to the *Bacillus* sp. proliferation. The detection of calcium oxalate compounds like *whewellit*e and *weddellite*, can be attributed to the metabolic activity of the microorganisms which colonise these paintings.

Regarding the pink/dark orange colouration of the inner walls of Évora Cathedral, the results revealed that this phenomenon can be attributed to the presence of carotenoid compounds produced by the metabolic activity of microorganisms. Furthermore, the study revealed that *Rhodotorula* sp. yeast is the main agent involved in this process.

The combined spectroscopic approach using SEM, Raman and FTIR-ATR allowed the pigments and support matrix characterisation of the mural paintings, alteration products and the microflora proliferation, proving to be a useful methodological approach for the biodeterioration assessment in artworks.

CHAPTER III

Culture-dependent methods and molecular approaches to access microbial communities

The results presented in this chapter were published in the following scientific paper:

Rosado T, Mirão J, Candeias A and Caldeira AT (2014) Microbial communities analysis assessed by pyrosequencing - a new approach applied to conservation state studies of mural paintings. *Analytical and Bioanalytical Chemistry* 406:887-895.

1. Overview

The knowledge about the microbial communities present in mural paintings is of utmost importance to develop effective conservation and mitigation strategies. This chapter describes a methodological approach for the detailed characterisation of microorganisms thriving in mural paintings by combining culture-dependent methods that allow the identification of microorganisms capable of growing in laboratory conditions and to obtain high cell densities for further studies, and culture independent methods, such as Denaturing Gradient Gel Electrophoresis (DGGE) and pyrosequencing. The combined use of culture dependent methods and DGGE does not give enough information to fully investigate the diversity and abundance of microorganisms present in wall paintings. Pyrosequencing, a novel molecular technique, used here for the first time in this area of research, allowed to identify a large number of microorganisms, confirming some already identified by the cultivation-dependent methods such as fungi of the genera *Aspergillus*, *Cladosporium* and *Penicillium*, but also providing a great contribution in the identification of several genera and species, unprecedented identified in these artworks, giving also a detailed overview of contaminants which was not possible with the other approaches. The results obtained on several mural painting samples have shown a strong relationship between the most deteriorated areas of the paintings and higher microbial contamination.

2. Introduction

Microorganisms like bacteria, fungi, algae and lichens can play an important role in the biodegradation of cultural heritage, together with ageing, the chemical structure of the substrate and the environmental conditions such as humidity, temperature, pH, and light (Garg *et al.*, 1995; Pangallo *et al.*, 2009b). Development of microorganisms on mural paintings may cause aesthetic and/or structural damages such as pigment discolouration, stains and biofilms formation on the painted surface, cracking and disintegration of paint layers, and, degradation of binders resulting in detachment of the paint layer (Ciferri, 1999; Capodicasa *et al.*, 2010; Pepe *et al.*, 2011a).

Although the involvement of microorganisms in the degradation process is well acknowledged, the specific role of the different groups and species that compose the microbial communities is not yet well understood because methodologies tend to identify only easily cultivable and omit slow growing and uncultivable microorganisms. Identification of the microbial diversity present in cultural heritage is a crucial step to develop and apply correct conservation and mitigation methodologies and to prevent further contaminations (Ramírez *et al.*, 2005).

The traditional way to identify the microbial diversity is based on the cultivation of microorganisms in specific nutrient media, but, this approach detects less than 1% of the microbial communities present on the Earth (González and Saiz-Jiménez, 2005). To understand the phenomena that promote the degradation of mural paintings it is important to know as much as possible the microbial population that colonise these artworks. The use of culture-independent techniques like molecular approaches, based on nucleic acids detection, allows the differentiation of microorganisms within complex microbial communities, since each microorganism holds unique sequences (Portillo and Gonzalez, 2009).

Cultivation-independent methods enable to detect uncultivable microorganisms giving a more complete view of the microbial communities present in a certain sample than traditional cultivation techniques (Schabereiter-Gurtner *et al.*, 2001a; Justé *et al.*, 2008). Thus, molecular fingerprinting techniques like denaturing gradient gel electrophoresis (DGGE) have been used to determine and identify the genetic diversity of natural microbial communities present in mural paintings (Rölleke *et al.*, 1996 ; Gurtner *et al.*, 2000a; Piñar *et al.*, 2001).

DGGE technique has the advantage of directly profiling microbial populations present in specific ecosystems by separating PCR products that have originated with universal primers, on the basis of the melting domain of the DNA molecules (Muyzer *et al.*, 1993; Rantsiou *et al.*, 2005; Justé *et al.*, 2008).

The detection of microorganisms is mainly based on the small subunit ribosomal DNA (rDNA) genes, 16S rDNA for prokaryotes and 18S rDNA for eukaryotes. Ribosomal DNA (rDNA) is the most commonly employed target for PCR amplification prior to DGGE because they are present in every living organism and they contain variable and highly conserved regions which allow to distinguish between organisms on all phylogenetic levels (Ercolini, 2004; González and Saiz-Jiménez, 2005; Santos *et al.*, 2009).

DGGE separates amplified rDNA fragments of the same length but with different base pair sequences (Rölleke *et al.*, 1996 ; Malik *et al.*, 2008). Double-strand DNA fragments are subjected to an increasing denaturing environment as they encounter increasing concentrations of the denaturing agents and partially melt in discrete regions called ‘‘melting domains”, and, depends on the hydrogen bonds formed between the GC and AT base pairings and the attractions between neighbouring bases of the same strand. GC pairs are much more stable to denaturation than AT pairs. This technique uses a chemical gradient of urea and formamide created within an acrylamide gel. Usually, the PCR products applied in a DGGE gel are obtained by PCR amplification using a GC-rich tail at the 5’-end of one primer, generally composed by about 40 bases like as 5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G and it will be continued by the priming sequence complementary to the target DNA to be amplified. This GC rich tail is highly resistant to chemical denaturation (Ercolini, 2004; Gonzalez and Saiz-Jimenez, 2004). This technique provides information about the microbial diversity in the samples and by excising individual DGGE bands from the gel and reamplifying the DNA, it is possible to get sequence information of single community members. Thus, DGGE represents a powerful tool for monitoring microbial communities present in wall paintings and other cultural assets (Möhlenhoff *et al.*, 2001; Hoshino and Morimoto, 2008).

Pyrosequencing, a next generation sequencing technology, allows high-throughput sequencing and is revolutionising the study of microbial diversity. This methodology has been applied to identify mammal species, to study microbial diversity in soils, freshwater, human guts, wastewater treatment facilities (Karlsson and Holmlund, 2007; Jones *et al.*, 2009; Roh *et al.*, 2009; Acosta-Martínez *et al.*, 2010; Nam *et al.*, 2011; Vaz-Moreira *et al.*, 2011; Ye and Zhang, 2011; Hu *et al.*, 2012). More comprehensive information about the microbial communities contributing to the degradation of mural paintings are needed, so in this work pyrosequencing was envisaged to access microbial diversity.

Pyrosequencing technology is a non-electrophoretic real-time ssDNA sequencing method based on the detection of released pyrophosphate during nucleotide incorporation in the DNA-strand. The DNA synthesis is catalysed by four kinetically well-balanced enzymes: DNA

polymerase, ATP Sulfurylase, Luciferase and Apyrase (Ronaghi, 2001; Langaee and Ronaghi, 2005; Ahmadian *et al.*, 2006a; Trama *et al.*, 2007; Edlund and Allen, 2009; Petrosino *et al.*, 2009; Fakruddin *et al.*, 2012; Leite *et al.*, 2012).

This approach has never been applied to mural paintings biodegradation assessment. A single study was found in the literature for the application of this technique in cultural heritage research and relates to the characterisation of algal and fungal community living on sandstone buildings in Belfast (UK) (Cutler *et al.*, 2013).

The strategy adopted in this study combined culture-dependent methods and molecular approaches: DGGE and 454-pyrosequencing, to investigate the diversity and abundance of microorganisms present in the wall paintings. Moreover, it is intended to compare the results obtained with each approach and assess the microorganisms found by culture-dependent/independent methods.

With this innovative application, we hope to contribute to deepen the knowledge about the microbial populations that colonise mural paintings.

To develop this study, two mural paintings inserted in different environmental conditions were selected: *Santo Aleixo* Church (Figure III-1A) and Oval Room of *Condes de Basto* Palace (Figure III-1B).

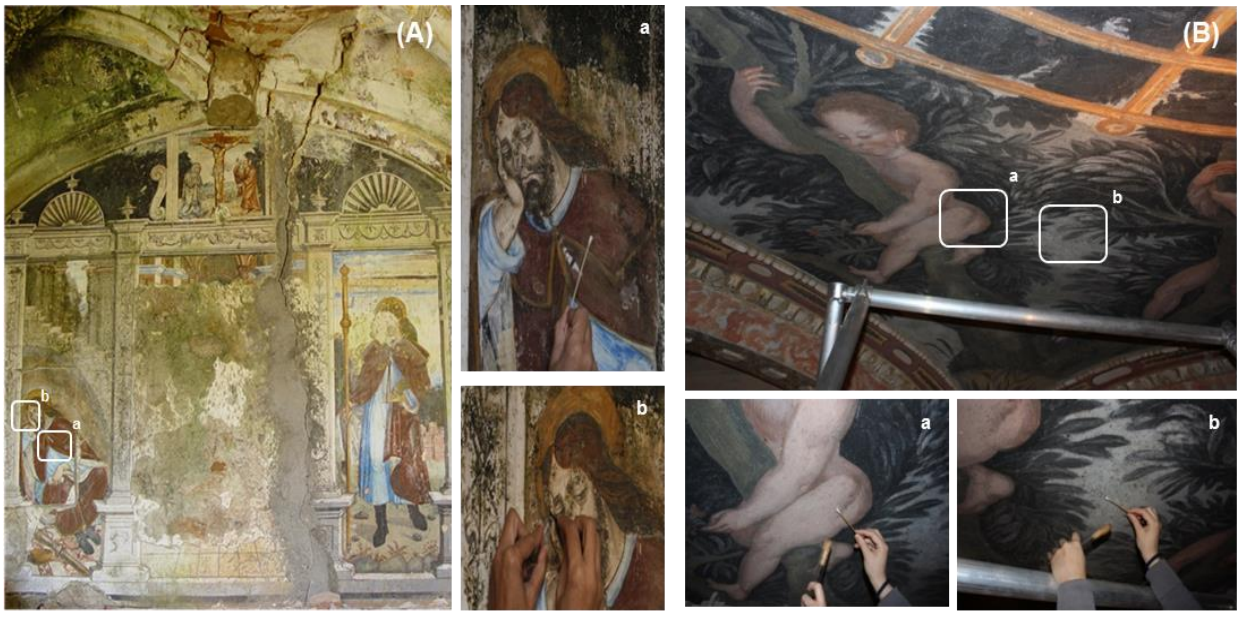


Figure III-1. Sampling location in mural paintings from *Santo Aleixo* church (A) and Oval Room of *Condes de Basto* Palace (B).

The *Santo Aleixo* Church is an abandoned church in a rural area, near Montemor-o-Novo (Southern Portugal), that has suffered severe structural damages with partial collapse of the ceiling but that holds important renascence frescoes (Figure III-1A). The other case study is the mannerist mural paintings (2nd half of the 16th century) from the Oval Room of *Condes de Basto* Palace (Figure III-1B) in the world heritage town Évora (Southern Portugal). This palace has been inhabited continuously till present days.

3. Experimental Section

3.1. Sampling process

The sampling places for analysis were carefully chosen according to the level of degradation observed in the two cases (A - *Santo Aleixo* Church and B - Oval Room of *Condes de Basto* Palace) selected for this work (Figure III-1), and, ensuring the representativeness of the paintings. In the sampling process was collected only the minimum amount essential for the different analyses, using non- and micro-invasive methods (Annexe C-C4 and C5). Samples were collected under aseptic conditions with sterile swabs and scalpels, placed in a suspension of transport MRD medium (Maximum Recovery Diluent, Merck) and conserved at 4ºC until utilisation. Mortars microfragments (50 mg) were also collected using sterile scalpels and microtubes.

3.2. Evaluation of microbial contamination in mortars

The mortar microfragments collected were analysed by scanning electron microscopy (SEM), and the samples were coated with gold or used as such (Balzers Union SCD030), and observed in a Hitachi Scanning Electron Microscope S-3700N. The accelerating voltage was 18–20 kV. Microanalysis of the selected samples was performed using the same scanning electron microscope coupled with a Bruker XFlash 5010 energy dispersive X-ray spectrometer (SEM-EDX). This technique allowed the observation of the mortars microstructure and morphology, and, microbial contaminations as well as the elemental composition (point analysis and 2D mapping).

3.3. Culture-dependent methods

Serial dilutions (10^{-1}-10^{-3}) of the samples recovered were prepared and inoculated (100 µL) in selective media (Annexe A) such as Nutrient Agar for bacteria isolation, Yeast Extract Peptone

Dextrose Agar for yeasts development, Malt Extract Agar and Cook Rose Bengal to isolate filamentous fungi. The cultures were incubated at 30°C for 24-48h for the development of bacteria, and for 4-5 days at 28°C for fungal growth. To detect slow growing microbial populations the inoculated Petri dishes stayed in incubation for longer period of time. Each different colony observed was picked up to obtain pure cultures, stored at 4ºC and periodically peaked to maintain the cultures active.

The characterisation of the microbial isolates was performed based on the observation of macroscopic features of the colonies such as texture and colour of the colonies and in micro-morphology of the hyphae and reproductive structures (in the case of spore isolates). The preparations made for fungal isolates were stained with methylene blue, observed with a 20x and 50x objective with an optical microscope Leica DM 2500P and digitally recorded by a Leica DFC290HD camera photo. The bacterial isolates were carried out with Gram stain and observed in the same optical microscope with a 100x objective lens.

3.4. Culture-independent methods

3.4.1. Denaturing gradient gel electrophoresis

3.4.1.1. DNA amplification

Metagenomic DNA was extracted with NucleoSpin 740945 DNA Extraction kit (Macherey-Nagel, Düren, Germany) and was used as template for PCR amplification. PCR reactions were carried out in a final volume of 50 µL containing reaction buffer 10x supplied with $MgCl_2$ 25 mM, dNTPs 2 mM, primer sets (A e B) 0.4 µM, Taq DNA polymerase 5 U and DNA extracted (1 µL). The amplification started with a denaturation step of 5 min at 94ºC, followed by 40 cycles of denaturation for 1 min at 94ºC, annealing for 1 min at 50ºC, extension for 2 min at 72ºC ended with a final elongation step of 6 min at 72ºC, using a Robocycler (MJ Mini Bio-Rad). The integrity of the PCR products was checked by 1.2% agarose gel electrophoresis (Annexe B-B2), containing ethidium bromide 10 mg/mL, at 90 V, and visualised under UV light (Bio-Rad).

A partial sequence of 18S rDNA gene was amplified using primer pair A: NS1 (5'-GTAGTCATATGCTTGTCTC-3') / GCfung (5'-CGCCCGCCGCGCCCCGCGCCCGGCCCG CCGCCCCCGCCCCATTCCCCGTTACCCGTTG-3') and for 16S rDNA the primer pair B: 518F-GC (5'- CGCCCGCCGCGCGGGGGGGG-3') / 785R (5'-CTACCAGGGTATCTAATCC-3') (Muyzer *et al.*, 1993; Duong *et al.*, 2006; Mühling *et al.*, 2008).

3.4.1.2. DGGE gel analysis

DGGE analyses of the PCR products were carried out in polyacrylamide gels (8% (m/v) acrylamide-bisacrylamide (37.5:1)) with a gradient between 30% and 50% created by 0 to 80% denaturant (Annexe B-B1), consisting of urea and formamide, using a DGGE K-2401. Electrophoresis was performed in TAE 0.5x (Annexe B-B3) at a constant voltage of 100 V, 60°C during 8 h for bacteria and 22 h for fungi. Following completion of electrophoresis, gels were stained in an ethidium bromide solution (10 mg/mL) and documented using a transilluminator (Uvitec mod STX 20 M).

3.4.2. Pyrosequencing

3.4.2.1. DNA extraction

DNA from mural painting swabs was extracted with QIAamp® DNA Stool Mini kit (Qiagen, Hilden, Germany). Briefly, the swabs were incubated in ASL buffer for 30 min at 37ºC and 10 min at 95ºC; then glass beads were added to the suspensions and submitted to disruption and homogenization using the TissueLyser (Qiagen). The lysates were centrifuged and purified according to the standard procedure for pathogen detection of the kit.

3.4.2.2. Amplification of rDNA

DNA from each sample was used as template for amplification of the V3V4 region of the bacterial 16S rDNA and ITS2 region for the fungal population study.

The V3V4 region was amplified with the forward primer 5'–ACTCCTACGGGAGGCAG-3' and the reverse primer 5'–TACNVRRGTHTCTAATYC–3'.

The ITS2 region was amplified with the primers ITS2_F 5'-GCATCGATGAAGAACGC-3' and ITS2_R 5-'CCTCC GCTTATTGATATGC-3',

The forward primers contain an upstream 454 Life Science's titanium sequencing adaptor (5'-CGTATCGCCTCCCTCGCGCCATCAG-3') and a TAG sequencing with 8 nucleotides which allows the pooling of multiple samples for pyrosequencing. Reverse primers also contain an upstream 454 Life Science's titanium sequencing adaptor (5'-CTATGCGCCTTGCCAGCCCGCTCAG -3').

Two independent replicate reactions were done for each region of each sample with 1X Advantage 2 Polymerase Mix (Clontech, Mountain View, CA, USA), 1x Advantage 2 PCR Buffer,

0.2 µM of each PCR primer, 0.2 mM dNTPs (Bioron, Ludwigshafen am Rhein, Germany), 5% DMSO (Roche Diagnostics GmbH, Mannheim, Germany) and 2 µl of DNA. The following PCR programs were used: an initial denaturation at 94ºC for 4 min followed by 25x (ITS2)/30x (V3V4) cycles of denaturation at 94ºC for 30 s; annealing at 44ºC (V3V4)/50ºC (ITS2) for 45 s and extension at 68ºC for 60 s, and a final extension step at 68ºC for 10 min. All the amplifications were carried out in a MyCycle Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA). The amplified products were purified with AMPure XP beads (Agencourt, Beckman Coulter, USA) followed by quality assessment of nucleic acid on 1.2% (w/v) agarose gel and quantification by fluorescence using the PicoGreen dsDNA quantitation kit (Invitrogen, Life Technologies, Carlsbad, California, USA).

3.4.2.3. Emulsion PCR and massive parallel sequencing

The amplicons were clonally amplified by emulsion PCR, and immobilized onto beads, each bead carrying a single DNA molecule. The bead-bound library is then emulsified with the amplification reagents in a water-in-oil mixture, creating millions of microreactors, where a single-fragment PCR occurs. Resulting DNA library beads are loaded into the wells of a PicoTiterPlate (PTP) device. Once in the Genome Sequencer FLX Instrument (454 Life Sciences, Roche), the fluidics system delivers sequencing reagents across the wells of the plate, along with the four DNA nucleotides, added sequentially in a fixed order. During the nucleotide flow, millions of copies of DNA bound to each of the beads are sequenced in parallel. When a nucleotide complementary to the template strand is added into a well, the polymerase extends the existing DNA strand by adding nucleotide(s). Addition of one (or more) nucleotide(s) generates a light signal that is recorded by the CCD camera in the instrument, signal strength being proportional to the number of incorporated nucleotides. The software converts the light signals into nucleotide information generating the final sequencer reads.

3.4.2.4. Data analysis

The microorganisms present in each sample were identified with a bioinformatics pipeline developed at Biocant. Briefly, raw pyrosequencing readings were separated according to barcode identifiers and processed through quality filters to remove sequences that did not have a complete forward primer; had less than two undefined nucleotides and were shorter than 100 bp. Additionally, the 3’ ends were trimmed for average qualityscore ≤ 15, based on a seven bases

window. After filtering, reads were trimmed for the A and B sequence adaptors and the barcode. The high quality sequences were clustered together by uclust v2.1 (Edgar, 2010) with a similarity of 97%. The clustered sequences were then assembled by Cap3 (Huang and Madan, 1999) to produce OTU (Operational Taxonomic Units). The OTU were searched by NCBI BLAST against RDP, release 10 update 24 (Ribosomal Database Project) with a cut-off of 1e-50 to identify the *taxa*. Chimeras were identified by BLAST, through the confirmation of whether different fragments of the same OTU matched only the same hit. To improve the accuracy of the results a bootstrap method was included, where OTUs were replicated 100 times and changed in 10% by seqboot application from PHYLIP package (Felsenstein, 1993). Only sequences with 70% bootstrap support of the same taxonomy were identified.

4. Results and Discussion

4.1. Microbial contamination

This work focused on the application and comparison of different bioanalytical approaches for the characterisation of microbial populations, present in two different mural paintings with visible signs of contamination but in different degradation status, as can be seen in Figure III-1. In a first approach, microfragments of mortar were analysed by scanning electron microscopy, a technique that provides an image of high magnification and resolution and so allows to infer about the microstructure of the painting and the existence of biological contamination. SEM micrographs (Figure III-2A-D) of the mortar microfragments show evident indicators of biological contamination like filamentous fungi and hyphae proliferation within the mortars, which may explain the presence of cracks and detachments in the paint of case A (*Santo Aleixo* Church) and the colour alterations in the panels of case B (*Condes de Basto* Palace). It is also possible to visualise the typical microstructure of lime mortars with crystallites of calcite.

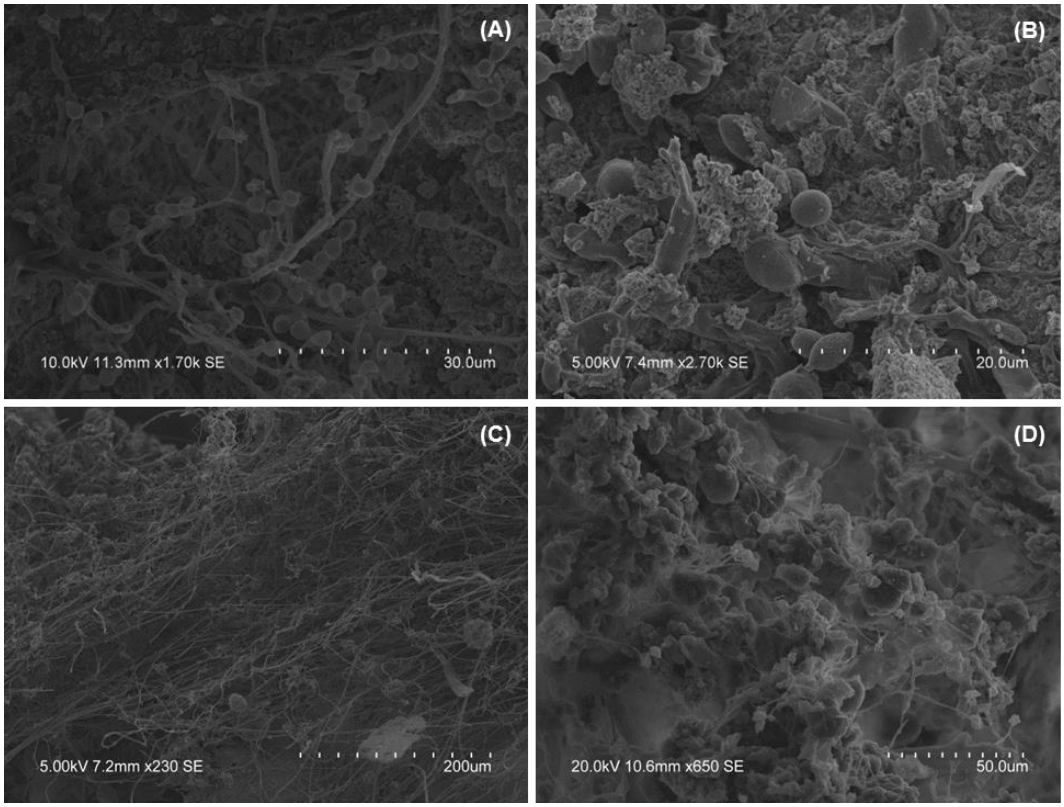


Figure III-2. SEM micrographs of the microfragments of mortars. Filamentous fungi and hyphae penetrating in the microstructure of the mortars (A, B) and superficial proliferation (C, D).

EDX analysis (Figure III-3) of these structures confirm the concomitant presence of elements characteristics of organic material such as carbon, sulphur, oxygen and nitrogen compatible with the presence of microbial contamination in the paintings.

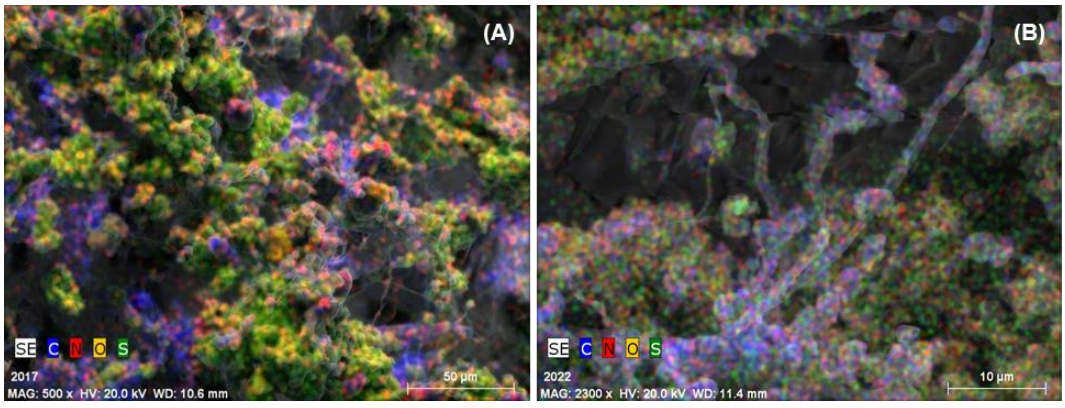


Figure III-3. SEM micrograph and EDX 2D mapping of mortars microfragments with representation of elemental maps of Carbon (C), Oxigen (O), Nitrogen (N) and Sulfur (S). Microample removed from Oval Room of *Condes de Basto* Palace (A) and *Santo Aleixo* Church (B).

Microbial proliferation on wall paintings has been associated to several degradation effects. Some microorganisms, like fungi and bacteria, have the ability to survive and thrive in extreme conditions (extremophile behaviour) including in the presence of heavy metals as is the case of some pigments present in mural paintings. Filamentous fungi development in the paintings lead the hyphae penetration within the mortar structure, promoting the proliferation of these microorganisms in depth, affecting the cohesion of the structure facilitating the appearance of some cracks and hence detachment of some fragments, which leads loss of some structures, or even, in extreme cases the entire work. Apart from structural effects, the biological activity of the microorganisms in the surface of the paintings can also induce chromatic alterations due to products excretion resulting from their metabolic activity or due to biofilms formation. For example, some microorganisms can induce irreversible stainings and chromatic alterations (see Chapter II), due to their ability to produce pigmented compounds, like carotenoids, as described in the Chapter II (Rosado *et al.*, 2013c). On the other hand, calcium oxalates have been reported in degraded areas of mural paintings due to the metabolic activity of the microorganisms, which secrete oxalic acid that reacts with calcium compounds present on the surface. Their formation can occur as a defence mechanism of the microorganisms in situation with excess of calcium, to prevent the toxicity to the cell (Rosado *et al.*, 2013a).

Therefore, microbial activity contributes to deterioration of mural paintings, and its interaction with physico-chemical mechanisms is considered central to understanding the long term deterioration, as well as knowing the agents that colonise these artworks.

In order to characterise the microbial population present in mural paintings several analyses using culture-dependent and independent techniques were adopted.

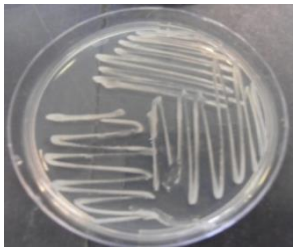
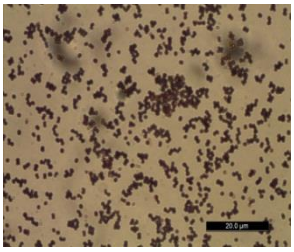
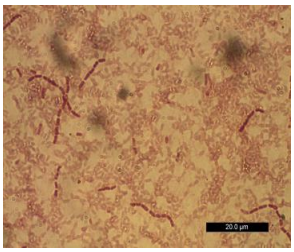
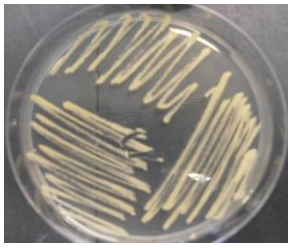
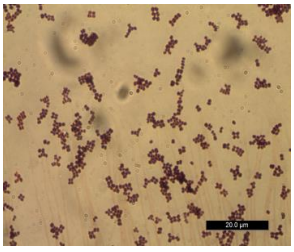
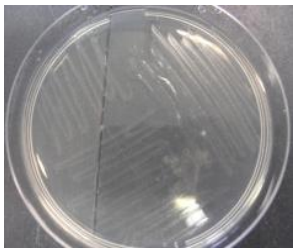
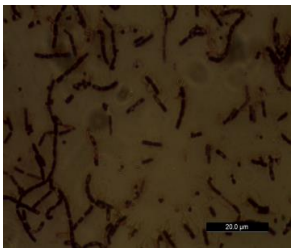
4.2. Culture-dependent methods

While cultivation methods give information about the microorganisms able to grow on a culture medium, molecular approaches provide information of the DNA sequences. Combining the information obtained with these different methods it is possible to know with more detail the microbial diversity that colonise these art works.

Isolated microorganisms from the paintings were characterised taking into consideration their macroscopic (colour, size and morphology) and microscopic features (type of reproductive structures and colour of colonies in the case of bacteria). The predominant microflora isolates were bacteria, yeasts and filamentous fungi (Garg *et al.*, 1995; Ciferri, 1999; Jurado *et al.*, 2008; An *et al.*, 2009; Pangallo *et al.*, 2009b; De Felice *et al.*, 2010; Pepe *et al.*, 2010; Laiz *et al.*, 2011).

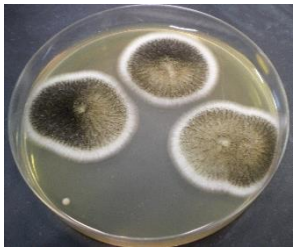
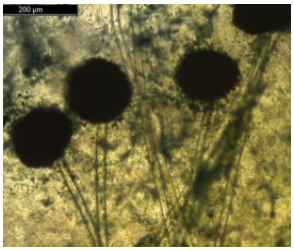
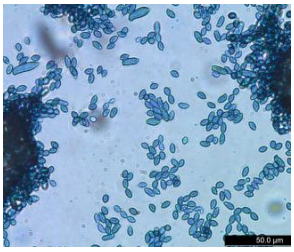
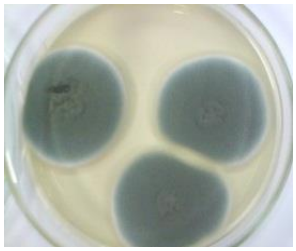
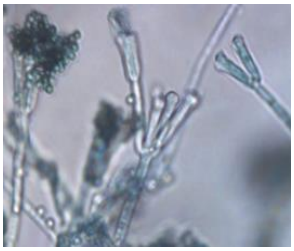
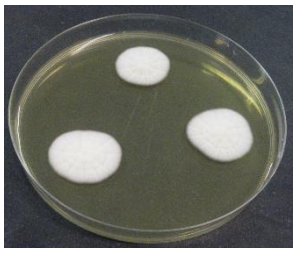
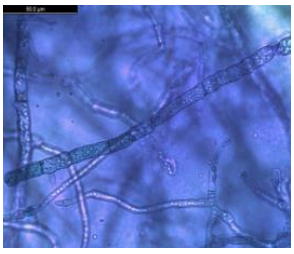
The identification of the bacterial population (Table III-1) isolated is difficult to perform based on the macroscopic and microscopic features, however it was possible identify that there are different bacteria, composed by cocci and bacilli morphology, being possible to identify some *Bacillus* sp. strains.

Table III-1. Identification of bacterial isolates obtained by culture-dependent methods.

Macroscopic features of colonies	Microscopic features	Identification
		Cocci (Gram-negative)
		Bacilli (Gram-positive)
		Cocci (Gram-positive)
		*Bacillus* sp. (Gram-positive)

In the case of fungal population was possible to identify several cultivable fungi of the genera Aspergillus, *Cladosporium*, *Penicillium*, but also unidentified fungi named sterile and non-sterile mycelia.

Table III-2. Identification of fungal isolates obtained by culture-dependent methods.

Macroscopic features of colonies	Microscopic features	Identification
		*Aspergillus* sp.
		*Cladosporium* sp.
		*Penicillium* sp.
		Mycelium

Although this approach does not enable a full characterisation of the microbial community, since some microorganisms do not have the ability to grow under *in vitro* conditions, is extremely

important because it gives a first scan on the cultivable microflora and allows to obtain high density of cells for simulation assays or biocide tests, essential steps for the development of adequate conservation methodologies.

4.3. Microbial communities

Considering that not all microorganisms have the ability to grow under *in vitro* conditions, the proposed research strategy envisaged the application of DGGE and pyrosequencing to the same collected samples.

DGGE was employed to assess the structure of microbial communities in samples without cultivation. The purity and integrity of the metagenomic DNA amplified was analysed by agarose gel electrophoresis (Figure III-4). It is possible to observe fragments with 300 bp and 400 bp, for bacteria and fungi respectively.

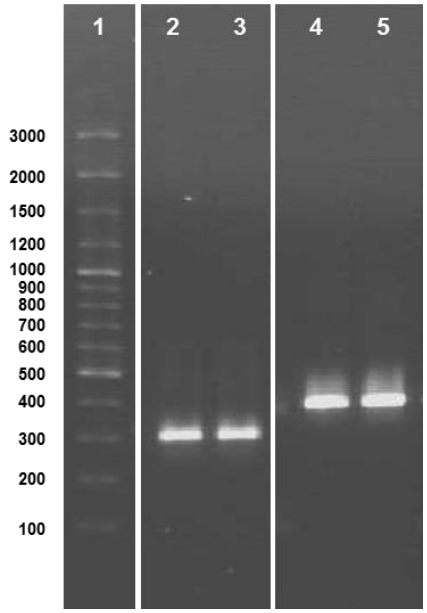


Figure III-4. Agarose gel electrophoresis of metagenomic DNA extracted from *Santo Aleixo* Church (Sample A) and *Condes de Basto* Palace (Sample B). Legend: 1 - 100 bp Ladder; 2, 3 - PCR product of bacterial amplification of Sample A and B, respectively; 4, 5 - PCR product of fungal amplification of Sample A and B, respectively.

The PCR products obtained were separated by DGGE and the results revealed the presence of several distinguishable bands, most likely derived from different fungal and bacterial species constituting the population of each sample. It is possible to observe that exist more fungal diversity (Figure III-5a), due to the presence of a larger number of bands, in comparison with the bacterial

population detected (Figure III-5b). These results are also correlated with those obtained by culture-dependent methods, where fungal isolates prevail.

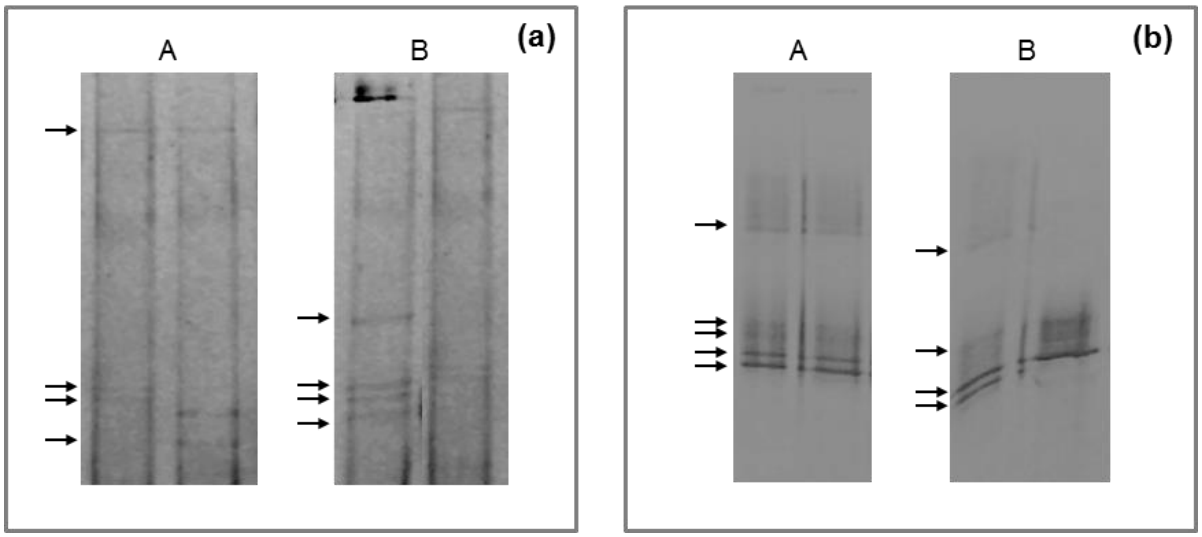


Figure III-5. DGGE fingerprints of the amplified fungal (a) and bacterial (b) DNA of the samples A - Oval Room of *Condes de Basto* Palace and B - *Santo Aleixo* Church.

DGGE represents a powerful tool for monitoring microbial communities and examining population dynamics (Gonzalez and Saiz-Jimenez, 2004; González and Saiz-Jiménez, 2005; Malik *et al.*, 2008; Pepe *et al.*, 2011), but it does not allow full identification of the microorganisms present in the samples. Strategies for sequenciation of separated bands can be applied but are highly time consuming and the DNA obtained correspond frequently to a mix DNA, conducing to ambiguous identification. Pyrosequencing on the other hand, is a powerful novel technique that complements the results obtained by the aforementioned methods, allowing the full identification of the microbial population and was used on this work for the first time, as to the authors' knowledge, to biodegradation studies of artworks.

Amplicons for the V3V4 and ITS2 regions were generated for the samples using primers of conserved regions and submitted to pyrosequencing in the 454 sequencing platform as described on the Experimental Section. The number of sequences obtained for each sample is listed in Table III-3.

Table III-3. Massive parallel sequencing general results. Raw sequences correspond to the number of sequences obtained after sequencing and before data processing.

Sample	Raw Sequences	High quality sequences	OTU (3%)
A_V3V4	15,887	12,061	319
A_ITS2	6,985	6,314	74
B_V3V4	1,742	1,064	264
B_ITS2	9,200	8,318	334

Sequences in each sample were grouped to generate consensus sequences, named OTU (operational taxonomic unit). An operational taxonomic unit is the consensus sequence containing sequences that are no more than 3% different from each other, which is generally considered to define a microbial specie. The number of generated OTU found in Table III-3 shows that sample B (*Condes de Basto* Palace) is a little bit greater in fungal contamination than bacterial population and in sample A (*Santo Aleixo* Church) the opposite is observed. The OTUs were assigned a taxonomic ID by searches against data in public databases. To better understand the meaning of these values, the results for each sample were subjected to non-parametric statistical analysis by determination of CHAO parameter. Figure III-6 shows rarefaction curves for the samples and the respectively CHAO.

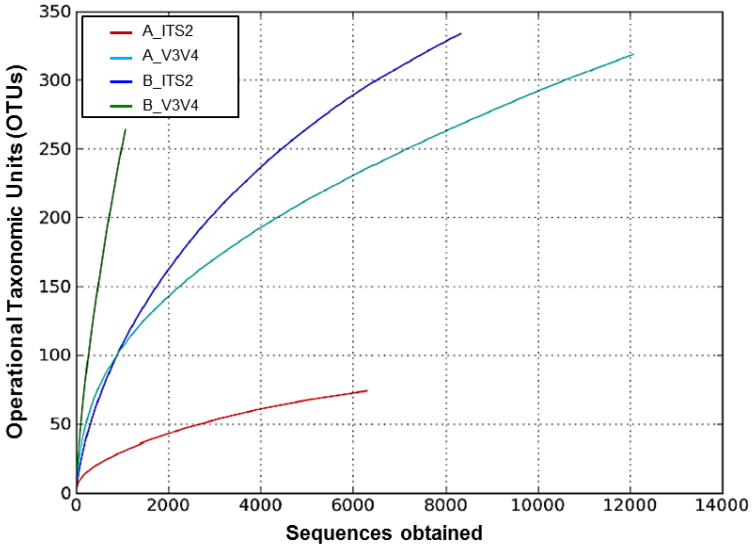


Figure III-6. Rarefaction curves of the sequenced samples at 3% difference level.

This analysis estimates the coverage of sequencing in samples, by determining the expected number of independent sequences and the number of independent sequences actually obtained in each sample. The results are summarised in Table III-4. These results, although unexpected taking into consideration the different environment and state of conservation of both mural paintings studied, show that identified species found in sample A and B are very similar.

Table III-4. Statistical analysis of sequencing results.

Sample	Raw Sequences	OTU (3%)	High quality sequences
A_V3V4	581.52	319.00	54.86
A_ITS2	93.89	74.00	78.81
B_V3V4	678.55	264.00	38.91
B_ITS2	488.22	334.00	68.41

The microflora present on the two case studies is divided into three kingdoms: Bacteria (53%), Fungi (41%) and Viridiplantae (5%), in the total of 303 microorganisms identified, which bacterial population is slightly higher than fungal diversity.

This approach allowed the identification of more than one hundred and thirty genera and sixty different species. Namely, for the fungi:

Cladosporium, *Penicillium*, *P.daleae*, *P.digitatum*, *P.corylophilum*, *P.glabrum*, *Aspergillus*, *Cystoderma*, *Hypholoma*, *Tubaria*, *Pholiota*, *Armillaria*, *Physalacria*, *Chondrostereum*, *Schizophyllum*, *Coltricia*, *Fuscoporia*, *Hyphodontia*, *H.alutaria*, *H.radula*, *H.nothofagi*, *H.nespori*, *Phlebia*, *Radulomyces*, *Vesiculomyces*, *Russula*, *Amphinema*, *Hyphodontiella*, *Lactarius*, *Cyphellostereum*, *Stereum*, *Skeletocutis*, *Ganoderma*, *Tyromyces*, *Antrodia*, *Postia*, *Coriolopsis*, *Rhodotorula*, *R.mucilaginosa*,

and for the bacteria:

Bacillus, *Catenibacterium*, *Anaerococcus*, *Roseburia*, *Veillonella*, *Atopostipes*, *Dolosigranulum*, *Granulicatella*, *Aerococcus*, *Abiotrophia*, *Streptococcus*, *Lactobacillus*, *L.delbrueckii*, *Leuconostoc*, *L.citreum*, *Marinococcus*, *Virgibacillus*, *Geobacillus*, *G.stearothermophilus*, *Thermicanus*, *Staphylococcus*, *Salinicoccus*, *S. halodurans*, *Paenibacillus*, *Streptomyces*, *S.clavuligerus*, *Actinomyces*, *Nocardia*, *Rhodococcus*, *Corynebacterium*, *Arthrobacter*,

Micrococcus, *Kocuria*, *Rothia*, *Blastococcus*, *Geodermatophilus*, *Bifidobacterium*, *B.bifidum*, *Oligella*, *O.urethralis*, *Haemophilus*, *Pseudoxanthomonas*, *Pseudomonas*, *Sphingomonas*.

The results show that pyrosequencing has the potential to be an important tool in this field which will allow to revolutionise the knowledge about the microbiological colonisers of mural paintings.

5. Conclusions

This work encompassed different approaches to characterise the microbial population that colonise mural paintings, which enabled the creation of an analytical methodological strategy to address the present limitations in microbiological agents studies in the field of cultural heritage research (Figure III-7).

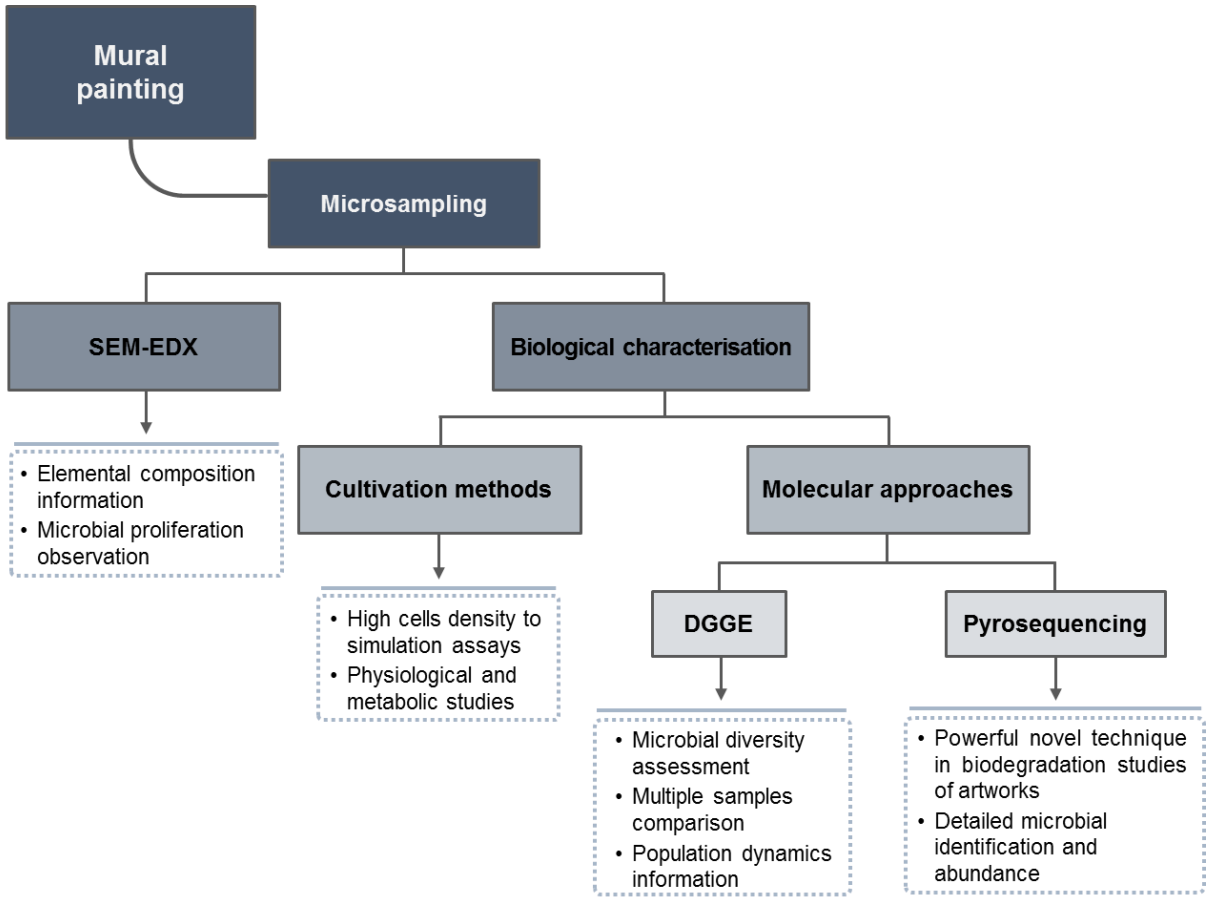


Figure III-7. Methodological strategy defined to mural paintings biodegradation/biodeterioration studies.

A very important point in this study was the application of pyrosequencing, which provided an important and exhaustive description about the microbial population that develops on the mural paintings and allowed to expand the knowledge about them, giving a detailed overview of the contaminants that was not possible with the other techniques. In fact, culture dependent methods and DGGE are useful tools in the characterisation of the biodeteriogenic agents however constitute incomplete approaches to investigate the diversity and abundance of microorganisms present in wall paintings.

CHAPTER IV

Biodeteriogenic agents monitorisation

The results presented in this chapter were published in the following scientific paper:

Rosado T, Pires M, Mirão J, Martins MR, Candeias A and Caldeira AT (2013) Enzymatic monitorization of mural paintings biodeterioration, *International Journal of Conservation Science* 4: 603-612.

1. Overview

Biodegradation/biodeterioration of mural paintings is an important research field that needs novel approaches to fully understand their mechanisms and effects. In this work, the presence of microorganisms and their biological activity were investigated by extra and intracellular enzymatic monitorisation. The enzymes arylsulphatase, β-glucosidase, dehydrogenase and phosphatase were used as biomarkers of the microbial metabolic activity, and the viability cellular assays revealed a relationship with the degradation levels of the paintings. In this way, the metabolic activity of the microbial population can be correlated with the contamination levels detected and with biodegradation/biodeterioration status of the paintings. Therefore, enzymatic approaches constitute good biomarkers to be applied in this research field and are useful to detect biodeteriogenic agents.

2. Introduction

Mural painting is not only a form of art but also a way to learn more about our ancestral traditions. Unfortunately, some of these artworks have suffered alterations, which can lead to an incalculable loss. The damages usually found in deteriorated mural paintings are promoted by several factors, however the biological agents assume a role of utmost importance and it is necessary to give special attention to them. Therefore, biodegradation/biodeterioration is a serious risk to Cultural Heritage, which needs the application of effective and fast methods in order to identify the microorganisms involved in this process and to assess their biodegradation and biodeterioration ability (Pangallo *et al.*, 2009a). The term biodeterioration is defined as unwanted alteration in a material caused by the activity of biological agents. Biodeteriogenic organisms have the ability to use a substrate to sustain their growth and reproduction, producing alterations (Sequeira *et al.*, 2012). Several microorganisms can grow on various materials, causing their biodegradation and biodeterioration. In fact, it is a complex process that illustrates the interaction of living microorganisms with substratum and environment (Dakal and Cameotra, 2012). Some microorganisms have the capacity to degrade mural paintings and their biodeteriogenic ability, in synergy with other physical and chemical agents, may increase the damages (Cappitelli *et al.*, 2006; Rolón and Cilla, 2012).

The microbial flora present in artworks, like mural paintings, may result from the successive colonisations by different microorganisms. Their biological attack occurs at favourable temperature and relative humidity conditions for the development of microorganisms and spores present on the paintings, and, each coloniser agent has different ways to compromise these structures (Nugari *et al.*, 1993b; Borrego *et al.*, 2010). Thus, microorganisms that grow on mural paintings may origin structural damages involving different processes, such as cracking, exfoliation of paint layers, formation of paint blisters and detachment of the paint layer from the support, or, aesthetic damages which involves the pigment discoloration and stains. It is believed that aesthetic damages occur earlier than structural damages and can precede serious corruption of the materials, being these damages strongly linked (Sarró *et al.*, 2006; Santos *et al.*, 2009).

The study of microflora involved in biodegradation/biodeterioration processes of artworks, was usually based on DNA-dependent methods or in isolation procedures that were mainly useful to provide information about the presence of microbial communities (Gonzalez and Saiz-Jimenez, 2004; González and Saiz-Jiménez, 2005; Rosado *et al.*, 2013a; Sterflinger and Piñar, 2013 ; Rosado *et al.*, 2014a; Rosado *et al.*, 2014c), however, the physiological/biological potential of these microorganisms has not been explored in this field (Pepe *et al.*, 2011). Therefore, in this

study, enzymatic activities were taken into account, to understand the role of their metabolic activity on the biodegradation/biodeterioration process.

The enzymes ability to recognize specific molecules as substrates has led to the proposal of enzyme-based analytical approaches. Thus, different enzymes like: arylsulphatase, dehydrogenase, *β*-glucosidase and phosphatase were chosen to assess the physiological features of the predominant mural painting colonisers and to evaluate their biodegradative and biodeteriorative potential. Arylsulphatase, *β*-glucosidase and phosphatase enzymes, hydrolyse and catalyse specific reactions involved in the biogeochemical transformations of carbon (C), phosphorus (P) and sulphur (S). These enzymes regenerate inorganic nutrients from organic materials and have been reported as the rate-limiting step in the nutrient cycling process. On the other hand, organic phosphorus (P) must be mineralised into inorganic orthophosphate (PO_4^{3-}) ions to be assimilated by many organisms. Only enzymes produced by plants and/or microorganisms are able to hydrolyse organic P into phosphates. Dehydrogenase enzyme allows to detect viable organisms and can be considered an accurate measure of the microbial oxidative activity (Taylor *et al.*, 2002; Pozo *et al.*, 2003; Stege *et al.*, 2009).

To access the presence of microorganisms and evaluate their effect in the mural paintings degradation/deterioration, different enzymes were monitored and cell viability assay was for the first time applied in artworks, in order to develop biomarkers that may give information about the degradation assisted or not by deterioration process.

3. Experimental Section

3.1. Microorganisms selection

Several microbiological specimens, previously isolated from mural paintings, with significant alteration signs, were selected to investigate the role of the microorganisms in the alteration processes that affect these artworks. Thus, *Aspergillus* sp., *Cladosporium* sp., *Penicillium* sp. and *Rhodotorula* sp. (microorganisms from HERCULES-Biotech Laboratory, Évora University), the main fungi associated to mural paintings colonisation, were analysed individually and as a community, by the mixture of these microorganisms, in order to simulate mix cultures that proliferate in mural paintings.

3.2. Sampling process

Mortar microfragments from contaminated historical mural paintings were collected with sterile scalpels and microtubes, in two areas of the painting with different degradation/deterioration levels.

3.3. Analysis of mortars microfragments

In order to assess the degree of degradation/deterioration of the support and the type of colonising microorganisms, the mortar microfragments collected were analysed by scanning electron microscopy (SEM). The samples were gold sputtered (Balzers Union SCD030) and then observed under a scanning electron microscope (Hitachi 3700N) operated at high vacuum with an accelerating voltage 10–20 kV in secondary electron mode.

3.4. *In vitro* simulations of mortars colonisation

To evaluate the role of the microorganisms in the mural painting degradation/ deterioration, a combinatory strategy was used:

a) Development of liquid cultures with high cells density of pure cultures: *Aspergillus* sp., *Cladosporium* sp., *Penicillium* sp. and *Rhodotorula* sp., and, a mix culture combining these microorganisms (Figure IV-2);
b) Simulation assays in order to evaluate the influence of these microorganisms on real sterilised mortars, inoculated with each microbial isolated (*Aspergillus* sp., *Cladosporium* sp., *Penicillium* sp. and *Rhodotorula* sp.) and with a mix culture with each microorganisms previously mentioned.

Fresh fungal cultures were prepared in solid medium MEA (Malt Extract Agar). Fungal suspensions were prepared washing each slant with 2 mL of NaCl 0.85% solution. The suspensions from pure cultures and a mixed culture obtained by the combination of the 4 isolates were inoculated in 100 mL of Malt Extract liquid medium and incubated at 28ºC in an orbital shaker at 150 rpm (Heidolph unimax 1010), during 15 days (Annexe A).

For the simulation assays, mortar samples were sterilised, 1000 mg were distributed in Petri dishes and inoculated with 0.2 mL of the suspension prepared as the same form mentioned above (pure and mixed cultures) and incubated at 28ºC during 15 days.

3.5. Enzymatic assessment

The enzymes arylsulphatase, β-glucosidase, phosphatase and dehydrogenase were monitored in the assays of liquid cultures (a), in the simulations assays (b) and in real mortar samples.

Arylsulphatase activity was assayed according to the method of Tabatabai and Bremner (1970) (Tabatabai and Bremmer, 1970). The liquid (0.3 mL) and solid (0.1 g) samples were incubated 2 h at 20ºC with 0.5 M acetate buffer pH 5.8 and 0.2 mL of 2 mM p-nitrophenyl sulphate (PNS). The reaction was stopped by adding 0.1 mL of 0.5 M NaOH, and immediately centrifuged for 15 min at 10,000 rpm. The amount of p-nitrophenol (p-NP) released from PNS was measured spectrophotometrically (Hitachi, U-3010) in the supernatant at 405 nm.

β-Glucosidase activities were also evaluated according to Tabatabai and Bremner (1970) (Tabatabai and Bremmer, 1970). The liquid (0.1 mL) and solid (0.1 g) samples were incubated with modified universal buffer (Annexe B-B4) pH 6.0 and 0.2 mL of 2 mM p-nitrophenyl β-D-glucoside, during 1h at 37ºC. The reaction was stopped by adding 0.1 mL of 0.5 M NaOH and centrifuged for 15 min at 10,000 rpm. The amount of p-nitrophenol released was measured in the supernatant at 405 nm.

The enzymatic activity of the Phosphatase was evaluated according to the method of Tabatabai and Bremner (Tabatabai and Bremmer, 1969). The liquid (0.1 mL) and solid (0.1 g) samples were incubated at 37ºC for 1 h modified universal buffer (MUB) pH 5.0 and 0.2 mL of 115 mM p-nitrophenyl phosphate (p-PNP). The reaction was stopped by adding 0.1 mL of 0.5 M NaOH, and immediately centrifuged for 15 min at 10,000 rpm. The amount of p-nitrophenol released from PNP was measured in the supernatant at 405 nm.

Dehydrogenase enzymatic activity was determined according to Camiña (Camiña *et al.*, 1998; Taylor *et al.*, 2002). Cells disintegration was performed by sonification during 30 s, 5 times at 40/50 Hz. The liquid (0.1 mL) and solid (0.1 g) samples were incubated for 1h at 40ºC, in the dark, with 1 M Tris-HCl buffer pH 7.5 and 0.2 mL of 0.5% 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT). The reaction was stopped by adding 0.1 mL of ethanol:DMF (1:1), and immediately centrifuged for 15 min at 10,000 rpm. The amount of iodonitrotetrazolium formazan (INTF) released was measured spectrophotometrically (Hitachi, U-3010) in the supernatant at 490 nm.

These assays were performed in triplicate. A unit of enzyme activity (U) was defined as µmole of substrate hydrolysed or oxidized min^{-1} (Annexe D-D2), and per mg of protein (Annexe D- D1) for liquid assays or per mg of mortar for solid assays.

3.6. Statistical analyses

The results of the enzymatic activity monitored in the several assays mentioned above were reported as average ± standard deviation (SD). Data were evaluated statistically (Annexe D-D3) using the SPSS® 20.0 software for Windows Copyright©, Microsoft Corporation, by descriptive parameters and by *One-way* ANOVA in order to determine statistically significant differences at the 95% confidence level ($p<0.05$). The population variances homogeneity was confirmed by Levene test and multiple average comparisons were evaluated by Tukey test, being considered significant values those whose probability of occurrence is greater than 95% ($p <0.05$).

3.7. Microbial viability evaluation

Cell viability of the microbial population present in the mural paintings was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as previously described by Mosmann (Mosmann, 1983), being optimised in this work to be applied in artworks. Mortar microsamples (0.1 g) from mural paintings sites with different degradation levels were incubated with 0.5 mL of MTT stock solution (prepared in PBS at 5 mg/mL and after filtered to sterilise the solution), during 4h, in the dark, at room temperature. After this period, 1 mL of DMSO/ethanol (1:1) was added to dissolve the formazan crystals formed. The final suspension was centrifuged at 10,000 rpm for 15 min and the supernatant was spectrophotometrically (Hitachi, U-3010) analysed at 570 nm. Each assay was performed in triplicate.

4. Results and Discussion

The identification of the biological population that thrive in mural paintings is an important and necessary step, however, understand if these colonisers are metabolically active or not it is also a relevant approach that need to be exploited, in order to identify the most biodeteriogenic agents. In this way, several assays were carried out in microbial isolates, mixed cultures, simulated assays and in real mortar microfragments to gather as much information about the metabolic activity that these agents develop in mural paintings, almost without noticing them but after a period of time its effects are well visible and can be irreversible.

In this work several fungal strains were selected: *Aspergillus* sp., *Cladosporium* sp., *Penicillium* sp. and *Rhodotorula* sp. (Figure IV-1), isolated from different mural paintings (Garg *et al.*, 1995;

Ciferri, 1999; Sterflinger, 2010; Rosado *et al.*, 2013a; Rosado *et al.*, 2014a; Rosado *et al.*, 2014c; Rosado *et al.*, 2014e), in order to evaluate their role in the degradation process.

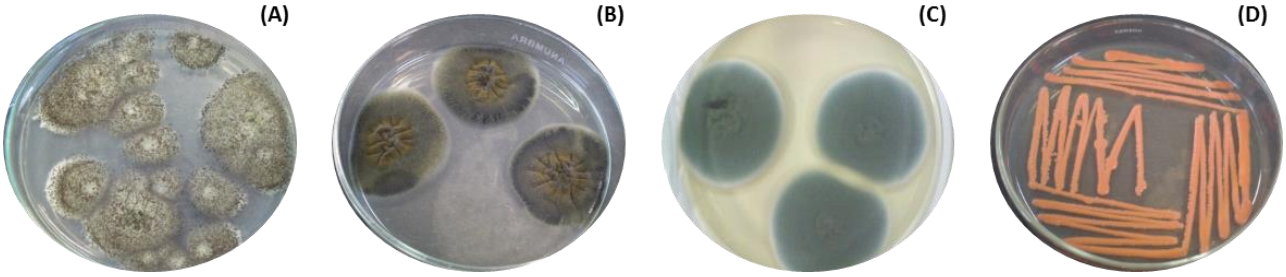


Figure IV-1. Microbiological agents commonly found in mural paintings: *Aspergillus* sp. (A), *Cladosporium* sp. (B), *Penicillium* sp (C) and *Rhodotorula* sp. (D).

4.1. Enzymatic assessment of liquid cultures

Several enzymatic assays were performed in order to understand if these enzymatic systems are active in each microbial isolate (*Aspergillus* sp., *Cladosporium* sp., *Penicillium* sp., *Rhodotorula* sp. and mix culture), and if they can be used as biological markers to monitor biodegradation/ biodeterioration (Figure IV-2). Therefore, the first approach intends to investigate if the enzymes arylsulphatase, β-glucosidase, phosphatase and dehydrogenase can be used for enzymatic monitorisation of the microorganisms usually found in mural paintings.

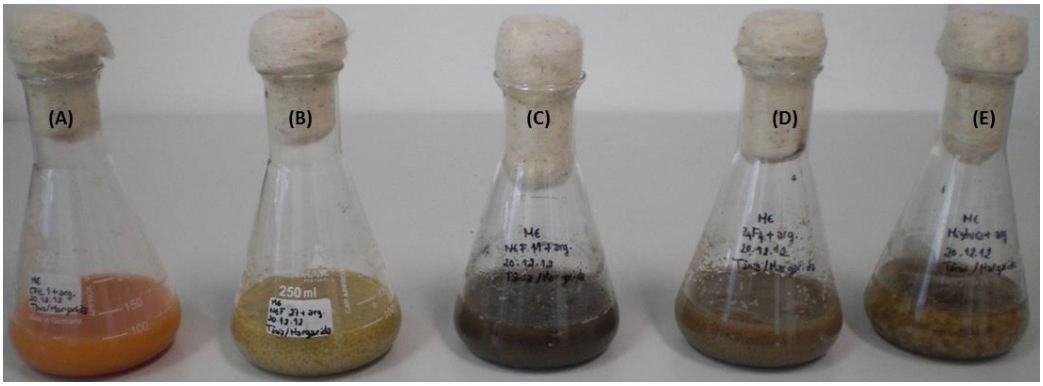


Figure IV-2. Liquid cultures of several microorganisms isolated from mural paintings: *Rhodotorula* sp. (A), *Cladosporium* sp. (B), *Penicillium* sp. (C), *Aspergillus* sp. (D) and a mixed culture (E) with these four microbial isolates.

These enzymatic systems have been used in soils, water and wastewaters quality/contamination studies (Bergstrom *et al.*, 2000; Pozo *et al.*, 2003; Klose and Ajwa, 2004; Jastrzębska and Kucharski, 2007; Floch *et al.*, 2009; García-Ruiz *et al.*, 2009; Kang *et al.*, 2009; Antunes *et al.*, 2011; Balestri *et al.*, 2013), being good indicators of biological activity, so this work intends to evaluate their potential in mural paintings degradation/deterioration.

Enzymatic monitorisation of arylsulphatase, β-glucosidase, phosphatase and dehydrogenase in liquid cultures (Figure IV-3) showed that the enzymatic systems are active in all the fungal strains tested. However, β-glucosidase reveal less activity than the other biological systems.

Analysing each enzyme individually it was possible to observe that:

In the case of arylsulphatase the microorganisms *Rhodotorula* and *Cladosporium* show higher enzymatic activity than the other agents tested. Relatively to β-glucosidase monitorisation, *Rhodotorula* is also the microorganism that reveal higher enzymatic levels, while in the case of dehydrogenase, *Cladosporium* stands out from the other microorganisms. On the other hand, *Cladosporium* and *Aspergillu*s reveal higher enzymatic activity in the phosphatase monitorisation. According these results, *Rhodotorula*, *Cladosporium* and *Aspergillus* seem to be easily detectable by enzymatic monitorisation. However, in the case of mix cultures, performed to simulate a microbial community, it is observed a decrease of the enzymatic activity comparatively to the microbial isolates, a trend that holds for all the enzymes tested, with the exception of the β-glucosidase enzyme which revealed an increase of 14% compared to *Rhodotorula*.

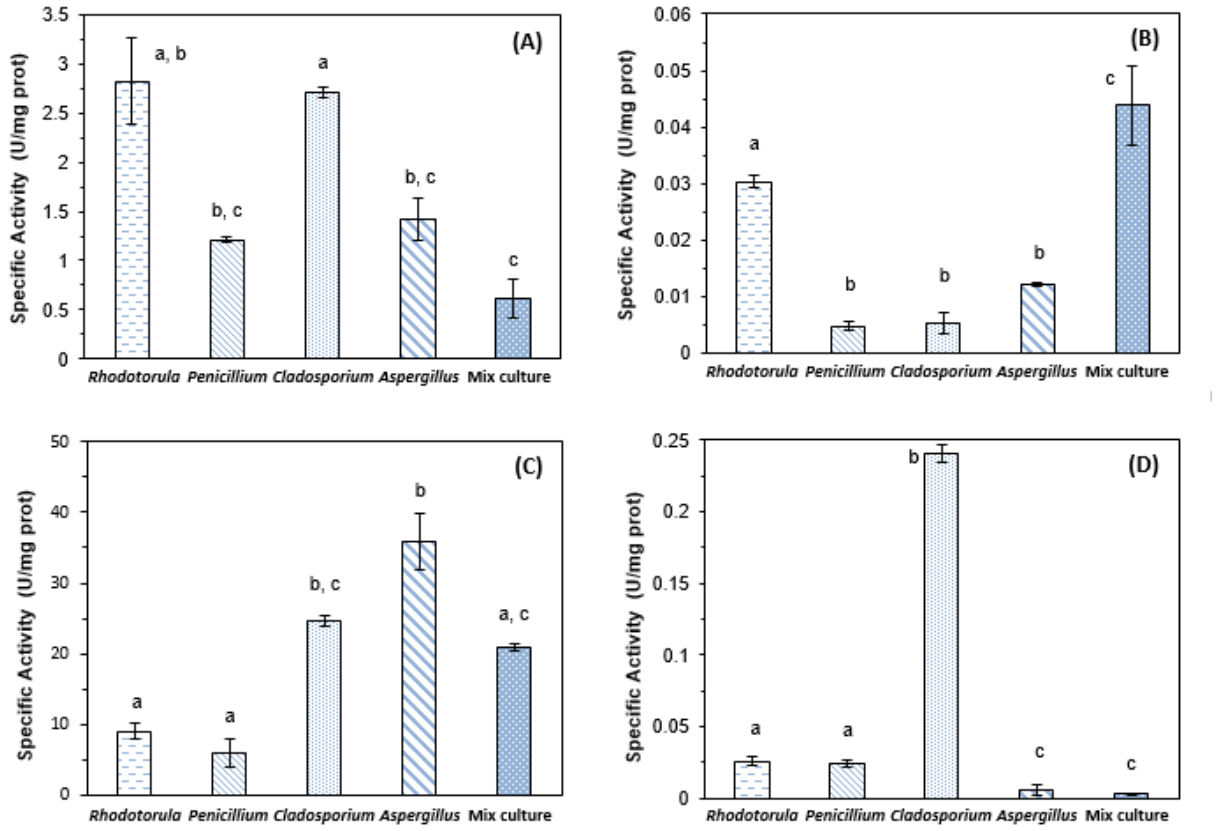


Figure IV-3. Enzymatic monitorisation of arylsulphatase (A), β-glucosidase (B), phosphatase (C) and dehydrogenase (D) in liquid cultures of predominant fungal strains isolated from mural paintings: *Rhodotorula* sp., *Cladosporium* sp., *Aspergillus* sp., *Penicillium* sp. and a mix culture of these microorganisms, performed during 15 days. Different letters (a-c) following the values indicate significant differences ($p<0.05$). Values of each determination represents means ± SD (n=3).

The activity of these enzymes is correlated to the compounds transformation that provide different components to the microorganisms development. In the case of dehydrogenase, their activity reflects the total oxidative activities of microorganisms and hence the presence of living cells (Huang *et al.*, 2012), and can be used as an indicator of the presence of metabolic active cells.

Thus, the results obtained allow us to verify that all fungi tested have the enzymatic systems studied active, suggesting that these enzymes are good biochemical markers to evaluate the metabolic activity of the coloniser agents of mural paintings.

4.2. Simulation assays on mortars

Once verified the presence of the enzymes arylsulphatase, β-glucosidase, phosphatase and dehydrogenase on liquid cultures, as well as their detection on mix cultures, simulation tests were carried out in mortars. These assays were used to mimic as much as possible, the real context where these microorganisms act. Accordingly, simulation assays, were performed on real sterilised mortars samples, inoculated with microorganisms aforementioned, allowing to verify their individual and combined action on the samples, and, infer about their biodeteriogenic capacity, clarifying the fungal impact in the mural painting biodegradation/biodeterioration.

Enzymatic monitorisation of arylsulphatase, β-glucosidase, phosphatase and dehydrogenase in mortar simulated assays (Figure IV-4) allowed to observe that the enzymes tested are active in the mortar samples inoculated with different microorganisms, showing that it is possible to monitor enzymatic activity in mortars.

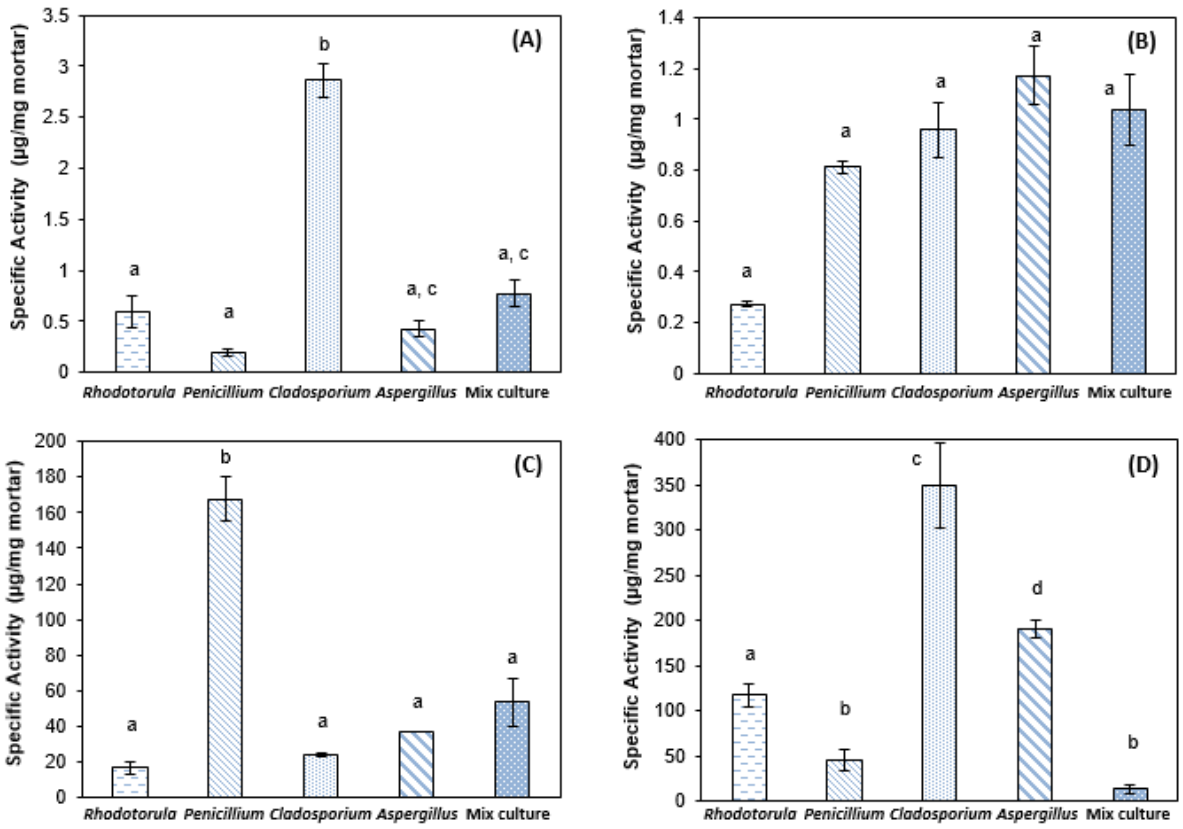


Figure IV-4. Enzymatic monitorisation of arylsulphatase (A), β-glucosidase (B), phosphatase (C) and dehydrogenase (D) in mortar simulated assays with the predominant fungal strains isolated from mural paintings: *Rhodotorula* sp., *Cladosporium* sp., *Aspergillus* sp., *Penicillium* sp. and a mix culture of these microorganisms. Different letters (a-c) following the values indicate significant differences ($p<0.05$). Values of each determination represents means ± SD (n=3).

The results showed that, for the enzyme arylsulphatase the fungi *Cladosporium* has the highest enzymatic levels. In the case of β-glucosidase, the filamentous fungi *Aspergillus*, *Cladosporium* and *Penicillium* are more active than *Rhodotorula*. As observed for arylsulphatase, the microorganism *Cladosporium* reveals greater activity for dehydrogenase. For this enzyme the fungus *Aspergillus* is also very active. In the case of phosphatase monitorisation, *Penicillium* exhibits the highest activity. Furthermore, for the mix cultures from mortar simulated assays was detected the same behaviour above described for liquid cultures, observing an enzymatic decrease for arylsulphatase, phosphatase and dehydrogenase. In addition, it is also important to emphasize that some microorganisms reveal increased activity in the presence of mortar fragments, probably due to the mortar constituents, which can be used by the microorganisms for their development, together with the nutrients obtained from the culture medium, promoting then higher development and consequently higher metabolic activity. On the other hand, in general, the enzymatic activity of the filamentous fungi was higher than the yeast tested. This result can be related with the higher ability of the filamentous fungi to proliferate in mortars and the different nutritional requirements of these distinct microorganisms. Moreover, a decrease of the metabolic activity observed for the microbial communities assays (mix cultures), probably can also be affected by the capacity of certain microorganisms to inhibit others. Another explanation for this behaviour can be due to nutritional requirements, wherein the development of some microorganisms can limit the growth of others.

4.3. Enzymatic monitorisation in real samples

In order to validate the applicability of these enzymatic systems as biodegradation/biodeterioration biomarkers, to be used in real mortar samples monitorisation, mortar microfragments with different visual alterations, removed from the highly degraded *Santo Aleixo* Church were analysed, to understand the effect of microbial proliferation in mural paintings and their impact in the degradation process. The different samples analysed were named: Low Deteriorated Sites (LDS) and High Deteriorated Sites (HDS) according to the degradation levels observed.

The results of the enzymatic assays of arylsulphatase, β-glucosidase, phosphatase and dehydrogenase, performed in real mortar samples with different contaminated levels, to evaluate the biological activity in mural paintings showed that samples from sites with larger signs of contamination present higher enzymatic activity (Figure IV-5). This effect is particularly relevant

to dehydrogenase, arylsulphatase and phosphatase, which revealed a noticeable increase in the places with higher alteration signs.

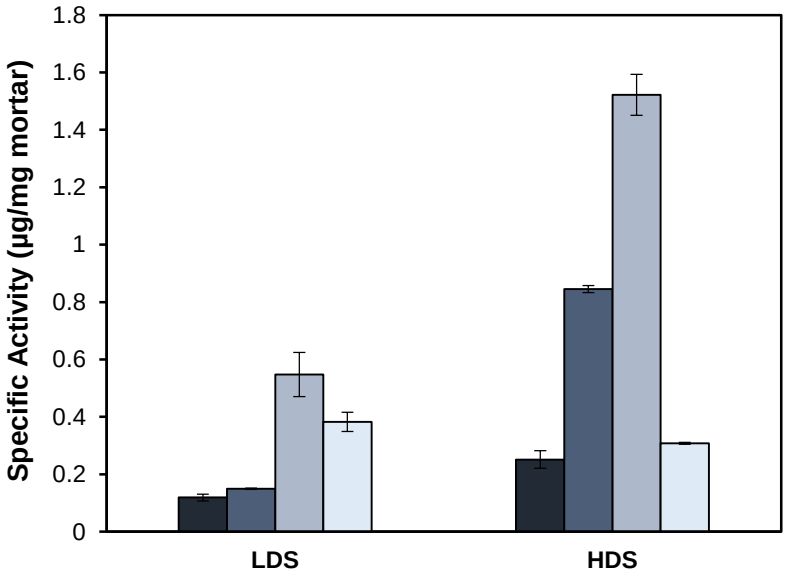


Figure IV-5. Enzymatic assays to evaluate biological activity in samples with different contaminated levels, using dehydrogenase (■), arylsulphatase (■), phosphatase (■) and β-glucosidase (□) as biochemical markers. LDS – Low Deteriorated Sites; HDS – High Deteriorated Sites.

Assays in real mortar samples that revealed the highest enzymatic activities were detected in samples from High Deteriorated Sites (Figure IV-5), which is located in the sites with greater degradation signals and concomitantly revealed high microbial colonisation, confirmed by SEM observation (Figure IV-6). An exception in this behaviour was detected for the enzyme β-glucosidase that presents little variability on LDS and HDS samples. The results showed that arylsulphatase, phosphatase and dehydrogenase can be used as biochemical markers in mural paintings, giving useful information about the biological activity of the microbial population, which can be correlated with the degradation status of the artwork.

The SEM analysis allowed a further insight on the presence of microbial communities thriving in the paintings and their capacity to proliferate within and/or penetrate inside the mortar structure. Results show that fungal proliferation conduces to penetration of mycelia structures in the microstructure of the mortars, promoting dissemination of these microorganisms in depth, whose behaviour seems to be correlated with cracks and the detachments observed in the painting (Figure IV-6). Thus, fungal proliferation appears associated to mortar structural damages and chromatic alterations.

The microbial degradation assisted or not by deterioration of paintings can be caused due to the hydrolytic activity of microorganisms to growth and/or also due to the damage that excretion metabolites inflict (Santos *et al.*, 2009). Moreover, the production of extracellular polymeric substances (EPS), mainly polysaccharides, surrounding the hyphae, promotes the adhesion to the substrate leading to biofilm formation (Zucconi *et al.*, 2012) that also contributes to the paintings degradation.

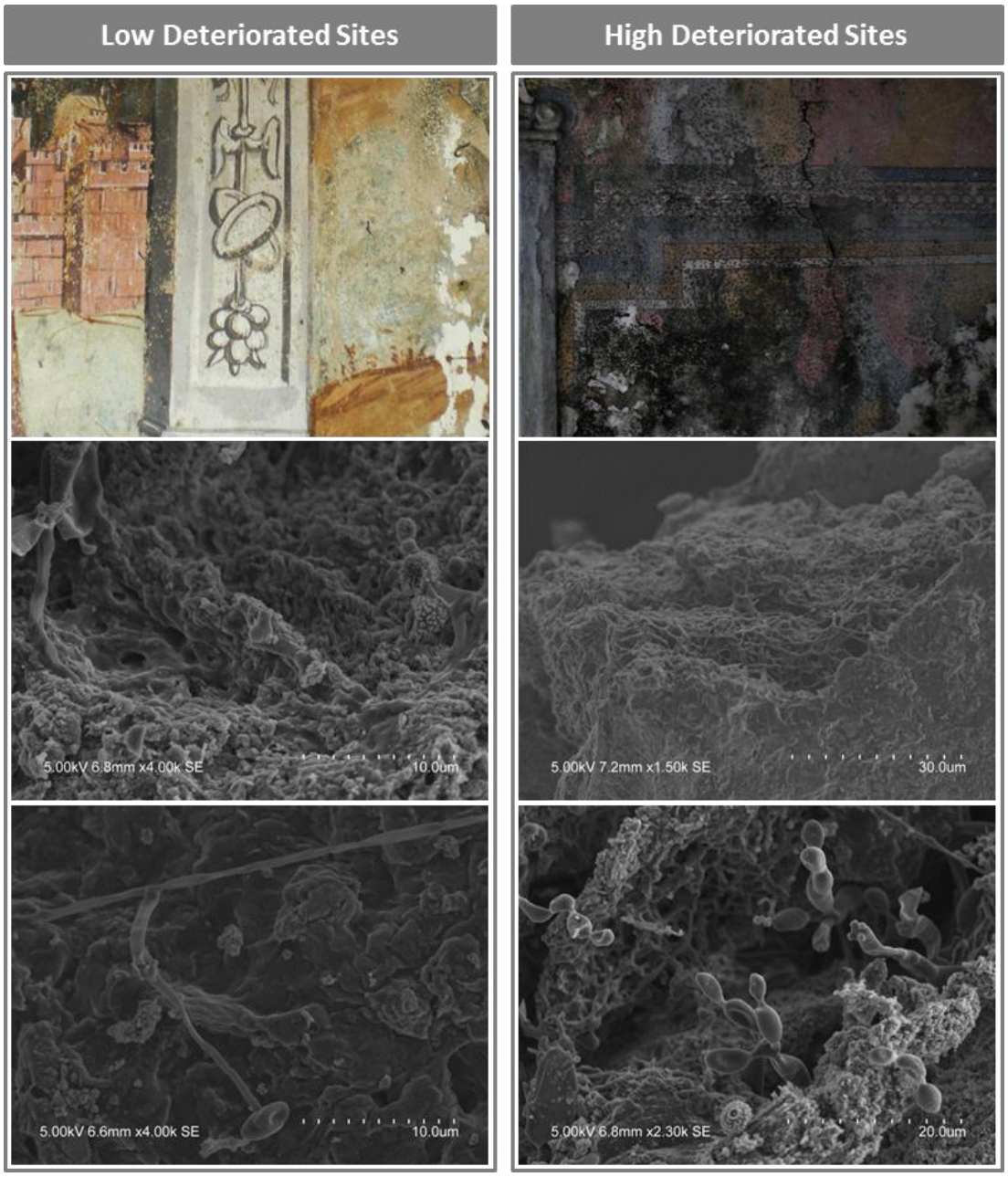


Figure IV-6. SEM analysis of samples from Low Deteriorated Sites (LDS) and High Deteriorated Sites (HDS).

The results showed that the combined approach using SEM analysis and metabolic activity measurement can be a useful methodology for the evaluation of microflora proliferation and the biodegradation/biodeterioration diagnosis of the mural paintings.

The enzymatic assays constitute good biomarkers for the biodegradation/biodeterioration assessment, giving a correlation with the degradation/deterioration levels of the paintings.

Therefore, the physical damages of the paintings, like cracks and detachment, are reported to microorganisms development, and, chemical decay of the mural paintings are associated to the metabolic activity of them, by assimilation or dissimilation processes. In the assimilation process, the microbial communities use the constituents of wall paintings as a carbon source through enzyme production, whereas in the dissimilation process, the decay is mainly by the excretion of waste products or secretion of metabolic intermediates including acids and pigments, promoting serious alterations in these artworks.

4.4. Microbial viability

Enzymatic assays can be affected by several parameters like temperature, pH and substrate. The assessment of total cells viability on the microsamples can constitute a quicker complementary methodology to biodeteriogenic agents detection.

A method, based on the cell viability, described by Mosmann (Mosmann, 1983), was adapted and optimised to monitor the biological activity in mortar microfragments from mural paintings (Rosado *et al.*, 2013b). This assay was originally developed to evaluate growth and survival of mammalian lymphoma cells, based on the transformation and colorimetric quantification of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]. It relies on the ability of living cells to reduce metabolically the MTT substrate in insoluble purple formazan crystals within the cells, which can be quantified by spectrophotometry. Formazan generated is proportional to the living cells present in the sample. These features can be taken advantage of in cytotoxicity or cell proliferation assays, which are widely used in immunology, toxicology, and cellular biology (Mosmann, 1983; Sieuwerts *et al.*, 1995; Freimoser *et al.*, 1999; Mota *et al.*, 2012) but never in Cultural Heritage biodegradation/biodetrioration studies

Cellular viability of the microbial population present on LDS and HDS samples from mural paintings were also used for the assays. The results are presented in Figure IV-7 and shows that samples from painting areas with low signals of degradation present low concentration of viable cells while samples from areas with evident signs of degradation have the opposite behaviour, *i.e.*, present high concentration of viable cells (Figure IV-7). These results are according to the

previous ones obtained by the enzymatic assays. This method is advantageous because it is simple, fast and very sensitive. Furthermore, the optimised methodology allows viability determination using only 100 mg of sample.

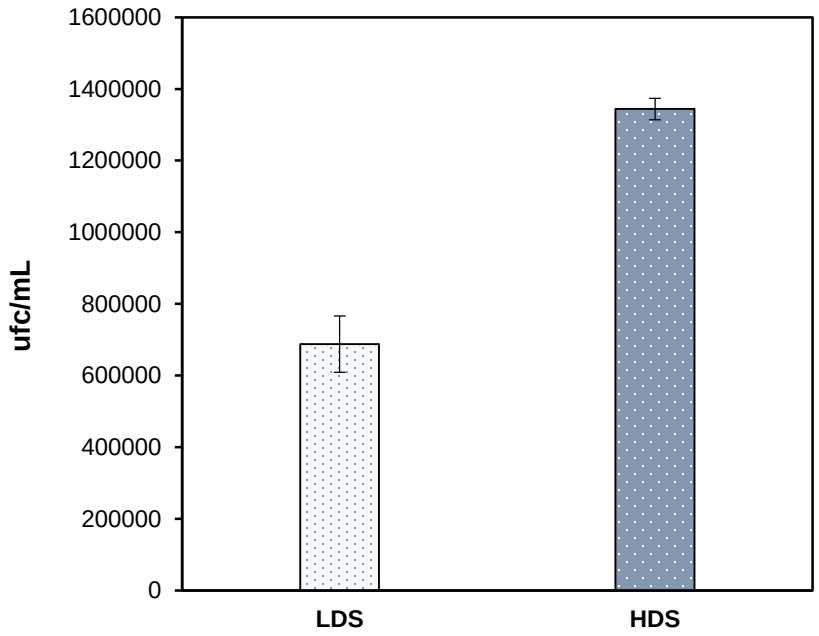


Figure IV-7. Cellular viability of the microbial population present in mural paintings. LDS - Low Deteriorated Sites; HDS - High Deteriorated Sites.

To validate the method response, sterilised mortar samples were inoculated with different concentrations of microorganisms. The results present a linear correlation between cellular viability and microorganisms concentration (ucf/mL). Thus, this approach can be used in mural paintings biodegradation/biodeterioration assessment to correlate degradation/deterioration status with metabolic active cells levels and consequently with microbial contamination degrees, constituting a very sensitive bioindicator.

5. Conclusions

Enzymatic systems like arylsulphatase, β-glucosidase, dehydrogenase and phosphatase constitute good biomarkers to assess biological activity in mural paintings and can be correlated with the biodegradation and biodeterioration status of the artwork.

Cell viability assays based on mitochondrial enzymes (MTT assays) were optimised in this work to be applied in mortar samples and constitutes an efficient real time method to assess metabolic activity in microsamples, which allows to infer about the active microbial contamination in the paintings.

The combined use of these techniques enabled the development of a novel methodological approach which represents an important contribution to artworks biodegradation/biodeterioration research, enabling an overview of the damages promoted by the microbial degradation and biodeteriogenic agents.

CHAPTER V

Mitigation strategies

Some results of this chapter were published in the following scientific papers:

Rosado T, Gil M, Caldeira AT, Martins MR, Dias C, Carvalho L, Mirão J and Candeias A (2014) Material characterization assessment of mural paintings: Renaissance frescoes from Santo Aleixo church, southern Portugal. *International Journal of Architectural Heritage* 8:1-18.

Rosado T, Gil M, Mirão J, Candeias A and Caldeira AT (2014) Biodeterioration assessment of the 16th century mural painting from *Casas Pintadas* in Évora, *Journal of Cultural Heritage* (*submitted*).

1. Overview

To ensure the longevity of the mural paintings and consequently the building where these are inserted, mitigation strategies must be developed in order to avoid the loss of these important landmarks. Two case studies were selected - *Santo Aleixo* Church (Montemor-o-Novo) and *Casas Pintadas* porch (Évora) which represent two completely distinct situations. The church of *Santo Aleixo* is almost destroyed, nevertheless integrates paintings of extraordinary beauty but in a status of extreme degradation. Regarding the paintings from *Casas Pintadas* some alteration signs were detected but at this moment these artworks are completely rehabilitated, due to the intervention that was submitted.

This chapter presents an integrated investigation on iconic mural paintings of Alentejo region encompassing material characterisation, biological contamination identification and *in vitro* and *in situ* biocidal treatments. All of these topics are very important steps to allow a well-defined remediation strategy and to attempt to obtain information for the conservation-intervention process to be the most faithful way possible.

To understand the effects of microorganisms involved in the biodeterioration/biodegradation process of cultural assets, and to define an efficient strategy to conserve and protect monuments and artworks from microbial colonisation, it is necessary their prior identification.

In vitro biocidal tests were performed against the main microbial mural painting colonisers, in order to select the most efficient commercial biocide to be applied in real situations. The antimicrobial assays revealed satisfactory inhibition results, whose action spectrum is noticeably enlarged by combined application of biocides.

2. Introduction

Biodeterioration/Biodegradation of Cultural Heritage is the result of interactions between living organisms, material support and environmental conditions (Nuhoglu *et al.*, 2006; Capodicasa *et al.*, 2010). The biological activity of microorganisms like bacteria, fungi, algae, and lichens, contributes to the deterioration of cultural assets, particularly if they are exposed to open air. Their interaction with physico-chemical properties of the materials is considered central to understand the long term deterioration (Ripka *et al.*, 2006; Herrera and Videla, 2009; Wiktor *et al.*, 2009). These microorganisms are able to obtain different elements (calcium, aluminium, silicon, iron and potassium) essential for their metabolism, by biosolubilisation of the materials (Nuhoglu *et al.*, 2006).

Taking into account the various external factors that affect the conservation status of the paintings, microorganisms play an extremely important role, which cannot be neglected. In mural paintings, the development of diverse organisms is supported by humidity, slight alkaline pH values and the presence of organic and inorganic nutrient sources (Altenburger *et al.*, 1996). On the other hand, the natural porosity of paintings makes their surfaces receptive to microbial spores and vegetative cells transported by airborne particles (Saarela *et al.*, 2004; Milanesi *et al.*, 2009).

Fungi are among the most harmful organisms associated to biodeterioration of organic and inorganic substances (Wiktor *et al.*, 2009). The destructive potential of these microorganisms is the result of mechanical and chemical processes, caused by mycelia penetration inside the plaster of the painting resulting in loss of cohesion and detachment of the paint layer, as well as paints discolouration due to products of their metabolism, secreted in the surface (Altenburger *et al.*, 1996 ; Rölleke *et al.*, 1996 ; Berner *et al.*, 1997; Herrera *et al.*, 2004; Milanesi *et al.*, 2006; Imperi *et al.*, 2007).

Therefore, it is crucial to develop efficient approaches to detect potentially harmful or destructive microorganisms, and strategies to conserve and eliminate their contamination. If procedures are taken to prevent their growth, biodegradation/biodeterioration can be avoided (Gurtner *et al.*, 2000 ; de los Ríos *et al.*, 2009; Wang *et al.*, 2011).

Consequently, for studying degradation/deterioration of artistic materials induced by environmental and biological agents, and thereafter proceed to a restoration, it is necessary the detailed knowledge of the materials originally employed by the artist (Milanesi *et al.*, 2009; Wiktor *et al.*, 2009). On the other hand, biocides application are a very important step to prevent and/or control microbial growth/ re-colonisation for one acceptable period of time (Urzì and De Leo, 2007; Fonseca *et al.*, 2010; de los Ríos *et al.*, 2012). However their application requires attention to

chromatic alterations, changes in water absorption capacity, permeability and surface tension (Tretiach *et al.*, 2007). Treatments with biocides should be tested on a small scale (*in vitro* test), but preferentially on the affected monument to determine their effectiveness against microorganisms, since some studies indicate that the biocides efficacy can be reduced significantly in the case of *in situ* applications compared to the sensitivity of the microorganisms observed in laboratory experiments (de los Ríos *et al.*, 2012).

In the case of artworks exposed to open air, usually occur fast recolonisation after restoration process (Nascimbene and Salvadori, 2008), thus the development of preservation strategies is urgent.

In this study, a biodegradation/biodeterioration assessment and mitigation measures were applied on two 16th century mural paintings of Évora region, which present completely different conservation status.

One of the paintings studied, the ancient Parish Church of *Santo Aleixo* (1531), is in an advanced degradation status. This Church is a building with simple structure, which has one of the most beautiful Renaissance Portuguese wall paintings, located in the wall of the main altar. It represents a false altarpiece with three scenes of *Santo Aleixo* life (Figure V-1). The building has been abandoned for almost 42 years resulting, among other factors, in the collapse of the nave roof, the appearance or deepening of structural cracks, and vandalism and theft of the central panel depicting Saint Alexius marriage.

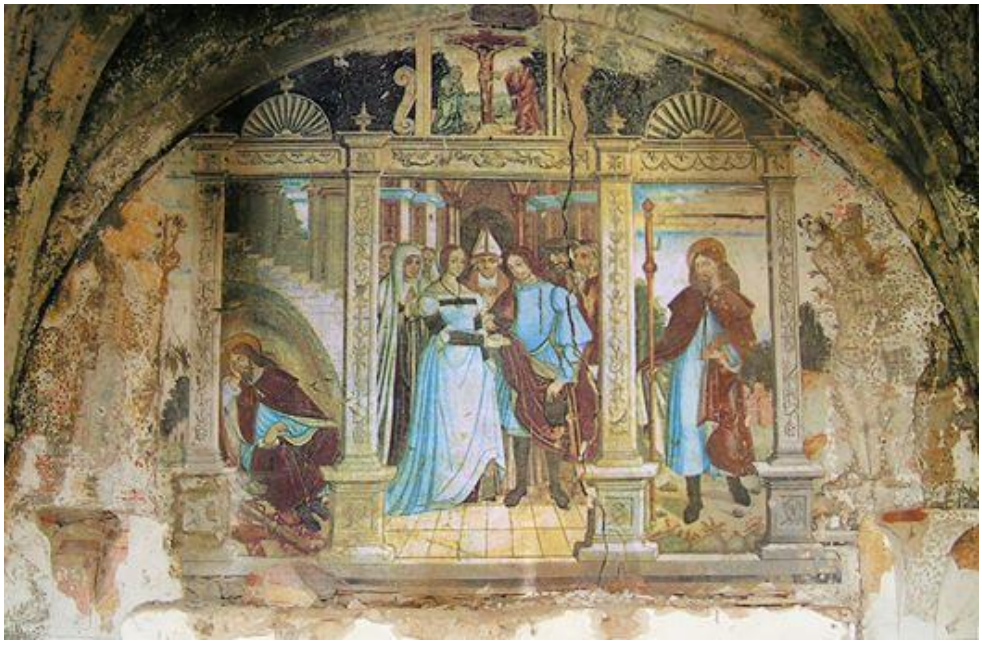


Figure V-1. Mural Painting of *Santo Aleixo church* (Adapted from Serrão, 2005).

The other case study used in this research work is the renaissance mural paintings from *Casas Pintadas* located in the garden of the Inquisition Palace in Évora (Portugal), classified as World Heritage by UNESCO, and, in 1950 as Asset of Public Interest by IGESPAR (*Instituto de Gestão do Património Arquitectónico e Arqueológico*). The paintings exhibited in *Casas Pintadas* (Figure V-2) show mythological and exotic scenes decorating a cloister and a small chapel. This space is all that remains of the Noble House belonging to Silveira Henriques family, Masters of the Horse of D. Afonso V and D. João II. However, in the Past they have been attributed to Vasco da Gama (Portuguese navigator), fact that has been proved to be a legend. The paintings in the cloister, of a great historical and artistic value, combine exoticism, originality and evocative power, constituting an iconic national and international mural composition (Caetano and de Carvalho, 2014).

Figure V-2. Mural Painting of *Casas Pintadas* located in the garden of the Inquisition Palace, Évora, before to the conservation-intervention process.

The main goal of this work was to obtain relevant data for art historians concerning the materiality of these unique paintings and to evaluate the main sources of degradation in order to produce a scientific and technical report for the regional authorities and the owners, to support a conservation-restoration intervention strategy for the future safeguard of these paintings.

Due to the great importance of these mural paintings a material study were performed, the biological contamination was evaluated and remediation strategies were studied in order to promote their longevity.

3. Experimental Section

3.1. Sampling process

The sampling process was performed on representative areas of the paintings, and, in areas with significant contamination and alterations signs, under the coordination of a Conservator-Restorer, using micro-invasive and non-invasive methods (Annexe C-C5 and C6).

Microsamples (samples with less than 1 mm^2) for chromatic layers characterisation were removed near paint losses or cracks to avoid further damage, using a small chisel, on several areas selected after *in situ* X-ray fluorescence spectrometry and colorimetric analysis, to allow a full characterisation of the paintings polychromy and support mortars (Annexe E-E1).

For microbiological assays (Annexe E-E1), samples were collected under semi-aseptic conditions with sterile swabs and scalpels, placed in a suspension of transport MRD medium (Maximum Recovery Diluent, Merck), until utilisation.

3.2. Material characterisation

Microsamples collected were incorporated in epoxy resin (Epofix Fix) and polished to allow cross-section analysis.

Optical microscopy observations were carried out in a Leica DM2500 microscope in reflected light and dark field mode and digitally recorded by a Leica DFC290 HD photo camera, enabling stratigraphy analysis and pigment morphology.

To allow microstructural characterisation of the paint layers and elemental composition (point analysis and 2D mapping), the paint cross-sections were used as such or coated with Au-Pd (Balzers Union SCD 030) and analysed with a HITACHI 3700N variable pressure scanning electron microscope (VP-SEM) coupled with a Bruker XFlash 5010 energy dispersive X-ray (EDX) spectrometer with an accelerating voltage of 20 kV.

Selected samples were further investigated by micro-Raman spectrometry and micro-X-ray diffraction to confirm pigment identification and alteration products. Raman spectra were measured on a Horiba Xplora confocal spectrometer, using 1% of the power coming from a 25

mW laser diode operating at a wavelength of 637.1 nm. This low power was necessary to preserve the sample of burning. To improve the signal-to-noise ratio, several spectra (between 10 and 20) were accumulated for an exposure time of 120 s for each. X-ray diffraction was performed in a Bruker D8 Discovery diffractometer with Gadds detector and Cu X-ray source operating. The detector diffraction image was converted to a diffractogram by integration of the diffraction pattern in the range of 3-70º and 0.02º steps.

3.3. Microorganisms isolation and characterisation

Samples collected for microbiological studies were mechanically shaken for 1h, and after serial dilutions (10^{-1} to 10^{-3}) were prepared and inoculated (100 µL), under aseptic conditions, in NA (Nutrient Agar), for bacteria isolation, in MEA (Malt Extract Agar) and CRB (Cook Rose Bengal) for filamentous fungi isolation, and, in YPD (Yeast Extract Peptone Dextrose Agar) for yeast growth (Annexe A). The cultures were incubated at 30°C for 24-48 h, and at 28°C for 4-5 days, to allow bacterial and fungal development, respectively. After this period, the plates stayed in incubation at the same temperature to detect slow microbial development. The several colonies developed were picked up to obtain pure cultures, and then stored at 4ºC.

The microbial population was characterised based on macroscopic features of the colonies, and, in micro-morphology of the reproductive structures, that were observed in the optical microscope Leica DM 2500P, and the images were acquired with the digital camera Leica DFC290HD.

3.4. Analysis of mortars biological contamination

Mortar microfragments were coated with gold (Balzers Union SCD030), and analysed by Scanning Electron Microscopy with an accelerating voltage of 10–20 kV in secondary electron mode, to evaluate the microbial proliferation.

3.5. Antimicrobial activities

The antimicrobial activity of several water soluble commercial biocides such as Preventol PN [sodium 2, 3, 4, 5, 6-pentachlorophenplate], Preventol R-80 [alkyl-benzyldimethyl ammonium chloride], NEW-Des [Streptamidina H, Igran 500FW [Terbutryn], Wikamol Murosol [tributiltin oxide], Panacide [4-chloro-2-[(5-chloro-2-hydroxyphenyl)methyl] phenol] and Linquad were evaluated against the predominant fungi isolated, under sterile conditions. The biocides were

tested at different concentrations against the several microorganisms isolates from *Santo Aleixo* and *Casas Pintadas* mural paintings. Cultures of these microorganisms were prepared in Malt Extract Agar (MEA) slant and incubated at 25ºC for 7 days.

Fungal spore suspensions were prepared by adding loopful of hyphae and spores in 5 mL of NaCl 0.85% solution. The suspension was filtered by sterilised triple gauze and incorporated (10^8 CFU) in MEA at 45ºC. Sterile filter paper discs (Macherey-Nagel 827 ATD) were placed on agar and impregnated with 20 µL of biocide. The Petri dishes were incubated at 28ºC for 4-5 days. Antimicrobial activity was evaluated accordingly to the inhibition halo formation developed around the disc. The measurement was performed in triplicate plates.

3.6. *In situ* biocides application

Preventol PN, Panacide and Linquad were applied in *Casas Pintadas* (combined applications), first in small areas and after in all affected zones, since no changes were detected. After these experimental applications, this local was subjected to a conservation intervention, together with a combined application of these biocides to prevent recolonisation.

4. Results and Discussion

Nowadays, the preservation of artworks has been a growing concern due to their historical and cultural importance.

In this way several measures have been proposed. However, some conservation-intervention processes undervalue the contribution of biologic agents in the processes of degradation, often performing restoration works without the elimination of these agents. These procedures often put in question the durability of the restoration process, whose degradation can be enhanced due to nutrients availability, which are used by the remain microorganisms for their growth and proliferation in these surfaces.

In this section two different mural paintings studies will be presented.

The cases selected are inserted in completely distinct context, environment and conservation status. In the case of *Santo Aleixo* Church the degradation level is very high, having a lot of detached fragments that can be used for laboratorial assays, being a particular situation, since, most of the times only a very limited number of microsamples can be collected. This fact contributed to perform several tests that usually are impossible to carry out with microfragments.

On the other hand, *Casas Pintadas*, presents a completely different situation from the above mentioned, having only some alteration signs due to the particular fact that these paintings are in an outdoor environment. These paintings suffered a conservation-intervention and this study was developed within this framework. Presently, the *Casas Pintadas* paintings are completely rehabilitated and it is possible to visit them.

4.1. *Santo Aleixo* Church case study

The mural paintings present in the *Santo Aleixo* church are currently in an advanced status of degradation, since the building has been abandoned for almost 42 years. The consequence of this abandonment reflects in the collapse of the nave roof, the appearing and/or deepening of structural cracks, vandalism and theft of the central panel depicting *Santo Aleixo* marriage.

The degradation progress of these paintings can be observed through photos acquired in different years (Figure V-3), when it is possible the identification of biological colonisation as the main responsible for paint layers and mortars degradation and disruption. Several brownish to greenish stains are visible in the paint surface and seem to be spreading a little bit further each year. Therefore, measures to avoid the complete destruction have to be taken. Nevertheless, it is necessary to understand the physicochemical properties of the mural paintings materials, including paint layers, grounds, and mortars to avoid the use of inadequate materials in restoration and also to take measures to control and eliminate biological proliferation.

Figure V-3. General view of the *Santo Aleixo* main altar mural paintings. Photographs taken in 1960, 2010, 2013 and 2014.

Comparing the alterations over the years, it is clearly visible that a total loss of these paintings can happen in a few years, as well as all information contained therein.

4.1.1. Identification of pigments and painting technique

Microanalysis by SEM-EDX allowed the identification of iron in all samples collected from red areas (Annexe E-E1), indicating that the red pigments used were very likely red ochres, clay-based earth pigment containing mainly hematite (Fe_2O_3) as chromophore species (Figure V-4). The EDX spectra are typical of ochre pigments and elemental maps allowed the association between Si and Al with Fe in the red layers. The different shades were obtained by mixing lime, for the light areas, and/or animal black pigment (C + $Ca(PO_4)_2$), in shadow areas.

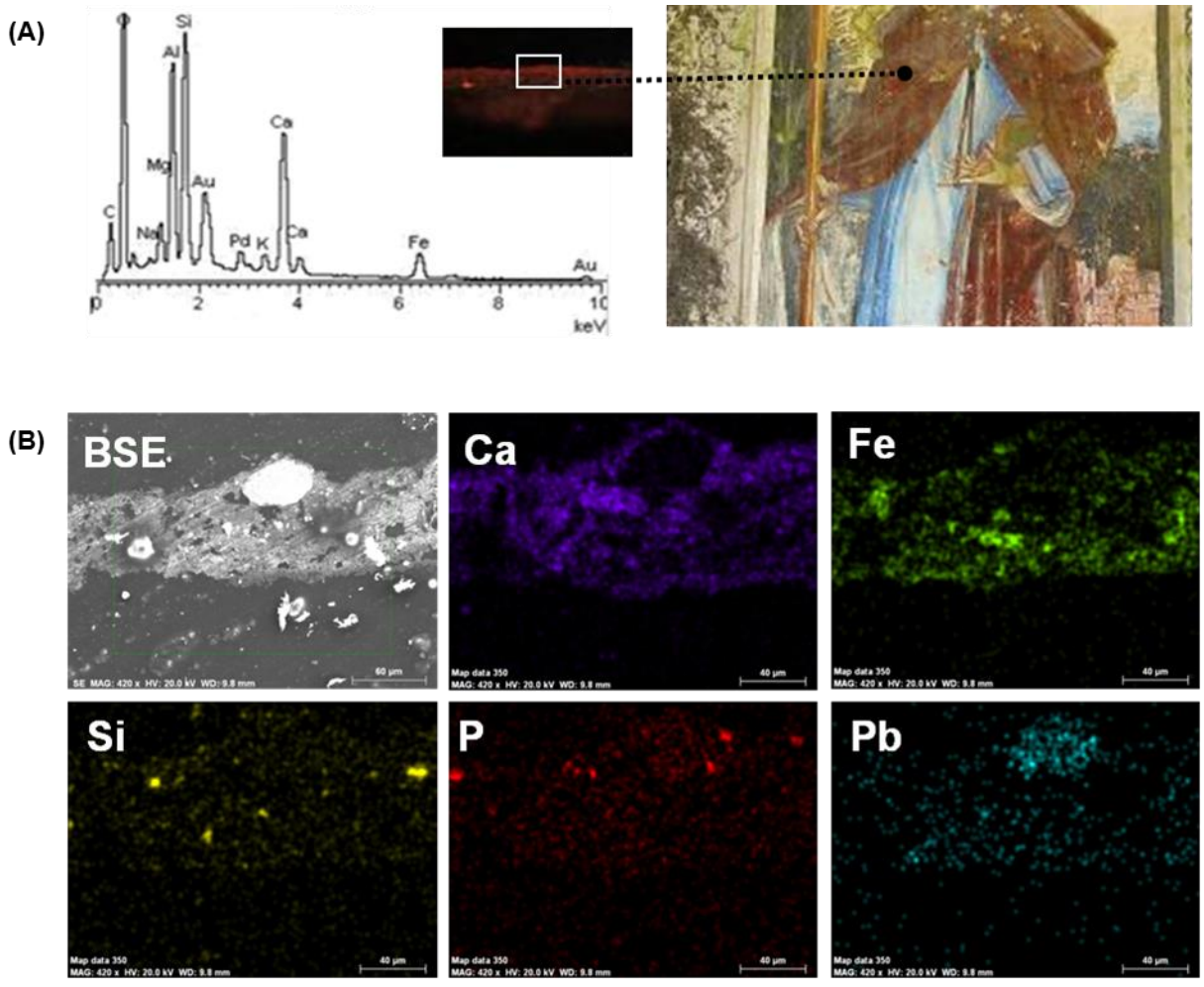


Figure V-4. Sample 42: (A) EDX spectrum of red pigment and (B) SEM micrograph in back-scattered mode and EDX 2D elemental maps of a cross-section from a red area.

Like for the red pigments, micro-analysis performed in the samples from yellow and brown areas (Annexe E-E1) allowed the identification of iron in all samples, indicating that yellow and brown pigments are yellow and brown ochres, pigments composed of clay minerals containing iron oxo-hydroxides such as goethite (FeOOH). EDX spectra obtained by scanning electron microscopy in Figure V-5 confirmed the association of Al and Si with Fe thus corroborating the use of ochre pigments.

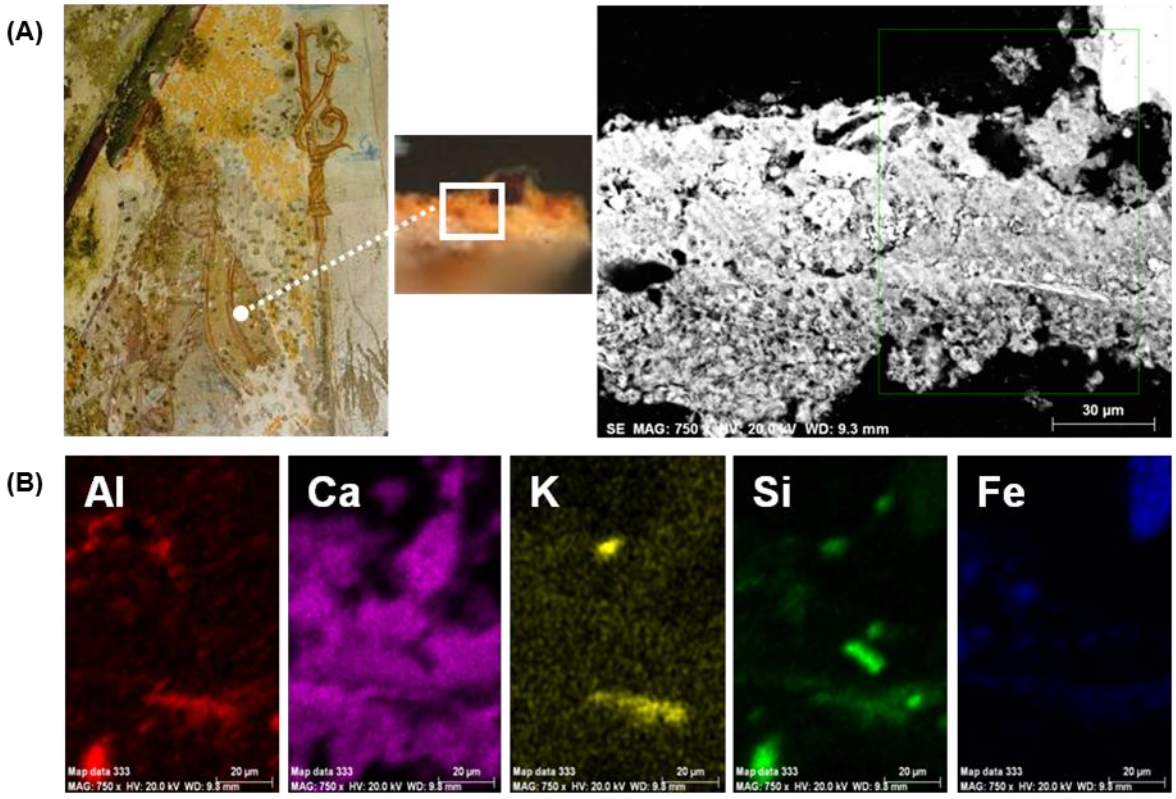


Figure V-5. Sample 8: SEM micrograph in back-scattered mode (A) and EDX 2D elemental maps (B) of a cross-section from a yellow-orange area.

Microchemical analysis of green samples (Annexe E-E1) detected the presence of copper, suggesting the use of a copper-based pigment, probably malachite. In Figure V-6, the EDX spectra and elemental mapping 2D confirmed the presence of Cu in green particles while the black areas are consistent with the use of animal black (presence of P and Ca, from *apatite*) just like was observed in the reds for the darker hues. Animal black pigment was also used intentionally as dark ground layer for the green foliage. Nowadays, almost all the green paint layers from these areas have disappeared.

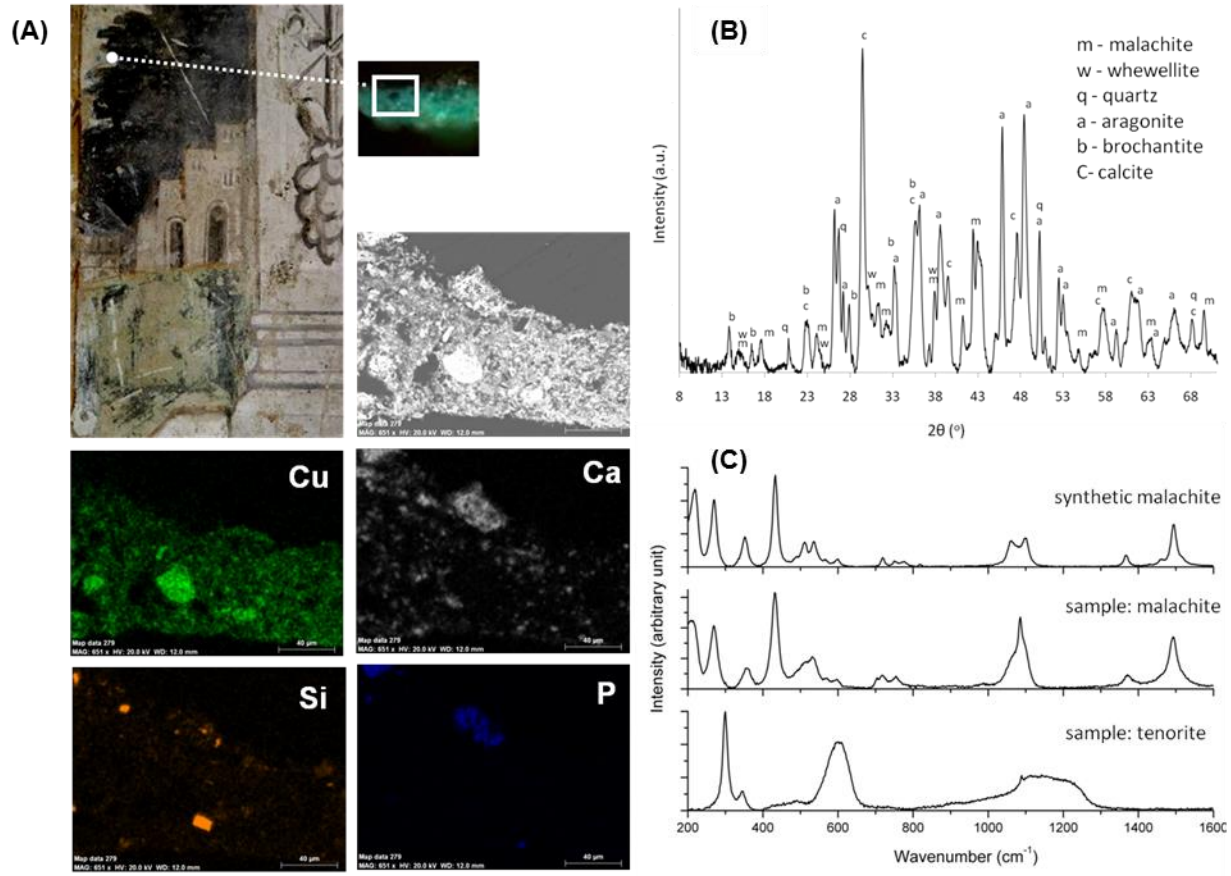


Figure V-6. Sample 4: (A) SEM micrograph in back-scattered mode and EDX 2D elemental maps of a cross-section from a green area (B) micro-X ray diffractogram of green particles and (C) micro-Raman spectra of green-black particles.

Micro-XRD and micro-Raman allowed the identification of the green pigment as malachite as well as the identification of degradation products namely *tenorite* (black copper oxide) and *whewellite* (calcium oxalate). Conversion of basic copper carbonates into *tenorite* is usually attributed to the exposure to an alkaline environment or warm situations. The paintings are sheltered from direct light exposure so the temperature does not seem to be the cause of its alteration. The most likely hypothesis is the painting technique since this pigment was mixed with a basic medium ($Ca(OH)_2$).

The presence of calcium oxalates indicates the action of oxalic acid ($H_2C_2O_4$) in the wall paintings. Oxalic acid could have promoted the decay of the basic green carbonates that were used by the painter. By reacting with calcium carbonate from the matrix and underneath mortars, it also continuously disrupts the material on which the artwork is based. This could be one of the reasons of the several chromatic lacunae present within the green decoration motifs.

As reported in Chapter II, the widespread formation of oxalates on paintings surfaces and on stone have been extensively studied (Pérez-Alonso *et al.*, 2006; Nevin *et al.*, 2008; Lluveras *et al.*, 2010; Rosado *et al.*, 2013a). However the origin of oxalates is still on discussion. Three hypotheses are currently appointed: a) metabolic products of biological activity (e.g. lichens); b) degradation of binding media (proteins and other organic materials) and finally, c) oxidation products of organic substances applied in conservation treatments. In the case study reported here, the first source seems to be the most likely origin (as noted previously in the discussion of biodegradation activity assessment). From the extensive green paint layers losses, the hypothesis b) cannot also be completely excluded although in the two paint layers analysed, the only binder found was calcite.

In the case of blue areas (Annexe E-E1), the only blue pigment (Figure V-7) used in *Santo Aleixo* main altar mural painting was the basic copper carbonate azurite. This pigment was identified by the optical properties under an optical microscope, by scanning electron microscopy (presence of copper) and confirmed by micro-XRD as illustrated in figure V-7B.

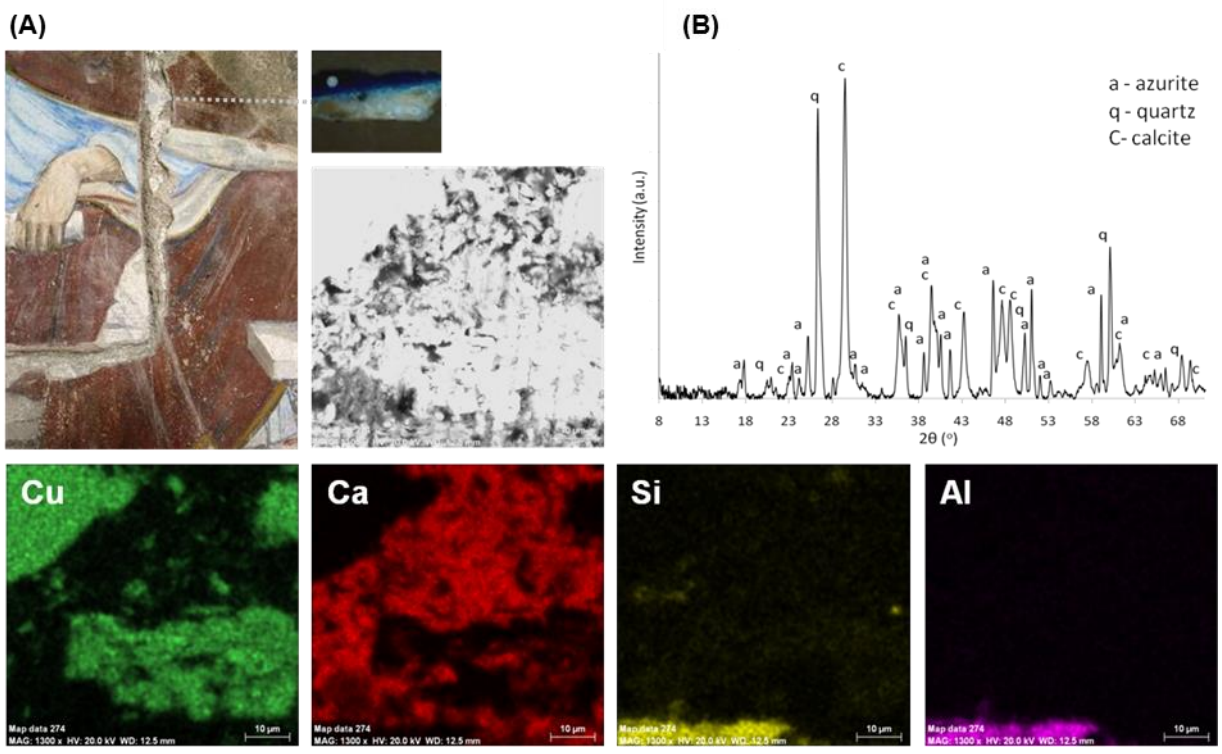


Figure V-7. Sample 12: (A) SEM micrograph in secondary electrons mode and EDX 2D elemental maps of a cross-section from a blue area and (B) micro-X ray diffractogram of blue particles.

In an alkaline environment it was also expected to find the black copper oxide (*tenorite*) as a degradation product. However, unlike malachite, in this case, azurite painted areas exhibit an extraordinary stability taking into consideration the fact that the pigment was laid down at *fresco*. One explanation for the stability of azurite painting may be due to the coarse nature of the pigment particles. Optical observation (Annexe E-E2) of the few black grains of tenorite present in the blue paint, shows that they have sizes smaller than 10 µm, which seems to corroborate this hypothesis (Gil *et al.*, 2011).

4.1.2. Microbiological study

The microbiological study allowed the isolation of 31 bacterial strains (eg Gram+ cocci, Gram+ bacilli, *Actinomycetes* sp.), five yeast strains and 53 filamentous fungi strains. The most predominant genera were *Penicillium* sp. and *Cladosporium* sp. *Aspergillus* sp., *Trichoderma* sp. and sterile micelia were also isolated. Figure V-8 presents macroscopic features of the colonies and microscopic morphology of the microorganisms both by optical microscopy and scanning electron microscopy.

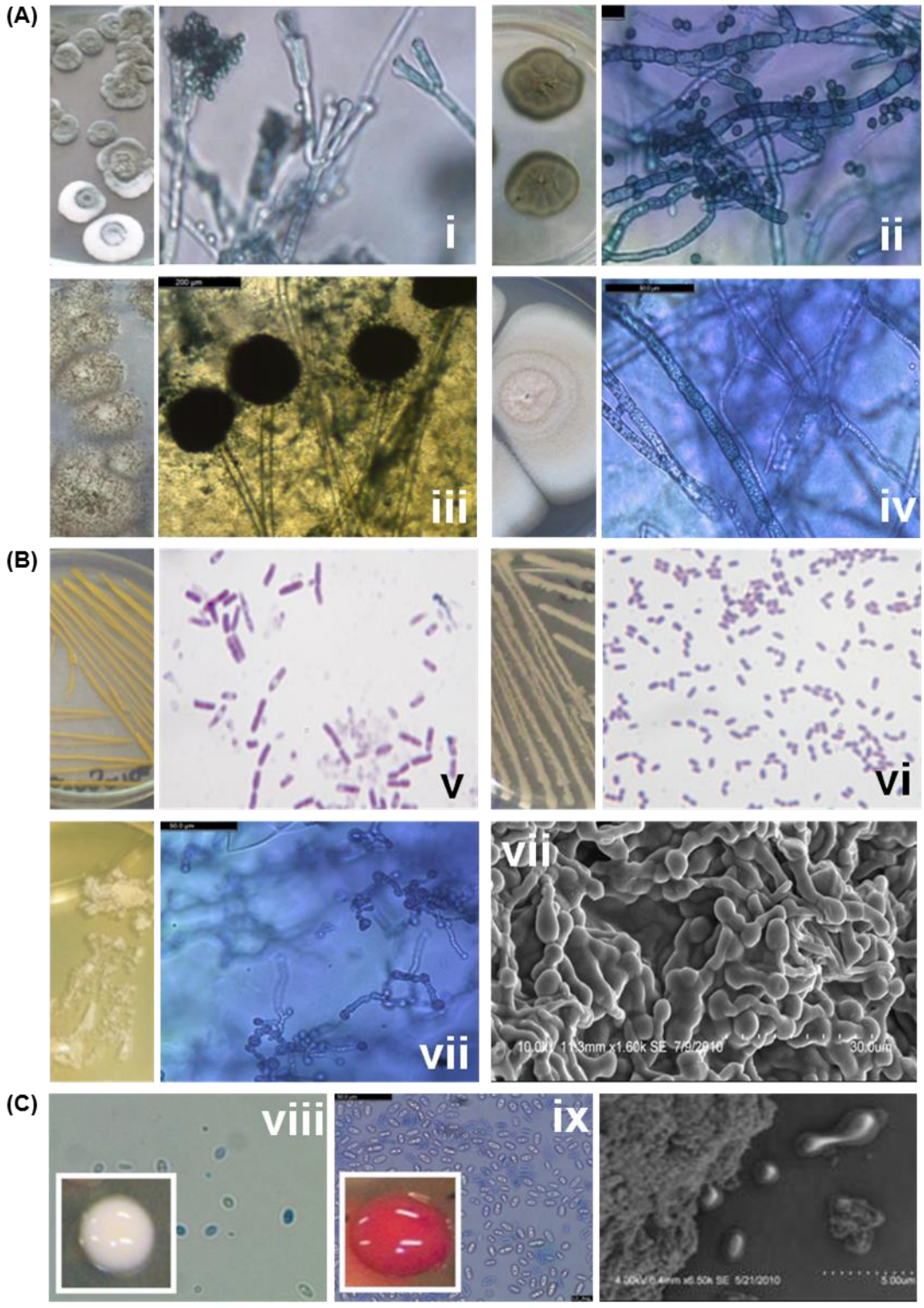


Figure V-8. Cultivable microbial population from *Santo Aleixo* church: (A) Main fungi isolation i - *Penicillium* sp.; ii - *Cladosporium* sp.; iii - A*spergillus niger;* iv- sterile mycelium; (B) Main bacterial isolates v - *Bacillus* sp., vi – Gram-positive cocci; vii – Actynomycetes, and, (C) Main yeast isolates viii, ix – unidentified yeast.

Additionally, sterile mortar samples were inoculated with the predominant isolates, and incubated during 1 month, in order to evaluate the proliferation ability of these microorganisms.

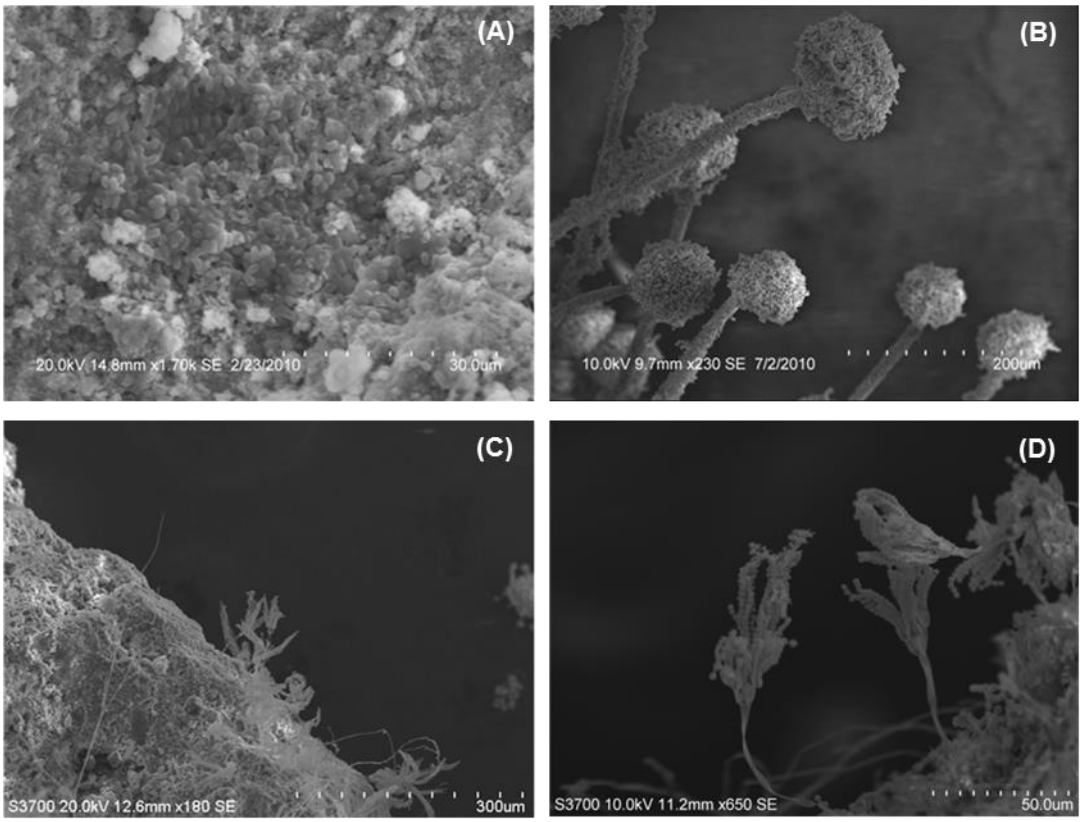


Figure V-9. SEM micrographs of mortars with bacterial contamination (A), reproductive structure of *Aspergillus sp.* grown (B) in the mortar, *Penicillium* sp. with reproductive structure and hiphae proliferation (C, D).

SEM observation in secondary electrons mode at high magnification and resolution allowed the observation of microorganisms showing that both bacterial and fungal population are capable of proliferating in the paintings (Figure V-9). Furthermore, fungal proliferation conduces to penetration of mycelia structures in the microstructure of the mortars, promoting dissemination of these microorganisms in depth (Figure V-9,B-D) while the bacterial growth occurs more superficially (Figure V-9A). These results complement the SEM analysis of the mortar microfragments collected in *Santo Aleixo* already showed in the Chapter IV.

4.1.3. Biocides treatment

The antimicrobial activity of several commercial biocides was evaluated against the predominant isolates found in the mural paintings. Table V-1 shows the inhibition halo results obtained against the main bacterial and fungal isolates.

Table V-1. Biocide activities against the main bacteria and fungal isolates from *Santo Aleixo* church.

	Inhibition halo (mm)				
	Preventol R-80 (5% v/v)	Preventol PN (0.6 % p/v)	Wikamol Murosol (1% v/v)	New Des (10% v/v)	Igran 500 FW (10% v/v)
***Actinomycetes* sp.**	29 ± 0.5	t.i.	t.i.	w.i.	38 ± 0.5
***Bacillus* sp.**	42 ± 1.5	51 ± 3.5	t.i.	w.i.	21 ± 0.5
***Pseudomonas* sp**	t.i.	t.i.	t.i.	w.i	29 ± 1.5
Cocci strain	t.i.	t.i.	t.i	w.i	w.i.
***Cladosporium* sp.**	30 ± 1.5	t.i.	35 ± 1.5	29 ± 2.0	w.i
***Penicillium* sp.**	37 ± 2.5	28 ± 1.5	49 ± 2.0	w.i.	w.i.
Aspergillus niger	29 ± 0.5	t.i.	26 ± 1.5	w.i.	w.i.
***Aspergillus* sp.**	24 ± 1.0	34 ± 1.2	31 ± 2.5	w.i.	w.i.
Sterile mycelium	32.9 ± 2.0	45.7 ± 1.0	32.2 ± 1.5	30.7 ± 2.5	n.d.

t.i. - total inhibition; w.i. - without inhibition; n.d. - not determined

The most effective biocides tested against bacteria were Preventol PN and Wikamol Murosol. Igram 500 FW which showed inhibition capacity for bacilli strains but were not active against cocci strains. The biocide New Des showed no inhibition capacity. For fungal communities, the most effective biocides were Preventol R-80 (inhibition zones ranging from 24 to 42 mm), Preventol PN (inhibition zones ranging for 28 mm to total inhibition) and Wikamol Murosol (inhibition zones from 26 to 49 mm). Igram 500 FW had no fungicide activity against the studied fungal strains.

Biodeterioration of mural paintings is caused by the attack of microorganisms which thrive and feed on the murals. Biocides used in chemical treatment exert their effect on the organisms in various ways, including oxidation, hydrolysis, denaturation, cell lysis, metabolic inhibition, and

alteration of membrane permeability (Warscheid and Braams, 2000; Allsopp *et al.*, 2004; Pepe *et al.*, 2010).

The commercial biocides used presented ability to inhibit the growth of all isolated microorganisms, showing satisfactory inhibition results particularly in a combined application of Preventol and Wikamol Murosol. In fact these two biocides belonging to the class of chlorophenols and organometalic compounds, respectively, act on cells by different mechanisms and their joint action can enhance a greater biocide activity.

This study allowed to perform a high number of studies, since these paintings are hugely degraded, and, in some cases with several fragments detached that can be used for simulation assays, giving useful information that can be used in other case studies.

4.2. Casas Pintadas in Évora case study

The other case study focused in this chapter is the sixteenth century mural paintings in the vaulted porch of the cloister of *Casas Pintad*as in Évora (Figure V-10). This study was developed under the framework of an integrated conservation-restoration intervention that took place during 2013, financed by *Fundação Eugénio de Almeida* and supervised by the Alentejo Regional Directorate for Culture. This intervention is one of the most complete examples of interdisciplinary research and encompassed the historical research, the material and biodegradation study for the support of the conservation-restoration intervention of this iconic national and international mural

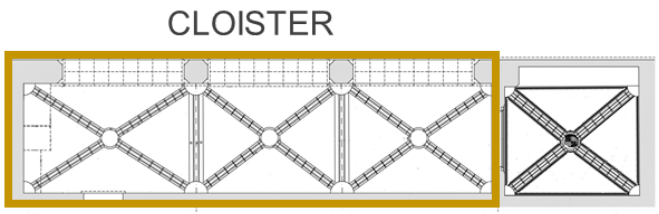


Figure V-10. General view of the mural paintings present in the cloister of *Casas Pintadas*.

The main problem of these paintings is their outdoor location, being exposed to several alteration agents that lead to deterioration/degradation mechanisms. One of the important agents that promote these alterations are microorganisms which gradually came to spread throughout the walls of the cloister, thus affecting, in various degrees, the whole surface of the paintings.

In this way, the identification of the biological agents and their short and medium term control was one of the most urgent operations, for preservation and future maintenance of these historical mural paintings.

4.2.1. Material characterisation

The material characterisation of mural paintings is an important step to understand an artist technique allowing a deeper knowledge on the pigments and mortars used and providing also crucial parameters for the conservation/restoration process and consequently contributing to their preservation. In this way, the strategy adopted started with *in situ* analysis which provided a global information about the mural paintings under study, allowing the careful selection of the collection points for the analytical and biological analysis.

These paintings showed a simple colour pallet composed by red, yellow, brown, blue and black pigments/shades. Stratigraphic analysis of the cross sections (Figure V-11), by optical microscopy and scanning electron microscopy enable the characterisation of these pigments.

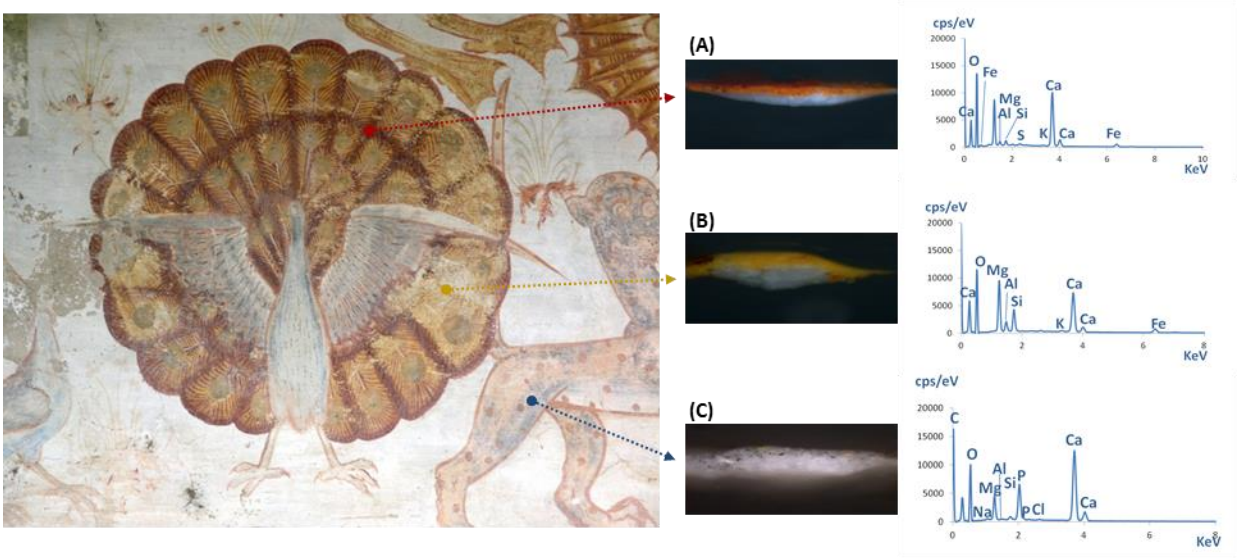


Figure V-11. SEM micrograph in back-scattered mode and EDX 2D elemental composition of a cross-section from red (A), yellow (B) and blue (C) areas.

Microanalysis by SEM-EDX of the red areas showed that red ochre is the pigment responsible for this colouration.

Ochres are composed by clay minerals (alluminosilicates) enriched in iron oxides and hydroxides, goethite (FeO(OH)) and hematite (Fe_2O_3) (Gil *et al.*, 2007). These compounds are easily identified by SEM-EDX by the presence of aluminium, silicon and potassium in their composition (Figure V-11A). Hematite (Fe_2O_3) is the responsible for the red colour of these pigments although these may also contain other chromophores in their composition, such as goethite (FeO(OH)) and manganese dioxide (MnO_2) that confer orange and brownish nuances (Gil *et al.*, 2009). Both on the upper painting as on the grotesque frieze, red ochres were used alone or in conjunction with bone black, a black pigment obtained from the burning of bones, and identified by the presence of Ca and P.

Like the red areas, yellow and brown colours were obtained with clay-based earth pigments, whose composition is similar to the red ochres, however the type and proportion of the iron compound present is different. In the yellow ochres, the chromophore responsible for the colour is mainly goethite, an iron hydroxide (FeO(OH)) compound (Figure V-11B). On the other hand, the brownish shades can be explained by the presence of manganese oxides, organic matter or others clay minerals (e.g. smectite).

The blue mineral azurite ($2Cu.CO_3.Cu(OH)_2$) was detected in the remaining blue layers of the flesh tones in the upper register of the cloister wall (Figure V-11C).

For the backgrounds of the grotesque frieze, currently dark bluish grey, however, no chromophore was identified. The absence of blue pigment particles in the samples analysed by optical microscopy and scanning electron microscopy, the detection of animal black and yet the presence of red and yellow ochres in the adjacent areas seem to indicate the possible use of an optical blue (Ashok, 1993).

In the black areas of the painting, the presence or absence of phosphates revealed the application of bone black or charcoal, respectively. Both pigments are artificially produced by calcination of organic matter (bones and wood).

4.2.2. Microbiological study

To assess the biodegradation of *Casas Pintadas*, samples from areas with obvious signs of alteration were analysed. This approach involved a detailed study in order to characterise the biological agents that promote degradation of the murals and to understand their propagation in the deteriorated areas.

Thereby, mortar microfragments were observed by SEM (Figure V-12) which confirmed microbiological contamination, showing the capacity of microorganisms to thrive in the paintings. Fungal hyphae proliferation in the microstructure of the mortars promote microbial depth dissemination (Figure V-12 A-F), fact that may explain the detachment and cracking observed in some areas of the painting.

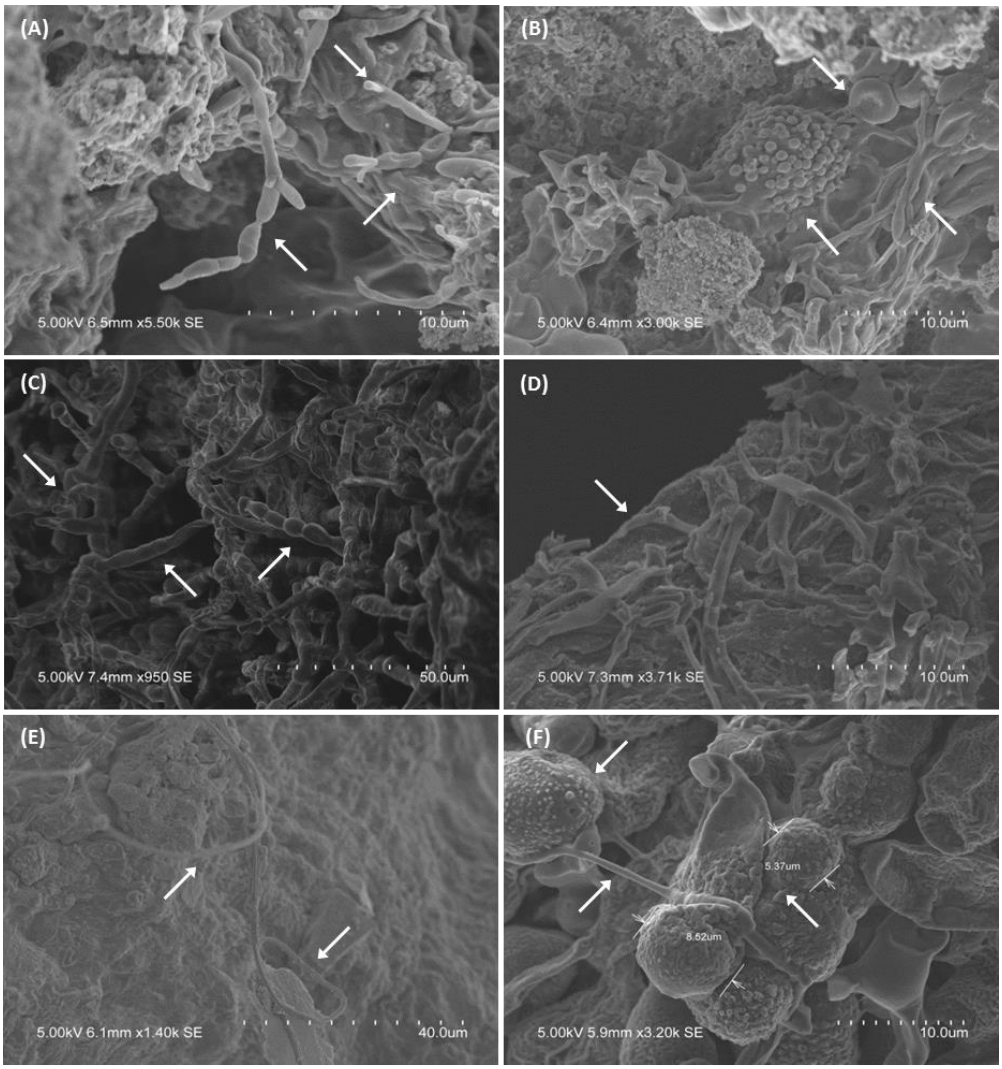


Figure V-12. SEM micrograph of mortar microfragments, evidencing filamentous fungi and hyphae proliferation in the surface of the mortar and the penetration of these microorganisms in depth.

In figure V-12D is evident the micellar structures of filamentous fungi**,** forming a biofilm on the surface of the mortar, covering some areas of the paint, which can induce pigment alterations due to the metabolic activity of the microorganisms or colour acquisition from the development of the

microorganisms in the surface of the walls. Biofilms are biological deposits of a highly hydrated gel of extracellular polymeric substances containing microbial cells and inorganic detritus that can drastically change the physicochemical characteristics of the environment in contact with the structural material and generally increase its aggressiveness (Herrera *et al.*, 2004; Harding *et al.*, 2009).

Once detected microbial proliferation in the paintings it was necessary to characterise this population, to identify the harmful microorganisms in the degradation process and the areas with the greatest contamination.

The microbiological study allowed the isolation and characterisation of several bacterial strains such as cocci and bacilli Gram-positive and *Actinomycetes* sp., yeast strains and filamentous fungi of the genera *Aspergillus*, *Cladosporium*, *Penicillium*, *Sporothrix*; microorganisms frequently founded in the mural paintings (Garg *et al.*, 1995; Gorbushina *et al.*, 2004; Sterflinger, 2010; Rosado *et al.*, 2013a; Rosado *et al.*, 2014a). In addition, other unclassified microorganisms namely mycelia and sterile mycelia were also isolated.

These results show a high microbial contamination in the paintings surface, fact that can be correlated with the damages observed, whose dissemination affected the structure and visual appearance of the paintings. Highly contaminated areas showed higher degradation levels, due to fungal proliferation as it was possible to detect by SEM analysis.

The paintings are located in an outdoor area of the building, exposed to variations of temperature, humidity and luminosity during the day and over the seasons of the year, which, together with the high biological contamination detected can promote chromatic alterations, cracking and detachment of some areas of the painting (Garg *et al.*, 1995; Ciferri, 1999; Gorbushina and Petersen, 2000 ; Milanesi *et al.*, 2006; Guiamet *et al.*, 2011).

The biological attack and biodeterioration processes are strongly influenced by water availability (Guiamet *et al.*, 2013). Since the paintings are in outdoor environment and are subject to extreme humidity due to the harsh winters in this location, this poses an important factor to take into account in a conservation strategy.

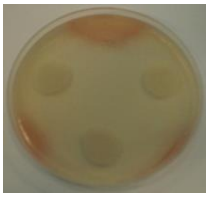
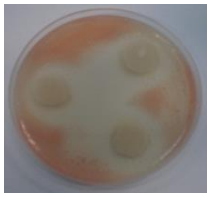
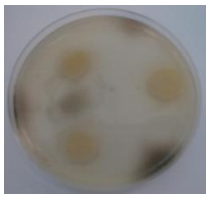
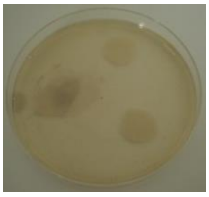
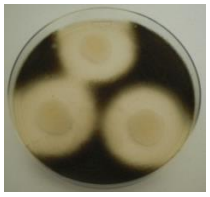
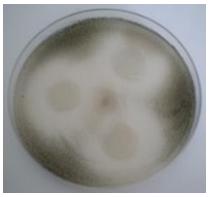
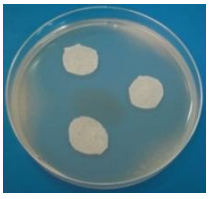
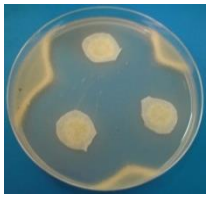
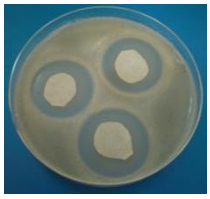
To ensure the longevity of the intervention and to avoid the fast recolonisation, remediation strategies were envisaged, using biocides treatment to control microbial proliferation.

4.2.3. Biocides application

To determine the efficacy of the biocides to eliminate the microorganisms which develop in these paintings, a serial dose laboratory tests were carried out with a high cell concentration of

fungi strains previously isolated from the wall paintings of the *Casas Pintadas*. The results are summarised in Table V-2 and analysed according to the inhibition halo formed in the cultures.

Table V-2. Effect of biocides against fungal isolates of *Casas Pintadas*.

	Preventol PN	**Panacide**	**Linquad**
Yeast			
Penicillium			
Sporothrix			
Cladosporium			
Aspergillus			
Mycelium			

Preventol PN was the most efficient biocide for inhibiting all fungal isolates. This biocide promoted almost total inhibition for all tested fungi except for the isolate *Aspergillus*. This compound has hydrophilic and hydrophobic chemical groups, able to disrupt the cell membrane structure of the microorganism causing leakage of intracellular materials (Ascaso *et al.*, 2002). The inhibition induced by Panacide was satisfactory for all tested microorganisms, being less effective for *Cladosporium*, however inhibits considerably their growth. Linquad produced satisfactory results, however showed lower inhibition capacity than the other biocides tested. This fact was evident for the fungal isolate whose identification was not possible so far, thereby calling it mycelium. The majority of the fungal isolates were inhibited with more efficiency by Preventol PN, followed by Panacide and then Linquad, except for *Cladosporium* wherein Linquad had greater inhibition capacity than Panacide.

Mixtures of biocides are frequently employed to allow an effective microbiological elimination, in order to avoid a quick recolonisation (Gaylarde *et al.*, 2011).

In this study the results show that the biocides have the ability to inhibit the growth of all isolated fungi, promoting good inhibition results particularly with a combined application of Preventol PN and Panacide in the wall paintings of *Casas Pintadas* (Figure V-13).

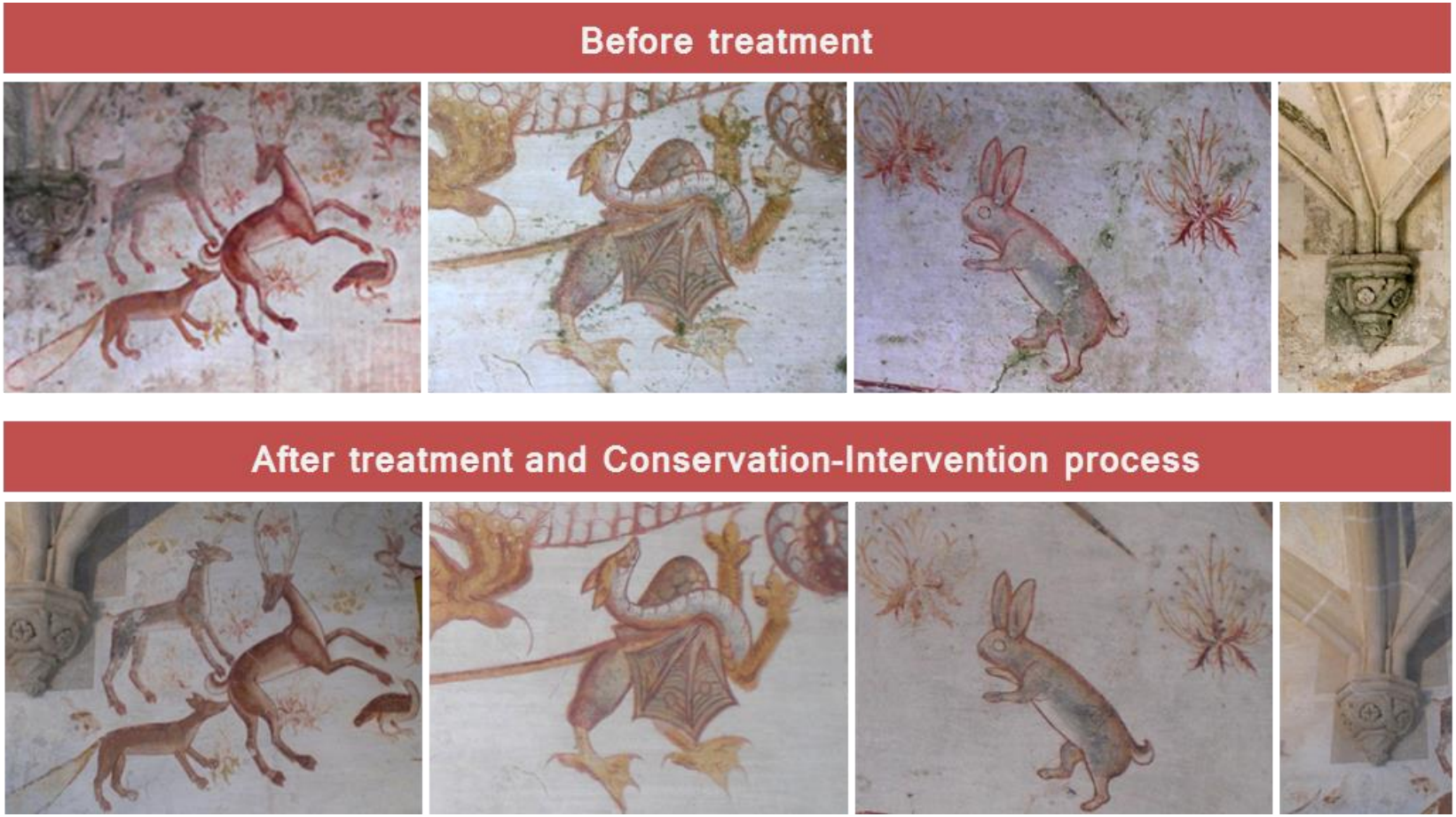


Figure V-13. Details of the mural paintings of *Casas Pintadas*, before and after treatment with biocide, and, intervention and restoration process.

The compounds considered in this work were able to eliminate and control microorganisms development and present low toxicity to humans. *In situ* application of biocides in these paintings did not promote chromatic alterations neither mortar damages.

During the conservation-restoration process, painting layers were fixed, holes were filled and some areas were retouched. One has to take into consideration that after the conservation-restoration process it is important to control and prevent possible recolonisation. Therefore, preventive conservation measures were taken and presently a long term *in situ* monitorisation is ongoing that encompasses weekly measures of T and RH in the paintings area, and monthly photogrametry/photographic assessment and collection of possible neoformation products (salts) and microorganisms.

5. Conclusions

The analytical methodology adopted, based on optical and scanning electron microscopy analyses and complemented with μ-XRD and μ-Raman, allowed the identification of the pigments palette used, as well as alteration products. This is a determinant step for the conservation-restoration intervention of these murals.

The microbial population detected in these paintings is responsible for the biodegradation on *Santo Aleixo* and *Casas Pintadas*, and may have an important role on the overall degradation process. Due to a wide microbial diversity present in these structures it was necessary to develop strategies to eliminate and prevent their proliferation. Biocides applications seem to be the answer for this problem, safeguarding the pigments and support matrix integrity. The greatest efficacy of biocides treatment is obtained for combined applications of these compounds once exist a wide variety of microorganisms.

The results show that to efficiently eliminate and control the development of the microorganisms actively involved in the biodegradation process it is crucial to have a deep knowledge of the processes and remediation solutions before the conservation-restoration intervention and after to develop preventive conservation monitorisation programs to ensure the longevity of the intervention.

CHAPTER VI

Concluding Remarks

This thesis developed a multidisciplinary approach to characterise the microbial population present in mural paintings and to identify the biodeteriogenic agents responsible for the degradation/deterioration of these heritage assets.

The results obtained reveal the involvement of the microorganisms in the paintings alterations, showing the active contribution of this agents in the mural paintings decay.

This work gives a substantial input in the biodegradation studies, providing a methodological advance in Portugal, for cultural heritage safeguard, having developed strategies and procedures that can constitute an experimental key to define protocols for cultural assets studies.

The early diagnosis and the development of strategies that characterise the biological agents, involved in the alterations of these artworks, and the effective elimination of these agents, are an added value to safeguard these heritage assets. The methodology used in this study and the strategies outlined proved to be very effective and promising in mural paintings decay prevention, and, can be adapted to other artworks for diagnostic and rehabilitation, contributing to the cultural heritage safeguard.

The methodology developed for biological characterisation under the framework of this research has proven that:

- The combined application of culture-dependent methods and molecular approaches are useful tools for microbial identification;
- Traditional cultivation methods allow to obtain high microbial cells concentration for simulation assays, and for metabolic and physiological tests;
- DGGE analysis allows multiple samples comparison enabling to discriminate different biological contamination levels;
- Pyrosequencing discriminate different biological contamination levels and reveal a strong potentialities for microbial identification from microsamples, proving to be revolutionary tool for the microbial knowledge in this field;
- The biological agents found in mural paintings were fungi of the genera *Aspergillus*, *Cladosporium*, *Penicillium*, *Cystoderma*, *Hypholoma*, *Tubaria*, *Pholiota*, *Armillaria*, *Physalacria*, *Chondrostereum*, *Schizophyllum*, *Coltricia*, *Fuscoporia*, *Hyphodontia*, *Phlebia*, *Radulomyces*, *Vesiculomyces*, *Russula*, *Amphinema*, *Hyphodontiella*,

Lactarius, *Cyphellostereum*, *Stereum*, *Skeletocutis*, *Ganoderma*, *Tyromyces*, *Antrodia*, *Postia*, *Coriolopsis*, *Rhodotorula and bacteria of the genera Bacillus, Catenibacterium*, *Anaerococcus*, *Roseburia*, *Veillonella*, *Atopostipes*, *Dolosigranulum*, *Granulicatella*, *Aerococcus*, *Abiotrophia*, *Streptococcus*, *Lactobacillus*, *Marinococcus*, *Virgibacillus*, *Geobacillus*, *Thermicanus*, *Staphylococcus*, *Salinicoccus*, *S. halodurans*, *Paenibacillus*, *Streptomyces*, *S.clavuligerus*, *Actinomyces*, *Nocardia*, *Rhodococcus*, *Corynebacterium*, *Arthrobacter*, *Micrococcus*, *Kocuria*, *Rothia*, *Blastococcus*, *Geodermatophilus*, *Bifidobacterium*, *Oligella*, *Haemophilus*, *Pseudoxanthomonas*, *Pseudomonas* and *Sphingomonas*;

- The metabolic activity assessment allowed the signalisation of the main biodeteriogenic agents involved in the mural paintings decay: *Penicillium* sp., *Cladosporium* sp., *Aspergillus* sp., *Rhodotorula* sp. and *Bacillus* sp.;
- Areas with high alteration signs are correlated with high microbiologic contamination;
- Cell viability assays revealed to be an effective fast way to monitor and quantify microbial metabolic activity;
- There is a strong relationship between the presence of microorganisms metabolically active and the areas highly altered;
- Oxalate compounds, *plattnerite* and carotenoids were the main alteration products detected in the studied damaged mural paintings and mortars;
- *Whewellite* and *weddellite* were found essentially in altered areas, as a result of biological metabolism, mainly caused by *Bacillus* sp. metabolic activity, inducing chromatic alteration by biofilm development and oxalate compounds formation;
- *Plattnerite* detected in altered carnation areas of the paintings results from the oxidation of lead-based compounds;
- Fungi of the genera *Penicillium* and *Cladosporium* seem to be related with *plattnerite* formation;
- *Rhodotorula* sp. was identified as a biodeteriogenic agent, responsible for the pink/dark orange stains, that cover the mortars in the inner walls of Évora Cathedral;

- Aesthetic damages like chromatic alterations and stains appearance seem to be particularly related with bacteria and yeast proliferation, however some fungi are also responsible for these damages;
- Structural damages like salt efflorescence formation, cracks and detachment of some mortar fragments are associated to fungal development due to the hyphae ability to proliferate in the microstructure of the mortars promoting microbial depth dissemination;
- Biocides application are very effective in microbial inhibition of all microorganisms tested;
- Preventol PN and Panacide show, in most of the cases, high inhibition capacity;
- Fungi of the genera *Cladosporium* were the microorganisms more resistant to the tested biocides;
- The combined application of biocides allows better inhibition levels, since the action spectrum is enlarged;
- In the case of a conservation-intervention process, the durability of the intervention is increased with biocides application due to its capacity to inhibit microbial development.

In order to increase the knowledge about the biological agents that colonise mural paintings further approaches could be considered.

An important step in the mural painting degradation/deterioration studies is the detection of the biological agents. However, it is also important to know if they are active or not. In this way comparative DGGE analyses of the total DNA and RNA from microbial communities should be applied, in order to estimate the biodeteriogenic potential in a fast screening. On the other hand, real time-PCR can also be a useful contribution, providing quantitative information.

Biological contamination should be regularly monitored, once the environmental conditions can be altered and the presence of inactive microorganisms represents a potential risk. Additionally, other biological agents can develop in the paintings. The application of Fluorescence *In Situ* Hybridisation, with specific primers for biodeteriogenic agents, can be taken into account to perform their monitorisation.

In the point of view of the mitigation strategies, the development and application of natural/novel biocides, environmental friendly and more effective should be an interesting alternative to the commercial compounds usually applied.

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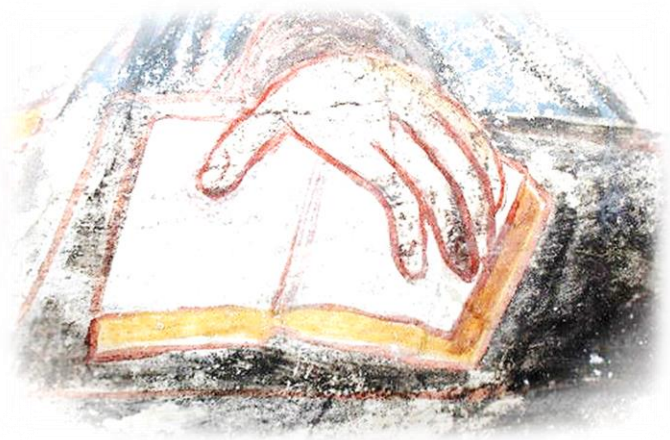

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ANNEXES

ANNEXE A. Culture medium composition

Table A-1. Composition of the several culture media used to microbiological development.

<table>
<tr><th>NA</th><th>NB</th><th>MEA</th><th>MEA</th><th>CRB</th><th>YEPD</th></tr>
<tr><td colspan="2">5 g/L Peptic digest animals</td><td colspan="2">30 g/L Malt extract</td><td>5 g/L Peptone</td><td>10 g/L Yeast extract</td></tr>
<tr><td colspan="2">1.5 g/L Beef extract</td><td colspan="2">5 g/L Peptone mycologic</td><td>10g/L Glucose</td><td>10g/L Peptone</td></tr>
<tr><td colspan="2">1.5 g/L Yeast extract</td><td colspan="2">20 g/L Glucose</td><td>1 g/L K_2HPO_4</td><td>20 g/L Dextrose</td></tr>
<tr><td colspan="2">5 g/L Sodium Chloride</td><td rowspan="4">15 g/L Agar</td><td rowspan="4">---</td><td>0.5 g/L $MgSO_4$</td><td rowspan="4">20 g/L Agar</td></tr>
<tr><td rowspan="3">15 g/L Agar</td><td rowspan="3">---</td><td>0.05 g/L Rose Bengal</td></tr>
<tr><td>0.1 g/L Chloramphenicol</td></tr>
<tr><td>15.5 g/L Agar</td></tr>
</table>

NA – Nutrient Agar; NB – Nutrient Broth; MEA - Malt Extract Agar; ME - Malt Extract; CRB - Cook Rose Bengal; YEPD - Yeast Extract Peptone Dextrose Agar

ANNEXE B. Solutions composition

B1. DGGE solutions

Stock solution 0%	Stock solution 80%
1.5 mL TAE 5x	1.5 mL TAE 5x
30 mL Acrylamide 40%	30 mL Acrylamide 40%
120 mL distilled water	30 mL distilled water
	48 mL Formamide
	50.4 g Urea

Solutions to gel preparation

Solution 30%	Solution 50%
7 mL Solution stock 0%	4.1 mL Solution stock 0%
4mL Solution stock 80%	6.9 mL Solution stock 80%
7.7 µL TEMED	7.7 µL TEMED
55 µL APS	55 µL APS

B2. TBE 10X (pH8)

Tris 890 mM

Boric acid 890 mM

EDTA 0.5 M

TBE 1x (pH8)

TBE 10x diluted 1/10 in sterile water

B3. TAE 50x

242g Tris

57.1 mL Acetic acid

EDTA 0.5M pH8.0

(for 1L)

TAE 0.5x

TAE 0.5x diluted 1/10 in sterile water

B4. Modified Universal Buffer (MUB)

12.1 g Tris

11.6 g Maleic acid

14.0 g Citric acid

6.3 g Boric acid

(for 1L)

ANNEXE C. Mural paintings samples collection

C1. *Santa Clara* Church, Sabugueiro

Figure C-1. Photographs of the sampling process performed in the *Santa Clara* Church.

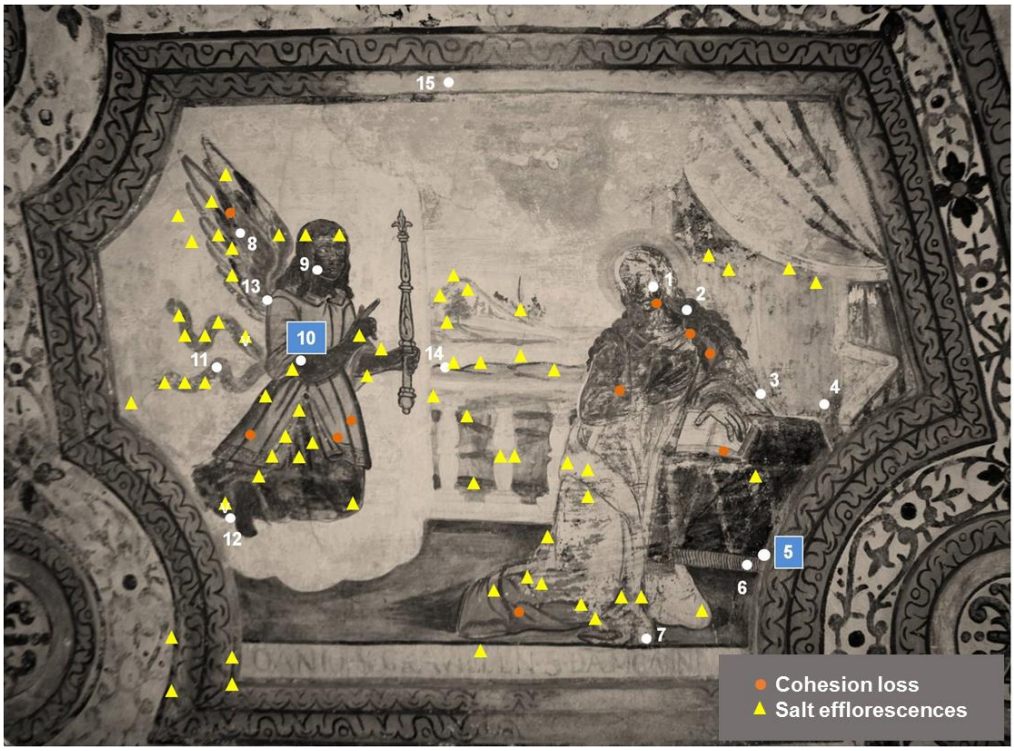

Figure C-2. Schematic representation of the salt efflorescence location and areas affected by cohesion loss in Panel 1 of the *Santa Clara* Church.

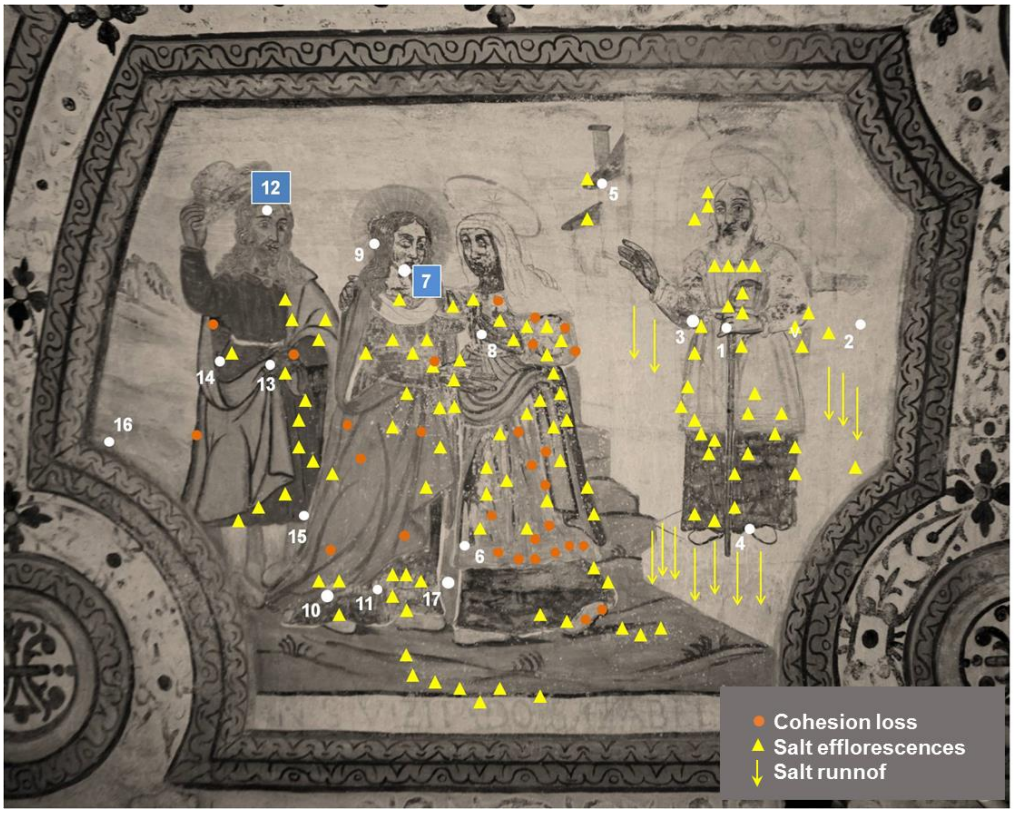

Figure C-3. Schematic representation of the salt efflorescence location and areas affected by cohesion loss in Panel 2 of the *Santa Clara* Church.

C2. Low Choir of *Nossa Senhora da Saudação* Convent, Montemor-o-Novo

Figure C-4. Photographs showing the collection of samples in the Low Choir of the *Nossa Senhora da Saudação* Convent.

C3. Évora Cathedral, Évora

Figure C-5. Examples of the sampling process performe in Évora Cathedral.

C4. *Condes de Basto* Palace, Évora

Figure C-6. Sampling process carried out in *Condes de Basto* Palace.

C5. *Santo Aleixo* Church, Montemor-o-Novo

Figure C-7. Photographs acquired during the sampling process performed in the *Santo Aleixo* Church.

C6. *Casas Pintadas*, Évora

Figure C-8. Photographs showing the sampling process performed in *Casas Pintadas*.

ANNEXE D. Enzymatic monitorisation

D1. Protein quantification

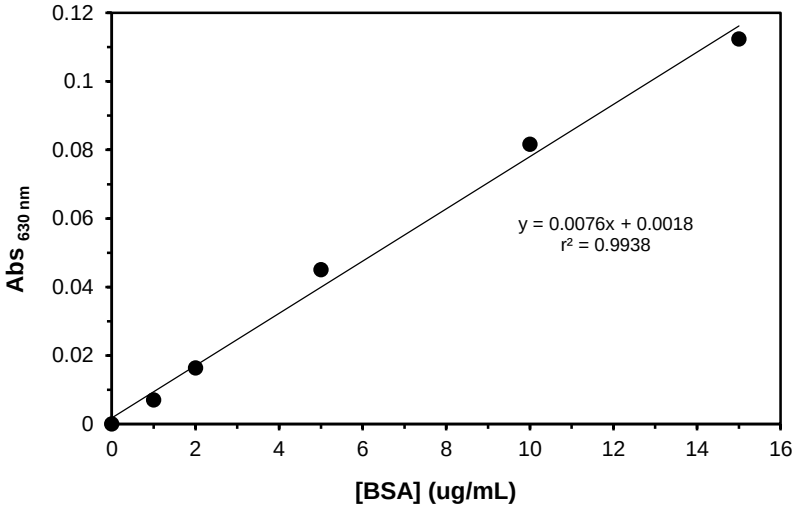

Figure D-9. Calibration curve for protein quantification.

D 2. Substrate quantification

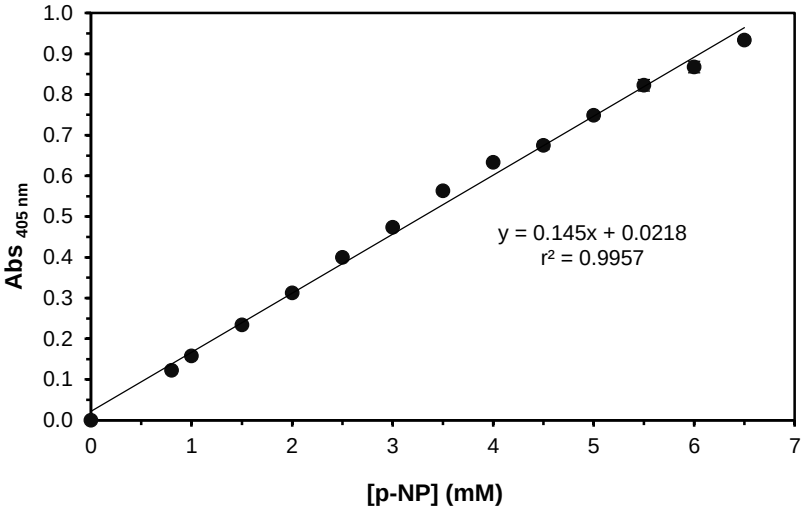

Figure D -10. Calibration curve of the p-nitrophenol (p-NP) for phosphatase quantification.

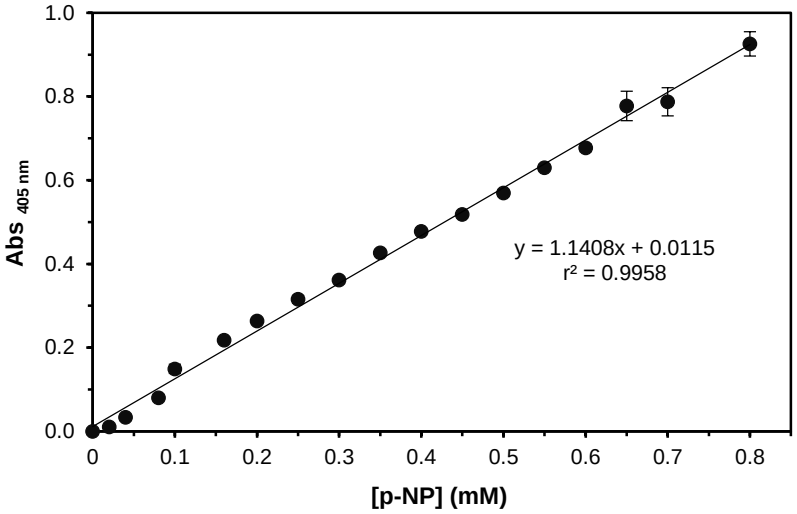

Figure D -11. Calibration curve of the p-nitrophenol (p-NP) for arylsulphatase quantification.

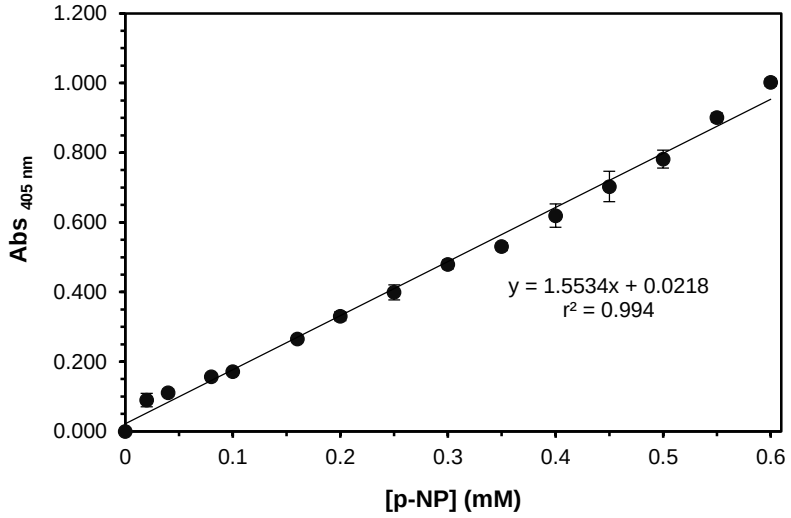

Figure D -12. Calibration curve of the p-nitrophenol (p-NP) for *β*-glucosidase quantification.

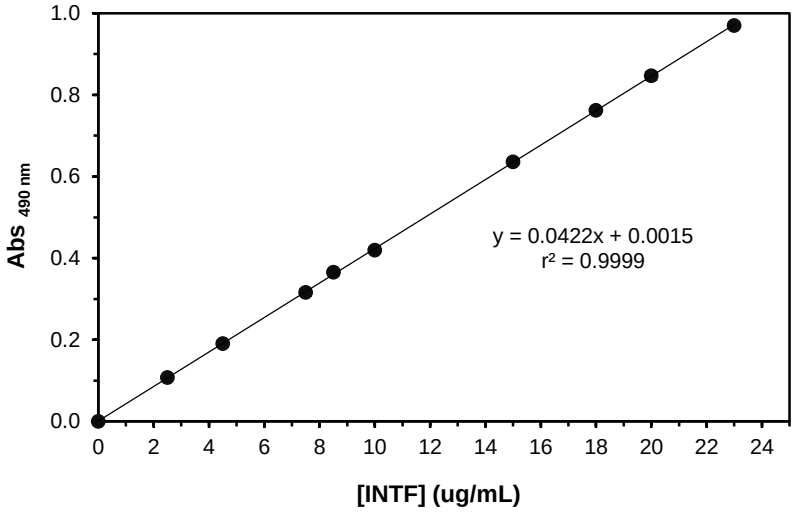

Figure D -13. Calibration curve of the iodonitrotetrazolium formazan (INTF) for dehydrogenase quantification.

D3. Statistical analysis

Table D-2. Analysis of variance (ANOVA) of the enzymatic activity of liquid cultures.

		Sum of Squares	df	Mean Square	F	Sig.
Arylsuphatase	Between Groups	11.496	4	2.874	14.641	0.000
	Within Groups	1.963	10	0.196		
	Total	13.459	14			
β-Glucosidase	Between Groups	3541.474	4	885.369	79.765	0.000
	Within Groups	110.997	10	11.100		
	Total	3652.471	14			
Phosphatase	Between Groups	1942.756	4	485.689	21.069	0.000
	Within Groups	230.521	10	23.052		
	Total	2173.277	14			
Dehydrogenase	Between Groups	0.124	4	0.031	2255.992	0.000
	Within Groups	0.000	10	0.000		
	Total	0.124	14			

Table D-3. Average multiple comparison of the enzymatic activity of liquid cultures, by Tukey HSD test.

Dependent Variable	(I) Activity	(J) Activity	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Arylsuphatase	*Rhodotorula*	*Penicillium*	1.24522*	0.36176	0.040	0.0546	2.4358
		Cladosporium	-0.61816	0.36176	0.470	-1.8087	0.5724
		Aspergillus	1.02694	0.36176	0.100	-0.1636	2.2175
		Mix cultures	1.79585*	0.36176	0.004	0.6053	2.9864
	Penicillium	*Rhodotorula*	-1.24522*	0.36176	0.040	-2.4358	-0.0546
		Cladosporium	-1.86338*	0.36176	0.003	-3.0540	-0.6728
		Aspergillus	-0.21829	0.36176	0.971	-1.4089	0.9723
		Mix cultures	0.55063	0.36176	0.572	-0.6400	1.7412
	Cladosporium	*Rhodotorula*	0.61816	0.36176	0.470	-0.5724	1.8087
		Penicillium	1.86338*	0.36176	0.003	0.6728	3.0540
		Aspergillus	1.64509*	0.36176	0.007	0.4545	2.8357
		Mix cultures	2.41401*	0.36176	0.000	1.2234	3.6046
	Aspergillus	*Rhodotorula*	-1.02694	0.36176	0.100	-2.2175	0.1636
		Penicillium	0.21829	0.36176	0.971	-0.9723	1.4089
		Cladosporium	-1.64509*	0.36176	0.007	-2.8357	-0.4545
		Mix cultures	0.76892	0.36176	0.281	-0.4217	1.9595
	Mix cultures	*Rhodotorula*	-1.79585*	0.36176	0.004	-2.9864	-0.6053
		Penicillium	-0.55063	0.36176	0.572	-1.7412	0.6400
		Cladosporium	-2.41401*	0.36176	0.000	-3.6046	-1.2234
		Aspergillus	-0.76892	0.36176	0.281	-1.9595	0.4217
Glucosidase	*Rhodotorula*	*Penicillium*	25.54609*	2.72026	0.000	16.5935	34.4987
		Cladosporium	25.06941*	2.72026	0.000	16.1168	34.0220
		Aspergillus	18.13342*	2.72026	0.000	9.1808	27.0860
		Mix cultures	-13.47619*	2.72026	0.004	-22.4288	-4.5236
	Penicillium	*Rhodotorula*	-25.54609*	2.72026	0.000	-34.4987	-16.5935
		Cladosporium	-0.47668	2.72026	1.000	-9.4293	8.4759
		Aspergillus	-7.41267	2.72026	0.119	-16.3653	1.5399
		Mix cultures	-39.02228*	2.72026	0.000	-47.9749	-30.0697
	Cladosporium	*Rhodotorula*	-25.06941*	2.72026	0.000	-34.0220	-16.1168
		Penicillium	0.47668	2.72026	1.000	-8.4759	9.4293
		Aspergillus	-6.93599	2.72026	0.155	-15.8886	2.0166

		Mix cultures	-38.54559*	2.72026	0.000	-47.4982	-29.5930
	Aspergillus	*Rhodotorula*	-18.13342*	2.72026	0.000	-27.0860	-9.1808
		Penicillium	7.41267	2.72026	0.119	-1.399	16.3653
		Cladosporium	6.93599	2.72026	0.155	-2.0166	15.8886
		Mix cultures	-31.60961*	2.72026	0.000	-40.5622	-22.6570
	Mix cultures	*Rhodotorula*	13.47619*	2.72026	0.004	4.5236	22.4288
		Penicillium	39.02228*	2.72026	0.000	30.0697	47.9749
		Cladosporium	38.54559*	2.72026	0.000	29.5930	47.4982
		Aspergillus	31.60961*	2.72026	0.000	22.6570	40.5622
Phosphatase	*Rhodotorula*	*Penicillium*	2.99333	3.92021	0.935	-9.9084	15.8951
		Cladosporium	-19.62218*	3.92021	0.004	-32.5239	-6.7204
		Aspergillus	-26.80943*	3.92021	0.000	-39.7112	-13.9077
		Mix cultures	-7.99367	3.92021	0.315	-20.8954	4.9081
	Penicillium	*Rhodotorula*	-2.99333	3.92021	0.935	-15.8951	9.9084
		Cladosporium	-22.61551*	3.92021	0.001	-35.5173	-9.7138
		Aspergillus	-29.80276*	3.92021	0.000	-42.7045	-16.9010
		Mix cultures	-10.98701	3.92021	0.106	-23.8887	1.9147
	Cladosporium	*Rhodotorula*	19.62218*	3.92021	0.004	6.7204	32.5239
		Penicillium	22.61551*	3.92021	0.001	9.7138	35.5173
		Aspergillus	-7.18725	3.92021	0.407	-20.0890	5.7145
		Mix cultures	11.62851	3.92021	0.083	-1.2732	24.5302
	Aspergillus	*Rhodotorula*	26.80943*	3.92021	0.000	13.9077	39.7112
		Penicillium	29.80276*	3.92021	0.000	16.9010	42.7045
		Cladosporium	7.18725	3.92021	0.407	-5.7145	20.0890
		Mix cultures	18.81576*	3.92021	0.005	5.9140	31.7175
	Mix cultures	*Rhodotorula*	7.99367	3.92021	0.315	-4.9081	20.8954
		Penicillium	10.98701	3.92021	0.106	-1.9147	23.8887
		Cladosporium	-11.62851	3.92021	0.083	-24.5302	1.2732
		Aspergillus	-18.81576*	3.92021	0.005	-31.7175	-5.9140
Dehydrogenase	*Rhodotorula*	*Penicillium*	0.00192	0.00303	0.966	-0.0080	0.0119
		Cladosporium	-0.21500*	0.00303	0.000	-0.2250	-0.2050
		Aspergillus	0.02010*	0.00303	0.000	0.0101	0.0301
		Mix cultures	0.02274*	0.00303	0.000	0.0128	0.0327
	Penicillium	*Rhodotorula*	-0.00192	0.00303	0.966	-0.0119	0.0080
		Cladosporium	-0.21692*	0.00303	0.000	-0.2269	-0.2070
		Aspergillus	0.01818*	0.00303	0.001	0.0082	0.0281
		Mix cultures	0.02082*	0.00303	0.000	0.0109	0.0308

	Cladosporium	*Rhodotorula*	0.21500*	0.00303	0.000	0.2050	0.2250
		Penicillium	0.21692*	0.00303	0.000	0.2070	0.2269
		Aspergillus	0.23510*	0.00303	0.000	0.2251	0.2451
		Mix cultures	0.23774*	0.00303	0.000	0.2278	0.2477
	Aspergillus	*Rhodotorula*	-0.02010*	0.00303	0.000	-0.0301	-0.0101
		Penicillium	-0.01818*	0.00303	0001	-0.0281	-0.0082
		Cladosporium	-0.23510*	0.00303	0.000	-0.2451	-0.2251
		Mix cultures	0.00264	0.00303	0.901	-0.0073	0.0126
	Mix cultures	*Rhodotorula*	-0.02274*	0.00303	0.000	-0.0327	-0.0128
		Penicillium	-0.02082*	0.00303	0.000	-0.0308	-0.0109
		Cladosporium	-0.23774*	0.00303	0.000	-0.2477	-0.2278
		Aspergillus	-0.00264	0.00303	0.901	-0.0126	0.0073

*. The mean difference is significant at the 0.05 level.

Table D-4. Analysis of variance (ANOVA) of the enzymatic activity of simulation assays.

		Sum of Squares	df	Mean Square	F	Sig.
Arylsuphatase	Between Groups	8.094	4	2.023	9.104	0.002
	Within Groups	2.222	10	0.222		
	Total	10.316	14			
β-Glucosidase	Between Groups	2.456	4	0.614	2.068	0.160
	Within Groups	2.969	10	0.297		
	Total	5.426	14			
Phosphatase	Between Groups	46031.099	4	11507.775	7.641	0.004
	Within Groups	15060.005	10	1506.001		
	Total	61091.105	14			
Dehydrogenase	Between Groups	219724.465	4	54931.116	92.910	0.000
	Within Groups	5912.294	10	591.229		
	Total	225636.759	14			

Table D-5. Average multiple comparison of the enzymatic activity of simulation assays, by Tukey HSD test.

Dependent Variable	(I) Activity	(J) Activity	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval Lower Bound	95% Confidence Interval Upper Bound
Arylsuphatase	*Rhodotorula*	*Penicillium*	0.39793	0.38492	0.834	-0.8689	1.6647
		Cladosporium	-1.67587*	0.38492	0.010	-2.9427	-0.4091
		Aspergillus	0.17072	0.38492	0.991	-1.0961	1.4375
		Mix cultures	-0.17478	0.38492	0.990	-1.4416	1.0920
	Penicillium	*Rhodotorula*	-0.39793	0.38492	0.834	-1.6647	0.8689
		Cladosporium	-2.07381*	0.38492	0.002	-3.3406	-0.8070
		Aspergillus	-0.22722	0.38492	0.974	-1.4940	1.0396
		Mix cultures	-0.57272	0.38492	0.591	-1.8395	0.6941
	Cladosporium	*Rhodotorula*	1.67587*	0.38492	0.010	0.4091	2.9427
		Penicillium	2.07381*	0.38492	0.002	0.8070	3.3406
		Aspergillus	1.84659*	0.38492	0.005	0.5798	3.1134
		Mix cultures	1.50109*	0.38492	0.019	0.2343	2.7679
	Aspergillus	*Rhodotorula*	-0.17072	0.38492	0.991	-1.4375	1.0961
		Penicillium	0.22722	0.38492	0.974	-1.0396	1.4940
		Cladosporium	-1.84659*	0.38492	0.005	-3.1134	-0.5798
		Mix cultures	-0.34550	0.38492	0.891	-1.6123	0.9213
	Mix cultures	*Rhodotorula*	0.17478	0.38492	0.990	-1.0920	1.4416
		Penicillium	0.57272	0.38492	0.591	-0.6941	1.8395
		Cladosporium	-1.50109*	0.38492	0.019	-2.7679	-0.2343
		Aspergillus	0.34550	0.38492	0.891	-0.9213	1.6123
β-Glucosidase	*Rhodotorula*	*Penicillium*	-1.23373	0.44491	0.111	-2.6980	0.2305
		Cladosporium	-0.68806	0.44491	0.558	-2.1523	0.7762
		Aspergillus	-0.90268	0.44491	0.320	-2.3669	0.5615
		Mix cultures	-0.76597	0.44491	0.464	-2.2302	0.6983
	Penicillium	*Rhodotorula*	1.23373	0.44491	0.111	-0.2305	2.6980
		Cladosporium	0.54567	0.44491	0.738	-0.9186	2.0099
		Aspergillus	0.33104	0.44491	0.941	-1.1332	1.7953
		Mix cultures	0.46776	0.44491	0.826	-0.9965	1.9320
	Cladosporium	*Rhodotorula*	0.68806	0.44491	0.558	-0.7762	2.1523
		Penicillium	-0.54567	0.44491	0.738	-2.0099	0.9186
		Aspergillus	-0.21463	0.44491	0.987	-1.6789	1.2496

		Mix cultures	-0.07791	0.44491	1.000	-1.5421	1.3863
	Aspergillus	*Rhodotorula*	0.90268	0.44491	0.320	-0.5615	2.3669
		Penicillium	-0.33104	0.44491	0.941	-1.7953	1.1332
		Cladosporium	0.21463	0.44491	0.987	-1.2496	1.6789
		Mix cultures	0.13672	0.44491	0.998	-1.3275	1.6009
	Mix cultures	*Rhodotorula*	0.76597	0.44491	0.464	-0.6983	2.2302
		Penicillium	-0.46776	0.44491	0.826	-1.9320	0.9965
		Cladosporium	0.07791	0.44491	1.000	-1.3863	1.5421
		Aspergillus	-0.13672	0.44491	0.998	-1.6009	1.3275
Phosphatase	*Rhodotorula*	*Penicillium*	-151.01577*	31.68596	0.005	-255.2969	-46.7347
		Cladosporium	-0.88547	31.68596	1.000	-105.1666	103.3956
		Aspergillus	-58.02958	31.68596	0.408	-162.3107	46.2515
		Mix cultures	-37.17512	31.68596	0.766	-141.4562	67.1060
	Penicillium	*Rhodotorula*	151.01577*	31.68596	0.005	46.7347	255.2969
		Cladosporium	150.13030*	31.68596	0.006	45.8492	254.4114
		Aspergillus	92,98619	31.68596	0.087	-11.2949	197.2673
		Mix cultures	113.84065*	31.68596	0.031	9.5595	218.1218
	Cladosporium	*Rhodotorula*	0.88547	31.68596	1.000	-103.3956	105.1666
		Penicillium	-150.13030*	31.68596	0.006	-254.4114	-45.8492
		Aspergillus	-57.14411	31.68596	0.422	-161.4252	47.1370
		Mix cultures	-36.28966	31.68596	0.780	-140.5708	67.9915
	Aspergillus	*Rhodotorula*	58.02958	31.68596	0.408	-46.2515	162.3107
		Penicillium	-92.98619	31.68596	0.087	-197.2673	11.2949
		Cladosporium	57.14411	31.68596	0.422	-47.1370	161.4252
		Mix cultures	20.85446	31.68596	0.961	-83.4267	125.1356
	Mix cultures	*Rhodotorula*	37.17512	31.68596	0.766	-67.1060	141.4562
		Penicillium	-113.84065*	31.68596	0.031	-218.1218	-9.5595
		Cladosporium	36.28966	31.68596	0.780	-67.9915	140.5708
		Aspergillus	-20.85446	31.68596	0.961	-125.1356	83.4267
Dehydrogenase	*Rhodotorula*	*Penicillium*	83.04909*	19.85329	0.013	17.7103	148.3879
		Cladosporium	-232.48726*	19.85329	0.000	-297.8261	-167.1485
		Aspergillus	-72.92749*	19.85329	0.028	-138.2663	-7.5887
		Mix cultures	99.88050*	19.85329	0.004	34.5417	165.2193
	Penicillium	*Rhodotorula*	-83.04909*	19.85329	0.013	-148.3879	-17.7103
		Cladosporium	-315.53635*	19.85329	0.000	-380.8751	-250.1976
		Aspergillus	-155.97658*	19.85329	0.000	-221.3154	-90.6378
		Mix cultures	16.83141	19.85329	0.909	-48.5074	82.1702

	Cladosporium	*Rhodotorula*	232.48726*	19.85329	0.000	167.1485	297.8261
		Penicillium	315.53635*	19.85329	0.000	250.1976	380.8751
		Aspergillus	159.55977*	19.85329	0.000	94.2210	224.8986
		Mix cultures	332.36776*	19.85329	0.000	267.0290	397.7066
	Aspergillus	*Rhodotorula*	72.92749*	19.85329	0.028	7.5887	138.2663
		Penicillium	155.97658*	19.85329	0.000	90.6378	221.3154
		Cladosporium	-159.55977*	19.85329	0.000	-224.8986	-94.2210
		Mix cultures	172.80799*	19.85329	0.000	107.4692	238.1468
	Mix cultures	*Rhodotorula*	-99.88050*	19.85329	0.004	-165.2193	-34.5417
		Penicillium	-16.83141	19.85329	0.909	-82.1702	48.5074
		Cladosporium	-332.36776*	19.85329	0.000	-397.7066	-267.0290
		Aspergillus	-172.80799*	19.85329	0.000	-238.1468	-107.4692

*. The mean difference is significant at the 0.05 level.

Table D-6. Analysis of variance (ANOVA) of the enzymatic activity of real mortars.

		Sum of Squares	df	Mean Square	F	Sig.
LCS	Between Groups	187.823	3	62.608	5.535	0.024
	Within Groups	90.498	8	11.312		
	Total	278.322	11			
HCS	Between Groups	490.107	3	163.369	12.769	0.002
	Within Groups	102.350	8	12.794		
	Total	592.458	11			

Table D-7. Average multiple comparison of the enzymatic activity of real mortars, by Tukey HSD test.

Dependent Variable	(I) Activity	(J) Activity	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
LCS	Desidrogenase	Arylsulphatase	-0.23319	2.74618	1.000	-9.0274	8.5611
		Phosphatase	-9.20126*	2.74618	0.041	-17.9955	-0.4070
		β-Glucosidase	0.03006	2.74618	1.000	-8.7642	8.8243
	Arylsulphatase	Desidrogenase	0.23319	2.74618	1.000	-8.5611	9.0274
		Phosphatase	-8.96807*	2.74618	0.046	-17.7623	-0.1738
		β-Glucosidase	0.26325	2.74618	1.000	-8.5310	9.0575
	Phosphatase	Desidrogenase	9.20126*	2.74618	0.041	0.4070	17.9955
		Arylsulphatase	8.96807*	2.74618	0.046	0.1738	17.7623
		β-Glucosidase	9.23132*	2.74618	0.040	0.4371	18.0256
	β-Glucosidase	Desidrogenase	-0.03006	2.74618	1.000	-8.8243	8.7642
		Arylsulphatase	-0.26325	2.74618	1.000	-9.0575	8.5310
		Phosphatase	-9.23132*	2.74618	0.040	-18.0256	-0.4371
HCS	Desidrogenase	Arylsulphatase	0.53731	2.92048	0.998	-8.8151	9.8897
		Phosphatase	-14.37221*	2.92048	0.005	-23.7246	-5.0198
		β-Glucosidase	0.59367	2.92048	0.997	-8.7587	9.9461
	Arylsulphatase	Desidrogenase	-0.53731	2.92048	0.998	-9.8897	8.8151
		Phosphatase	-14.90952*	2.92048	0.004	-24.2619	-5.5571
		β-Glucosidase	0.05636	2.92048	1.000	-9.2960	9.4088
	Phosphatase	Desidrogenase	14.37221*	2.92048	0.005	5.0198	23.7246
		Arylsulphatase	14.90952*	2.92048	0.004	5.5571	24.2619
		β-Glucosidase	14.96588*	2.92048	0.004	5.6135	24.3183
	β-Glucosidase	Desidrogenase	-0.59367	2.92048	0.997	-9.9461	8.7587
		Arylsulphatase	-0.05636	2.92048	1.000	-9.4088	9.2960
		Phosphatase	-14.96588*	2.92048	0.004	-24.3183	-5.6135

*. The mean difference is significant at the 0.05 level.

ANNEXE E. Case study of *Santo Aleixo*

E 1. *Santo Aleixo* Church sampling process - Chapter V

Figure E -14. Sampling location of *Santo Aleixo* Church (orange dots for material characterisation; outline yellow dots for biological agents assessment).

E2. Stratigraphic analysis of *Santo Aleixo* Church samples

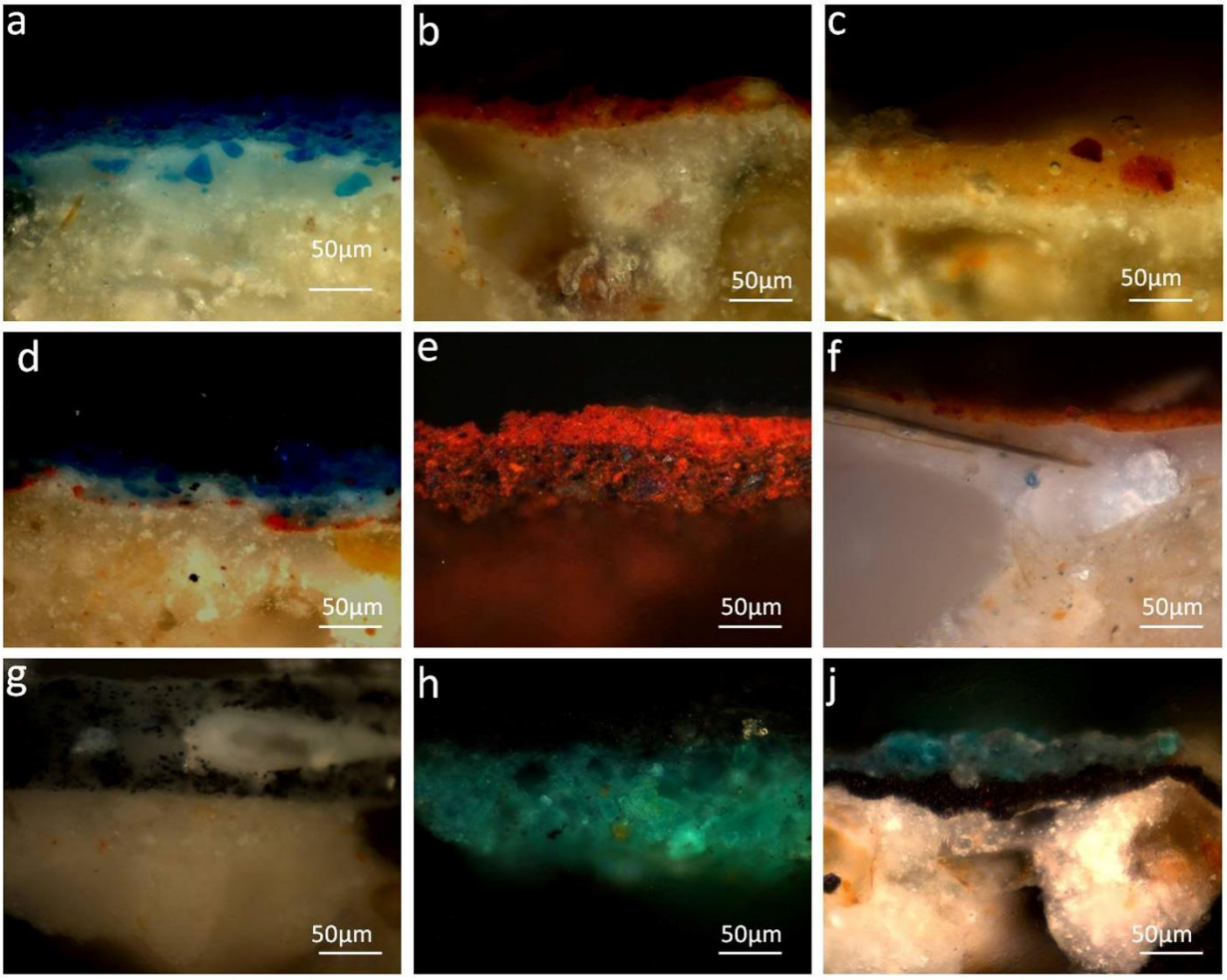

Figure E -15. Stratigraphies of paint cross-sections of Santo Aleixo paintings (a: sample 12; b: sample 15; c: sample 51; d: sample17; e: sample 42; f: sample 37; g: sample 22; h: sample 4; j: sample 1)

Table E-8. Summary of the analytical setup and stratigraphic analysis of the 11 paint layers, illustrated in the figure E-12, with the identification of the pigment present in each area.

sample ref.	layer nº	color	thichness (µm)	Microchemistry	EDS	XRD	µ-Raman
1	2	green	apr. 48-67	Cu based pigment	Cu,Ca		malachite
	1	black	apr. 30-40	bone black	P,Ca		_
4	1	greyish green	apr. 91	Cu based pigment	Cu,Ca	malachite,whewellite, quartz,aragonite,calcite	malachite and tenorite
8	1	yellow	apr. 12	Fe based pigment (yellow ochre)	Fe,K,Si,Al,Ca		_
12	2	blue	apr. 25-55	Cu based pigment	Cu,Ca	azurite, calcite,quartzo	
	1	light blue	apr.44	Cu based pigment	Cu,Ca		
13	1	red	apr.15-30	Fe based pigment (red ochre)	Fe,K,Si,Al,Ca		
15	1	brownish red	apr.5-30	Fe based pigment (ochre)	Fe,K,Si,Al,Ca		
17	3	dark blue	apr.55	Cu based pigment	Cu,Ca		
	2	blue	apr.39	Cu based pigment	Cu,Ca		
	1	red (preparatory drawing)	apr.15	Fe based pigment (red ochre)	Fe,K,Si,Al,Ca		
22	1	black	apr.117	bone black	P,Ca		
37	1	brown	apr.19	Fe based pigment (brown ochre)	Fe,Mn,K,Si,Al,Ca		
42	2	pinck	apr.19-54	Fe based pigment (red ochre)	Fe,K,Si,Al,Ca		
	1	blackish red	apr.21	Fe based pigment (red ochre)+ bone black	Fe,P,K,Si,Al,Ca		
51	2	orange	apr.12-30	Fe based pigment (ochre)	Fe,K,Si,Al,Ca		