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Biodeterioration in cultural assets – fungal contamination assessment in art objects and documents.

Tese de Doutoramento em Biociências, especialização em Microbiologia, orientada pelo Professor Doutor António Manuel Santos Carriço Portugal e co-orientada pela Professora Doutora Maria Filomena Meireles Abrantes de Macedo Dinis, apresentada ao Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra.

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Tese de Doutoramento apresentada à Universidade de Coimbra para obtenção do grau de Doutor em Biociências, especialização em Microbiologia, realizada sob a orientação científica do Professor Doutor António Manuel Santos Carriço Portugal do Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra e co-orientação da Professora Doutora Maria Filomena Meireles Abrantes de Macedo Dinis do Departamento de Conservação e Restauro da Faculdade de Ciências e Tecnologia da Universidade Nova de Lisboa.

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"O artista de agora, se quiser persistir, tem de ser um homem de acção. - Mas que acção? Ajudar a construir um mundo que o nega? Ajudar a destruir um mundo onde ele próprio já não vive? A acção dum artista é fazer a sua obra."

Miguel Torga *in* ***Diário*** **(1948)**

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Abstract

Historical and cultural items of our cultural heritage are stored, gathered, studied and restored in archives and museums, with the main objective of their preservation and protection. Fungal contamination inside the repositories represents a great challenge for scientists and restorers once it compromises not only the affected objects, as well as the storage environments and consequently the health of these professionals.

This multidisciplinary thesis addresses the risk assessment of fungal contamination in different affected objects, inside these repositories. The main objectives of this thesis were: 1) To determine the importance of fungi as biodeterioration agents in archive and museum environments; 2) To analyze different affected art objects and documents by implementing different methods for fungi identification; 3) To determine the possible consequences and risks associated with the identified fungal species; 4) To contribute to better preserve cultural heritage inside archives and museums' indoor environments.

This multitasked research begins with the study of the fungal diversity in eighty different parchment documents, belonging to five different collections, from the "Arquivo da Universidade de Coimbra". Molecular methods complemented with morphological identification were applied to identify all fungal organisms. In total, 230 isolates, belonging to 22 different genera and 42 different species, were obtained. The most frequent species were *Alternaria alternata*, *Aureobasidium pullulans*, *Cladosporium cladosporioides*, *Epicoccum nigrum*, *Penicillium citrinum*, *P. glabrum* and *P. spinulosum*. The Shannon-Wiener index was calculated for fungal diversity. A low species diversity was found in all parchments collections. Also, a Linear Model Regression analysis was calculated between the age of the documents, the number of species and number of isolates, confirming that time is significantly associated with species diversity; older collections generally presented a higher number of fungal species.

Art repositories are considered appropriate storage rooms for the preservation of artwork. Nevertheless, the presence of fungal colonies was unexpectedly detected on

wooden sculptures and paintings that were deposited inside the repository of the "Museu Nacional de Machado de Castro". In order to find a possible origin of the repository's contamination, spoiled art objects were sampled for fungal isolation and identification, along with seasonal indoor air sampling, for a one-year period. Molecular biology and morphological observation methods were used for the identification of fungal organisms. The species *Aspergillus versicolor*, *Chaetomium globosum*, *Penicillium copticola*, *P. citrinum*, *Phlebiopsis gigantea* and *Pithomyces chartarum* were isolated from wooden sculptures and paintings, and were also found in air-sampling isolates. The co-occurrence of the same fungal species in artworks and in air samples is demonstrative of cross-contamination. Although the number of airborne CFU was considerably low in all seasons, some fungal species with known biodeterioration capability and adverse human health effects were found. In light of this study, preventive measures are advised and considerations are made to apply potentially more effective approaches.

Fungal stains affecting paper documents and artwork represent a challenge to conservators worldwide and, to achieve effective cleaning treatments, the identification of the causative fungal species and respective colorants is paramount. An association between specific fungal stains and fungal species was intended. Twenty-three stains from three paper documents were sampled. *In situ* observation of fungal structures with optical and scanning electron microscopy was compared with the identification of collected samples by molecular biology tools. Correlations between the applied methodologies were achieved, varying from 13% to 64% of the samples within the three studied documents. Black and dark brown stains were associated with *Chaetomium globosum*, *C. murorum*, *Penicillium chrysogenum*, *P. commune*, *Myxotrichum deflexum* and *Stachybotrys chartarum*. *Eurotium rubrum* was isolated from a foxing stain and *Penicillium citrinum* was identified on light orange stains.

As little information about the fungal communities was obtained in the former study with culture-based methods, next-generation sequencing (NGS) was for the first time applied in canvas artwork, to deeply evaluate the fungal biodiversity in a very famous painting by the artist Paula Rego. Optical and Scan Electron Microscopy were

used to visualize suitable sampling points. In order to prevent any damage to the support, a non-invasive sampling method was developed to collect mycobiota by soft aspiration. Using NGS with *Illumina* platform, high values of fungal diversity were obtained, mainly in the points with greater contamination. The most representative orders were *Capnodiales*, *Eurotiales*, *Hypocreales*, *Russulales* and *Sporidiobolalles*. Genera such as *Aspergillus*, *Candida*, *Cladosporium*, *Penicillium*, *Tolypocladium*, *Rhodotorula*, *Stereum* or *Tricholoma* were identified in the DNA samples. High-throughput sequencing by *Illumina* coupled to non-invasive sampling allowed the identification of a complex fungal community.

The information provided in this thesis contributes to overcome fungal contamination in archives and art repositories assets', considering the development of appropriate preventive measures, and for future research on cultural heritage conservation.

Keywords: Fungi, Parchment, Collection, Diversity, Opportunistic species, biodeterioration, storage room, art contamination, conservation measures, Fungal stains, Paper conservation, Identification of fungi, SEM, DNA, Canvas Painting, Next-Generation Sequencing.

Resumo

Os objetos históricos e culturais do nosso património cultural são armazenados, reunidos, estudados e restaurados em arquivos e museus, com o principal objetivo da sua preservação e proteção. A contaminação por fungos dentro dos seus repositórios representa um grande desafio para cientistas e restauradores, uma vez que compromete não só os objetos afetados, como também os ambientes de armazenamento e consequentemente a saúde desses profissionais.

Esta tese multidisciplinar aborda a avaliação de risco de contaminação fúngica em diferentes objetos afetados, dentro desses repositórios. Os principais objetivos desta tese foram: 1) Determinar a importância dos fungos como agentes de biodeterioração em ambientes de arquivo e museu; 2) Analisar diferentes objetos e documentos de arte afetados, implementando diferentes métodos para identificação de fungos; 3) Determinar as possíveis consequências e riscos associados às espécies fúngicas identificadas; 4) Contribuir para melhor preservar o património cultural dentro dos ambientes interiores dos arquivos e museus.

Esta investigação multidisciplinar começa com o estudo da diversidade fúngica em oitenta documentos de pergaminho, pertencentes a cinco coleções, do Arquivo da Universidade de Coimbra. Foram aplicados métodos moleculares complementados por identificação morfológica, para identificar todos os organismos fúngicos. No total foram obtidos 230 isolados, pertencentes a 22 géneros e 42 espécies diferentes. As espécies mais frequentes foram *Alternaria alternata*, *Aureobasidium pullulans*, *Cladosporium cladosporioides*, *Epicoccum nigrum*, *Penicillium citrinum*, *P. glabrum* e *P. spinulosum*. Foi calculado o índice de Shannon-Wiener para a diversidade fúngica. Foi encontrada uma baixa diversidade de espécies em todas as coleções de pergaminhos. Além disso, foi calculada a Regressão Linear entre a idade dos documentos, o número de espécies e o número de isolados, confirmando assim que o tempo está significativamente associado à diversidade de espécies; coleções mais antigas apresentaram geralmente um maior número de espécies fúngicas.

Os repositórios de arte são considerados salas de armazenamento adequadas para a preservação de obras de arte. No entanto, a presença de colónias de fungos foi inesperadamente detetada em esculturas de madeira e pinturas, depositadas na reserva do Museu Nacional de Machado de Castro. A fim de encontrar uma possível origem da contaminação, os objetos de arte danificados foram amostrados para isolamento e identificação de fungos, juntamente com a amostragem sazonal de ar em ambiente fechado, por um período de um ano. Métodos de biologia molecular e observação morfológica foram utilizados para a identificação de organismos fúngicos. As espécies *Aspergillus versicolor*, *Chaetomium globosum*, *Penicillium copticola*, *P. citrinum*, *Phlebiopsis gigantea* e *Pithomyces chartarum* foram identificadas em esculturas e pinturas de madeira e também em isolados do ar. A co-ocorrência de espécies de fungos em obras de arte e em amostras de ar é demonstrativa de contaminação cruzada. Embora o número de UFC no ar tenha sido consideravelmente baixo em todas as estações, foram encontradas algumas espécies com conhecida capacidade de biodeterioração e com efeitos adversos para a saúde humana. À luz deste estudo, são aconselhadas medidas preventivas e são feitas considerações para aplicar abordagens potencialmente mais eficazes.

As manchas de fungos que afetam documentos em papel e obras de arte representam um desafio para conservadores em todo o mundo e, para obter tratamentos de limpeza eficazes, a identificação das espécies fúngicas e dos respetivos corantes é fundamental. O principal objetivo foi associar a cada mancha as respetivas espécies de fungos. Vinte e três manchas de três documentos em papel foram amostradas. A observação *in situ* das estruturas fúngicas, com recurso a microscópio ótico e microscópio eletrónico de varrimento, foi comparada com a identificação realizada por técnicas de biologia molecular nas amostras recolhidas. Foram obtidas correlações entre a observação *in situ* e os isolados fúngicos identificados para 13% a 64% das amostras. As manchas negras e de tom castanho-escuro foram associadas às espécies *Chaetomium globosum*, *C. murorum*, *Penicillium chrysogenum*, *P. commune*, *Myxotrichum deflexum* e *Stachybotrys chartarum*. A espécie *Eurotium rubrum* foi isolada de uma mancha de *foxing* e a espécie *Penicillium citrinum* foi identificada em manchas de tom laranja claro.

Como foram obtidas poucas informações sobre as comunidades fúngicas no primeiro estudo com métodos de cultura, aplicou-se "next-generation sequencing" (NGS) pela primeira vez em pintura de tela, para avaliar a biodiversidade dos fungos numa obra da artista Paula Rego. Utilizou-se microscopia ótica e eletrónica de varrimento para visualizar pontos de amostragem adequados. A fim de evitar qualquer dano ao suporte, um método de amostragem não invasivo foi desenvolvido para recolher micobiota por aspiração suave. Usando NGS com a plataforma Illumina, foram obtidos valores elevados de diversidade fúngica, principalmente nos pontos com maior contaminação. As ordens mais representativas foram *Capnodiales*, *Eurotiales*, *Hypocreales*, *Russulales* e *Sporidiobolalles*. Géneros como *Aspergillus*, *Candida*, *Cladosporium*, *Penicillium*, *Tolypocladium*, *Rhodotorula*, *Stereum* ou *Tricholoma* foram identificados nas amostras de DNA. A sequenciação de alto rendimento com Illumina acoplada a amostragem não invasiva permitiu a identificação de uma comunidade fúngica complexa.

As informações fornecidas nesta Tese contribuem para superar a contaminação fúngica em arquivos e repositórios de arte, considerando o desenvolvimento de medidas preventivas adequadas e para futuras pesquisas sobre conservação do património cultural.

Palavras-chave: Fungos, Pergaminho, Coleção, Diversidade, Espécies oportunistas, biodeterioração; repositório; contaminação de arte; medidas de conservação, Manchas de fungos; Conservação de papel; Identificação de fungos, SEM; DNA; Pintura em tela; "Next-Generation Sequencing".

Thesis outline

This thesis is divided into a general introduction, followed by chapters 1- 4 and a general discussion and conclusions. In chapter 1, the diversity evaluation of fungal species identified in collections of parchment documents is analyzed, given the cultural and historical importance of these items. In chapter 2, a case study of fungal contamination inside a painting reserve in the “Museu Nacional de Machado de Castro” is described, highlighting the necessity of guidelines for protective measures of the art pieces in these kinds of facilities in our country. Chapter 3 describes an experimental preliminary stage, to discriminate fungal species responsible for the appearance of colored spots on cellulosic materials, where morphological and molecular methods for fungi identification where applied. In chapter 4, a Next Generation Sequencing technique was applied to identify fungal communities’ in spots on a painting of a famous Portuguese artist, in one of the first studies to use NGS in the context of painting biodeterioration.

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List of abbreviators

A	Adenine
AT	Annealing temperature
AUC	Arquivo da Universidade de Coimbra
A_w	Water activity
bp	base pairs
C	Citosine
CCU	Close Control Unit
CFU	Colony Forming Unit
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
e.g.	*exempli gratia* ("for example")
G	Guanine
HEPA	High-Efficiency Particulate Air
HVAC	Heating, Ventilation and Air Conditioning
i.e.	*id est* ("that is")
ITS	Internal Transcribed Spacer
MEA	Malt Extract Agar
MgCl2	Magnesium cloride
MNMC	Museu Nacional de Machado de Castro
ml	mililiter
NCBI	National Center for Biotechnology Institute
NGS	Next-Generation Sequencing
OM	Optical Microscope
OTU	Operational Taxonomic Unit
PBS	Phosphate Buffered Saline
PCR	Polimerase Chain Reaction
PDA	Potato Dextrose Agar
RNA	Ribonucleic acid
rDNA	ribossomic DNA
RH	relative humidity
rRNA	ribossomic RNA
SEM	Scanning Electron Microscope
T	Temperature
T	Timine
UV	Ultra Violet
%	percentage
°C	degree Celsius
µl	microliter

General Introduction

General Introduction

Biodeterioration - concept and major considerations

The term Biodeterioration means, by definition, any kind of biological damage caused by organisms in any type of materials. Hueck (2001) defines it as "any undesirable change in the properties of a material caused by the vital activities of an organism". Urzí and Krumbein (1994) defined it as the irreversible loss of value and/or information of an object of art following the attack by living organisms. This has direct implications in worldwide historical assets or outside located cultural heritage, which are at risk when these organisms are capable of causing damage (sometimes irreversible) to important historical and cultural objects and artifacts. The biological attack present in some archeological areas, buildings, churches, museums, statues and many cultural items are the consequence of the different stress response of materials due to several factors, such as: geographical position, environmental conditions, micro-climatic exposure, type and dimension of the organism involved, different degrees of pollution, typology of materials and consequent status of conservation (Tiano 2002, Urzi and De Leo 2008). Also, the animal or vegetal origin of these objects, as well as their preservation along time, are relevant facts of their vulnerability for biodeterioration, but it is also important to be aware of the possible damage to new type of materials such as, polymers, plastics, cosmetics, textiles, etc, that are somewhat indispensable to an everyday lifestyle.

Biodeterioration is distinct from biodegradation as the former involves the use of living organisms that somehow benefit from any process or material. The use of living organisms such as fungi or bacteria, to convert a waste material into to a more useful or a less toxic product, is intended as biodegradation, which is a desirable process. As such, it is possible to say that biodeterioration has a negative effect on the material and biodegradation has a positive effect (Allsopp et al. 2004).

Another important issue is to ascertain the difference between biodeterioration and pathology. They both result as a consequence of the damage by a living organism,

but in the case of a pathogen attack, there is usually a reaction from the living tissues and there is always an effort to limit or remove the pathogen. In the case of materials, such reaction does not happen; a major difference which separates biodeterioration of materials from animal or plant pathology (Allsopp et al. 2004).

Biodeterioration is a harmful process involving the undesirable activity of an organism spoiling a material and there is more than one type of Biodeterioration:

1- Physical or Mechanical Biodeterioration- the organisms cause disruption or deformation of the material by growth or movement and do not use it as nutrient source. This is case of microorganisms' proliferation on rock layers or plant roots affecting the foundations of a building or bird strikes on aircrafts;

2- Aesthetic Biodeterioration - the presence of organisms or their dead body, excreta or metabolites generates an unacceptable appearance to the material, which indeed does not affect it at all. In this case microorganisms, such as fungi and bacteria, create a surface layer that is described as surface accumulations of the organic products with biological activity, known as biofilms;

3- Biochemical Assimilatory Biodeterioration- the organism directly uses the material as nutrient source by means of enzyme activity products for growth and energy production. This is the most easily understood form of biodeterioration, and happens when biochemical mechanisms of fungi or bacteria break down cellulose, or insect larvae consumes stored fruit and food;

4- Biochemical Dissimilatory Biodeterioration- in this type of biodeterioration, the material is consequently affected by chemical damage but not directly from the organism nutrients intake. As a result of the digestion process of the material, organisms excrete waste products that may interfere and damage the material itself. Its effect is, most of the time, difficult to understand because it occurs in parallel with the assimilatory biodeterioration process.

In the extent of understanding biodeterioration as a concerning issue in modern times, the biggest deal for a conservator/restorer and for researchers is to recognize the type of biodeterioration and the organism, or organisms, responsible for the support

contamination, as well as to apply the correct method for the treatment of the objects, without risking the cultural and historical value of the piece (Tiano 2002; Allsopp et al. 2004).

Fungi: contribution for biodeterioration

Fungi belong to a unique kind of eukaryotic organisms. They belong to a separate kingdom because they are described with the following characteristics: eukaryotic; heterotrophic; have apical growth (as form of hyphae or as single cells as yeasts); generally have a haploid genome; have walls composed by chitin and glucans; absorb soluble nutrients through the cell wall and the plasma membrane; and produce spores. Different fungal species have different roles in the ecological process: Pathogenic, when fungal species act as animal or plant pathogens; Symbiotic, for example when some species have a direct relation with plant roots to trade plant absorbed minerals for synthesized nutrients for fungi; and Saprophytic, when fungal species have a degrading and decaying function of materials in nature (Deacon 2006). Currently, there are five main divisions of fungi: the *Chytridiomycota* (many secondary aquatic fungi), *Glomeromycota* (symbiotic associations between plant roots and fungal species), *Zygomycota* (respinsable for the degradation of food products and are opportunists species in biodeterioration), *Basidiomycota* (also called mushrooms or toadstools, which comprise wood decaying species) and *Ascomycot*a (the most well known biodeterioration fungi belongs to this group) (Sterflinger and Pinzary 2012).

Today their functions and applications are studied and investigated further. So, their importance to humankind is crucial to discuss, because they have multiple applications: medicines and drug production (for example, penicillin, produced by the species *Penicillium chrysogenum*) or in the food industry (the species *Aspergillus niger* has a role here, or in the use of yeasts to produce beer and bakery). However, fungi have also a non-benefit side for humankind, since they allow food spoilage, produce toxins (mycotoxins) and could also act as pathogens or allergens. This seriously affects the human organism or can critically damage (sometimes irreversibly) most kind of materials, having a direct role in biodeterioration. In this way, fungi can be found

contaminating outdoor environments, such as architectural surfaces, facades and stone monuments, or at indoor environments, such as museums, depots, archives or private collections (Sterflinger and Piñar 2013). Here the so called hyphomycetes group, also known as "moulds", that includes species such as *Alternaria*, *Aureobasidium*, *Cladosporium* and *Epicoccum* spp., are quite responsible for most of the materials damaged in cultural heritage assets items (Sterflinger 2010, Sterflinger and Pinzary 2012). In fact, fungi associated to biodeterioration of organic and inorganic materials are the most harmful type of organisms. They have a metabolic versatility which empowers their effectiveness in colonizing different kind of substrata (wood, glass, stone, paper, parchment) that are present in cultural heritage depots and museums worldwide (Urzí and De Leo 2008). Also, they can tolerate desiccation, high salt concentrations and heavy metal compounds present in some inks and pigments over many variety of described substrata (Michaelson et al. 2010).

Fungi: Museum/Archive environments

Museums and Archives are privileged infrastructures that were designed and conceived for collecting, storing, rendering and restoring different types of librarian or artworks materials along several centuries, and have an overall goal of the protection of these materials from any type of damage (Karbowska-Berent et al. 2011; Grapek-Lejko et al. 2017). Currently, there are new modern buildings with proper monitoring *Heat, Ventilation and Air Conditioned* (HVAC) systems for more controlled environmental conditions inside these repositories (Montanari et al. 2012; Pinheiro et al. 2014).

The biodeterioration phenomenon takes no exception inside archive repositories because the stored materials are susceptible of being damaged by physical, chemical and biological agents. These situations can be determined by several factors such as: the chemical composition and the nature of the material itself; the climatic conditions and the exposure of the object; and also the frequency of the cleaning in these buildings (Sterflinger and Piñar 2013). With regard to biological deterioration in works of art, Urzí and De Leo (2008) described some important factors influencing biological establishment and colonization: Climatic and environmental factors (temperature,

relative humidity, sun, shadow, rain); Inorganic and organic pollutants (as C, N, S source and/or growth inhibitors); Surface bioreceptivity (nature of the material, conservation, length of exposure); and Treatments (Biocides, Surfactants and Hydrophobic compounds). In this way, environmental microorganisms have the capacity to deteriorate a variety of cultural heritage items and can be transported to indoor environments by human contact or attachment and by ventilation systems, for later settling on the support and consequently deteriorating the material (Guiamet et al. 2011; Borrego et al. 2012; Nunes et al. 2013). The restoration process may also contribute to increase the biodeterioration of a support by an organism. Materials used for retouching, restoration or cleaning and soaking procedures, can contribute to biological risk because they are freshly applied and have a high water content (Ciferri 1999; Tiano 2002).

Among the most problematic microorganisms in terms of deterioration, fungi, in form of spores or propagules, are known to cause great damage to historic and valuable cultural heritage materials because it has a high biodeteriogenic capacity for organic material (Anaya et al. 2016). Besides outdoor air contamination, fungal aerosols inside archives and libraries can be increased by spore dispersal with internal origin, as is the case inside contaminated materials or structures (walls, wall paint, floods, etc) (Karbowska-Berent et al. 2011; Šimonovičova et al. 2015; Polo et al. 2017). It is important to note that the spreading of fungal species inside these facilities is a risk for the objects as well as for the staff and visitors health, because some contaminant species of the indoor air are mycotoxins producers and can cause serious respiratory or skin disorders (Nielsen 2003; Fischer and Dott 2003; Piecková 2012). For this reason, it is always advisable to accomplish the guidelines for the indoor air quality and also have preventive conservation measures for the art pieces, which are, sometimes, still lacking (Cappitelli and Sorlini 2005; Sterflinger 2010).

After the first contact of the fungal spores and propagules with the support, comes the attachment to the surface. Then, the biodeterioration process begins as soon as the water availability present is sufficient to promote it (Szczepanowska and Cavaliere 2012). Consequent mycelia growth and pigment production occurs, which causes chromatic alterations in form of different colours, tonalities and textures on the support

(Borrego et al. 2010). This phenomenon is also known as foxing and is one of the major problems in fungal contamination of cultural heritage objects (Arai 2000; Tiano 2002; Corte et al. 2003).

Fungi: indoor ecological succession

Museum and archive depositaries store different cultural heritage objects and collections under standard conditions. As such, airborne fungal spores and structures can coexist with the artworks and with people in a simplified ecosystem, without causing great damage for a long time (Guiamet et al. 2011; Kadaifciler 2016). In the case of a change in the patterns of humidity, temperature and light, the lack of ventilation and the presence of a water vapour gradient on the materials' surface promote local water condensation points, with higher water availability for fungi than in the rest of an indoor environment (Sterflinger and Pinzari 2012; Sterflinger and Piñar 2013; Micheluz et al. 2015; Polo et al. 2017). In this situation, a special microniche is created, which is suitable for the proliferation of ruderal species, especially in places where the overall environmental conditions appear to be hostile to microbial life (Michaelson et al. 2010; Sterflinger 2010; Montanari et al. 2012; Polo et al. 2017).

Fungal species' establishment and colonization inside buildings and in proper storage facilities are described by some authors, particularly in archive environments, as possibly leading towards the formation of specified and specialized microniches on artwork. In terms of biodeterioration potential in cultural heritage, there are two distinct groups: 1- opportunistic fungi, that grow in almost any type of materials with sufficient humidity for growth and development. In this case, there is no enzymatic degrading action on the material and no use of it as a carbon source; 2- real "material pathogens", that are substrate specific and capable of degrading specific art works materials such as cellulolytic fungi in canvas or paper and keranolytic fungi in parchment or leather. Both groups of fungi can cause biodeterioration, but only the ones belonging to the second group are responsible for the decay of the material itself (Sterflinger and Pinzary 2012).

Art objects such as wooden sculptures, paintings (panel and wood board canvas) or ancient documents made by parchment or paper, are optimal nutritional sources for fungi because their raw material compositions are quite susceptible for degradation (Cappitelli and Sorlini 2005, Santos et al. 2009, Michaelson 2010, Kraková et al. 2012a). Fungi are able to produce a lot of different enzymes for each type of support even at low water activity. It is the case of cellulases, glucanases, keratinases, laccases, lignin-peroxidases, phenol oxidases, and many more. In fact, many fungal species have strong cellulolytic (genera *Alternaria*, *Botrytis*, *Chaetomium*, *Penicillium* or *Trichoderma*), proteolytic (genera *Aureobasidium*, *Epicoccum* or *Mucor*) and lipolytic activities (genus *Paecilomyces*) (Harkawy et al. 2011; Karbowska-Berent et al. 2011).

In these kind of environments, fungal dispersal and contamination has airborne origin, with typical seasonal variations, and a great number of spores can be accumulated in dust layers. Besides the chemical composition of the material itself and the environmental factors involved with it (humidity, temperature and airborne nutrients), water availability is the most important factor for the predominance of fungi (Steflinger and Pinzary 2012). Fungi are such remarkable organisms that some species can actually grow at lower water activity (A_w) such as *Eurotium halophilicum*, which can start its growth at A_w>0.6 (Montanari et al. 2012; Polo et al. 2017). Fungi deteriorating substrata are considered as prime colonizers and require a lower water activity (A_w<0.8) for growing. This is the case of some slow growing *Ascomycetes* and mitosporic xerophilic or xerotolerant species such as *Aspergillus versicolor*, *A*. *niger*, *Penicillium brevicompactum* or *P*. *corylophilum*. After this first attack, the substrate water activity suffers an alteration and it becomes possible for other species to grow. Here, the second colonizers, which are water stress resistant species (A_w 0.8-0.9), arise and their development occurs because of the establishment of newly unstable microniches, due to small changes in humidity patterns. This is the case of species such as *Alternaria*, *Cladosporium*, *Phoma* and *Ulocladium* spp.. Tertiary colonizers are species known as "water damage fungi", that need high water activity (A_w> 0,9) and thus are water indicating fungi. Species such as *Chaetomium* spp., *Epicoccum* spp. (produces coloured stains), *Stachybotrys* spp. (produces toxic compounds) and *Trichoderma* spp. (produces

strong odors) are examples of these type of moulds (Nielsen 2003; Montanari et al. 2012; Sterflinger and Piñar 2013; Kadaifciler 2016).

Types of studied objects

Parchment

The use of animal skin by humankind has had many applications, but parchment has had great importance because it has been the main writing support of its time. It is believed to have been invented in the second century BC in the ancient Greek city of Pergamon (modern Bergana, in Turkey), with the purpose of substituting papyrus (Capitelli and Sorlini 2005; Poulalakis et al. 2007). Parchment was more advantageous than papyrus because it was stronger, more durable and as a raw material (animal skins) it could be found almost everywhere. Also, it was possible to write on both sides of the object (Capitelli and Sorlini 2005). Until the invention of paper, it was the main vehicle of social and cultural transmission during the middle ages and, therefore, it is considered a very valuable source of historical-cultural data (Bower et al. 2010; Pangallo et al. 2010).

Parchment is made from untanned animal dermis skins, such as sheep, goat, lamb, pig and calves. Its main component is collagen, a highly resistant natural polymer, and also has a certain amount of keratin and elastin and a minimal amount of albumin and globulin (Tiano 2002; Axelsson et al. 2012). The manufacturing process of parchment is quite laborious: firstly it involves the scrapping of the skin for hair cleaning and removal, for further immersion in calcium hydroxide solution (bleaching) or in sodium or potassium chloride solutions (salt solutions prevented microbial attack during the first stage of manufacture); secondly the pH adjustment with ammonium chloride or sulfate with lime follows; and finally, the application of egg yolk, to remove any kind of loose grease, and egg whites, flour or milk to create a smooth material surface concludes the process, thus making a better writing or drawing support (Pinzary et al. 2012; Piñar et al. 2015 a).

Historical parchments stored in archives and libraries worldwide are important valuable artifacts that are damaged by biodeterioration. For that reason, efforts have

been made in the preservation of the material and also in the correct identification techniques of contaminant microbiological species, such as fungi (Kraková et al. 2012a; Troiano et al. 2014). The susceptibility of parchment to biodeterioration depends of the following factors: the raw material, its production method and the conditions of preservation. Just as other materials with a biological origin, and especially a treated or modified one, parchment is more vulnerable to the attack of microorganisms. As result of this biodeterioration, parchment loses some of its original properties, becoming deformed, with possible occurrence of white films, text fading and spots, in a phenomenon known as foxing (Arai 2000; Tiano 2002; Corte et al. 2003). Fungi with proteolityc activity can hidrolyze collagen fibres, modify inorganic components or produce pigments and organic acids which discolors parchment or cause indirect damage. *Zygomycetes* such as *Mucor* spp., *Ascomycetes* such as *Chaetomium*, *Fusarium*, *Gymnoascus* spp., and some mitosporic fungi such as *Acremonium*, *Aureobasidium*, *Aspergillus*, *Cladosporium*, *Epicoccum*, *Penicillium* and *Trichoderma* spp., are examples of proteolytic fungi responsible in damaging parchment (Sterflinger and Piñar 2013).

Paper

The invention of paper is usually associated to the Chinese court official Tsai Lun around 105 BC. In Middle East 8th Century, Arabs also had a remarkable role on this material, producing it in a different way by including linen in the raw materials and having paper mills. They also taught their paper-making art all over Europe and the Western world in the 12th century, and so becoming the most common writing material even in current times (Capitelli and Sorlini 2005). Until the 19th century, paper was handmade and had high quality because the raw material was made from linen and cotton rags that were carefully selected to extract cellulose, containing only a few impurities. After this period, paper changed its way of production, becoming machine-made in industrial manufacturing processes from the stem of wood, or wood pulp, containing a great amount of materials other than cellulose, as well as impurities, which worsen the quality of the so called modern paper. Thus, modern papers are more

propitious to microorganism attack and colonization than older ones (Tiano 2002; Capitelli et al. 2010).

Paper is primarily composed of cellulose and includes other components, from the manufacturing process, such as: lignin, hemicelluloses, pectins, waxes, tannins, proteins, gelatins, adhesives, sizings and mineral constituents. As such, it is a highly receptive material for microbial attack, since there is an available high water content. Accordingly, due to its hygroscopicity and composition, paper is a good carbon source for heterotrophic organisms, particularly to fungi (Capitelli and Sorlini 2005; Sequeira et al. 2012).

Fungi are considered the most common biodeteriogens of paper and paper-based materials, as they show a great tolerance to environmental conditions and can live with low water availability. Filamentous fungi are often associated with paper degradation through physical and chemical deterioration, since they are capable of disrupting cellulose fibers, by hydrolysis with cellulolytic enzymes, and producing stains or organic compounds responsible for paper discoloration, causing serious damage to historically cultural paper items, affecting the readability of the document most of the time (Michaelson et al. 2009; Sterflinger 2010). The staining issue on paper, usually in form of spots, has been currently denominated as foxing and is one of the major problems concerning fungal colonization on paper, especially under the suitable conditions of temperature, humidity and light, in archive and museum environments, where storage and maintenance conditions may not be efficient to correctly preserve these kinds of supports (Corte et al. 2003; Michaelson et al. 2010, 2013).

Many strains of micro-fungi were already isolated from books, documents and prints. In the case of cellulolytic fungi, many species of mitosporic fungi such as, *Alternaria* spp*., Aspergillus* spp*., Fusarium* spp., *Humicola grisea, Myrothecium verrucaria, Penicillium* spp*., Stachybotrys atra, Stemphylium* spp., *Trichoderma* spp*.* or *Ulocladium* spp., and others as *Cladosporium* spp. are commonly isolated (Tiano 2002; Mesquita et al. 2009; Jurado et al. 2010).

Painting canvas, wood board canvas and wooden sculptures

Art has the job of communicating the incommunicable, not the earthly. Mankind paintings, sculptures and other artwork are believed to have started in the pre-historical era, with great emphasis in various ancient civilizations (Egyptians, Greeks, Romans, etc). They were used to register some of their life events or important facts. From the Renaissance until the Impressionism period, art became culturally more valuable because it was a most representative way of human creation and interpretation of their situations and feelings. Here the artists used the colors, shades and shapes to enrich their work (McCrimmon 2017). This kind of artwork are priceless from an historical and cultural point of view. Also their materials and state of conservation could be at risk of biodeterioration and for that reason they are an important study object for conservators and scientists (Borrego et al. 2010, 2012; Sterflinger and Pinzary 2012).

Paintings can be made on different supports, such as canvas or wood, and are composed by a preparation layer and a paint layer. Their chemical composition depends on the mode of painting, the kind of paints used, the historical period and the author. In canvas and wood board paintings, the painted surface is composed by several colour layers and a protective varnish (Tiano 2002; Poyatus et al. 2017).

The organic and inorganic components in paintings represent a nutritive source and are suitable microniches for biological attack, which occurs when environmental conditions (temperature, humidity, light and pH) are favorable. In painted works of art, a part of the painting or all of its components can be affected by the biodeterioration processes. Other than the support material, some substances such as binders and additives (animal or plant glues, emulsifiers, plasticizers, thickeners) or components (egg, gums, waxes, oils, gypsum) and other materials used for different treatments of the support can increase its susceptibility (Santos et al. 2009; Capodicasa et al. 2010; Lopéz-Miras et al. 2012, 2013). Also dirt, dust and environmental surface contaminants are another important nutritive source. On the opposite side, the presence of heavy metals in some pigments, such as lead, zinc or chromium, can increase the resistance of the paint layer (Ciferri 1999; Tiano 2002). Fungal species are known for their thriving in works of art, such as canvas and wood board paintings or wooden sculptures. Fungal

growth on theses supports results in aesthetical (pigment production, stains), mechanical or physical (by hyphal penetration leading to cracking and disintegration of the painted surface, formation of blisters and degradation of the supports) and biochemical decay (Enzymes and organic acids production) (Ciferri 1999; Capodicasa et al. 2010; Sterflinger and Piñar 2013). Fungi are organisms that can degrade cellulose (main component of canvas paints) by producing cellulolytic enzymes that can hydrolyze it. Glucose and other metabolites are consequently produced and can be used by other fungi or other community microorganisms as a carbon source for growth and energy. This cellulolytic action explains the depolymerization and the loss of the fibers structure (Doménech-Carbó et al. 2007; Okpalanozie et al. 2016). In the case of the wood board paintings and wooden sculptures, along with the cellulolityc enzymes, some fungi can also produce lignolytic enzymes for lignin degradation on wood products composition (Pangallo et al. 2007; Kracová et al. 2012b).

Micro-fungi species that are frequently involved in deterioration of the canvas and woodboard paint layer are: *Alternaria*, *Aureobasidium*, *Aspergillus*, *Chaetomium*, *Cladosporium*, *Fusarium*, *Geotrichum*, *Memnominela*, *Mucor*, *Myrothecium*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Rhizopus*, *Scopulariopsis*, *Stachybotrys* and *Sordaria* spp. (Doménech-Carbó et al. 2007; Poyatus et al. 2017). For wood composition supports deterioration (wood-board paints, wooden sculptures and paint frame supports) three different groups of fungi are considered: Soft rot fungi, that grow in damp environments (with high humidity) and have a rapid and simple way of attacking, by growing their hyphae into the wood, leaving a characteristic signal from their enzyme activity in the form of rhomboidal cavities. They have little effect on lignin and need a very substantial amount of nitrogen levels for wood decay; Brown rot fungi, characterized for the typical brown colour in the decaying wood and for the brick-like cracking (irregular decay). This results from the degradation of cellulloses and hemicellulloses by an oxidative process (H_2O_2 production), being an efficient way of using the scarce nitrogen available in the wood; and White rot fungi, that are extremely efficient in using nitrogen and are remarkably lignin degrading organisms, by the production of a few specific enzymes (lignin-peroxidases, manganese-peroxidases, H_2O_2-generating enzymes, and laccases) that generate strong oxidants (Deacon 2006). Species such as *Alternaria* spp. and

Chaetomium spp. (Soft rot fungi) or *Merulis lacrymans* and *Coniophora puteana* (Brown rot fungi), or *Fomes* spp. and *Pleurotus* spp. (White rot fungi) can develop on the material surface or have internal support origin and produce exoenzymes that lead to the change in the material structure (Tiano 2002).

Fungi: identification techniques

As microorganisms, fungi are capable of the deterioration of documents and artwork, and for that reason it is very important to identify them as correctly as possible. Moreover, this identification step is crucial before any type of restoration process, since, most of the time, it could enhance the colonization and growth of different fungal species (Schabereiter-Gurtner et al. 2001).

For the isolation of fungal species, traditional cultivation methods are often used in these circumstances. Culture media such as PDA (Potato Dextrose Agar) or MEA (Malt Extract Agar) are commonly used to isolate different types of fungal strains, as they are considered general media. Also DG-18 (Dichlorane Glycerol Agar) is used to isolate xerophilic fungi, fungal species that grow at low water activity (Šimonovičova et al. 2004; Michaelson et al. 2009, 2010; Sterflinger 2010). The main advantage here is that more than 70% of the total fungi can be recovered with cultivation and therefore these methods are very useful in mycology (Sterflinger 2010). Generally materials' surface is scrapped with sterile cotton swabs, scalpels, tape or simply by adhering cellulose membranes, for later media cultivation. This next step's purpose is the isolation of fungal cultures and incubation for further morphological and molecular identification (Mesquita et al., 2009; Michaelson et al. 2010; Kraková et al. 2012a; Piñar et al. 2015a). Also biocollectors can be used for outdoor and indoor airsampling by air flow impactation onto culture media. Fungal contaminations are known to be mostly airborne and have proper seasonality characteristics, which in fact may help characterize the room/space environment of these strains origin (Medrela-Kuder 2003; Nielssen 2003; Sterflinger and Piñar 2013; Šimonovičova et al. 2015).

Application of Molecular biology in biodeterioration is emerging quickly and molecular techniques are now indispensable tools to detect fungal species present in many art objects relatively fast (Pangallo et al. 2007, 2009; Kraková et al. 2012 a,b; Piñar et al. 2015 a,b; Sanmartin et al. 2016; Kraková et al. 2017). The application and development of DNA sequencing for species identification allows a more correct and complete diversity evaluation of these individuals, which is also important to describe the community structures, or the influence of the surrounding environment and their consequences for the material (Michaelson et al. 2006; Poyatos et al. 2017). The Internal Transcribed Spacers (ITS regions) has been successfully used for this matter. These regions are nested in the nuclear rDNA repeat and possess a high variation between taxonomically distinct fungal species and even within the species, thus making the sequencing of the total ITS region a useful molecular tool for the identification of fungal species (Martin and Rygiewicz 2005; Michaelson et al. 2006; Schoch et al. 2012).

In spite of their effectiveness, culture-dependent methods still reveal a lower part of the colonizing fungal community in the materials. Direct DNA-based techniques allows the identification of single fungal species in spots, pigments or other foxing symptoms, without cultivation, which makes some non-cultivable species potential spoilers of cultural heritage material (Michaelson 2006, 2010; Piñar et al. 2015c). As such, other approaches are needed and some new protocols have been applied. One of the solutions is to combine culture-dependent with culture-independent methods (Lopez-Miraz et al. 2012, 2013) for a more wide perception of the colonizing microbiota. Unfortunately, in these cases, most of microfungi species will not grow on media, mainly because of the small sampled areas (spots), where they are present in very low amounts, in spite of their potential diversity. Another solution is to combine culture-independent methods (e.g. without cultivation) with SEM (Scanning Electron Microscope) microscopy approach, to first search for visually damaging fungal structures in the object for further identification of species (Piñar et al. 2015 a,b,c). In these last cases, the non-cultivable techniques were applied by direct DNA extraction for further DGGE (Denaturing Gradient Gel Electrophoresis) community analysis, as it allows the separation of a DNA mixture of different species. In this approach, PCR-amplified ITS regions are separated by DGGE fingerprinting and the most important bands are then

studied by the construction of clone libraries and sequencing, thus allowing a good understanding of the community (Michaelson et al. 2010; Rastogi and Sani 2011; Sanmartin et al. 2016). In spite of being one of the most used uncultured based methods in microbial communities' artworks identification, by rDNA library construction and Sanger sequencing, normally few sequences are obtained with the clone libraries analysis. Moreover, it is a time consuming methodology and it also has a high cost per sequencing unit (Lupan and Popescu 2012).

Recently, Next Generation Sequencing (NGS) has arised and its application provides large-scale sequencing technologies. NGS techniques make the sequence of entire genomes or specific areas of interest or small numbers of individual genes possible. Different NGS platforms are now available and they use different sequencing technologies, which allow the DNA sequencing of numerous small fragments in parallel. Bioinformatics analyses are further employed to assemble these fragments by mapping the individual reads. This technology generates a great number of reads at low cost with high speed, which can be useful for many applications, thus allowing an entire human genome sequencing in a single day or to investigate deeper layers of the microbial communities. In this context, it is essential to give, with high fidelity, the phylogenetic composition and functional diversity of studied microbial communities. In this kind of community studies, the sequencing of the rDNA (16S or 18S) hypervariable regions generates short fragments (100-350 bases), that are grouped in Operational Taxonomical Units or OTUs, which present sufficient phylogenetic information and are easily covered in short read lengths (Rastogi and Sani 2011; Benjati and Tarpey 2013; Kraková et al. 2017).

Places of study

"Arquivo da Universidade de Coimbra" (AUC)

The "Arquivo da Universidade de Coimbra" (AUC) is the depositary of the documentation produced and received by the University. It has come to hold a wide asset of documents either produced or received by the university. Since its founding by King D. Dinis in 1290, its documents reflect the history of the university in a unique way and its fundamental mission is the preservation, enrichment and technical treatment of its bibliographic and documentary heritage, support for teaching and research and the pursuit of its own cultural activity. The oldest document is the parchment of the "Colegiada de Guimarães" from the year 983.

The AUC main objectives are: a) conservation, enrichment, valorization, technical treatment and dissemination of the archival heritage of the UC and of the institutions of the district of Coimbra; b) support for university and extra-university education and research, providing access to its documentation and real or virtual information; c) the promotion of cultural activities, such as exhibitions, colloquia, conferences, study visits, debates, lectures and publications.

Today the AUC is a modern construction, built in the 1940's, and its staff works to prevent the deterioration of this valuable written heritage (Vasconcelos 1991, Nunes et al. 2013)

"Museu Nacional de Machado de Castro" (MNMC)

The "Museu Nacional de Machado de Castro" owes its designation in honor of the royal sculptor Joaquim Machado de Castro (1731-1822), a notable representative of Portuguese sculpture in the XVIII century, who lived in the reigns of D. José, D. Maria I and D. João VI.

The Museum opened to the public on October 11 of 1913, occupying the buildings that were built for the Episcopal residence from the 12th to the 18th century, adapting them to the museological function at the beginning of the XX century (between 1911 and 1913). It stands on the monumental "Criptopórtico" of the city of *Æminium* (century I), the most remarkable Roman construction conserved in Portugal and on which the Episcopal Palace was installed. There are still vestiges of the Romanesque church of S. João de Almedina, among them the cloister of the XII century. In the central courtyard, corresponding to the space of the Roman Forum, a renaissance loggia by Filippo Terzi (16th century), offers one of the most beautiful views over the city of Coimbra.

Reopened entirely at the end of 2012 - following the project of requalification and expansion of the architect Gonçalo Byrne, the "Museu Nacional de Machado de Castro" currently has the necessary conditions to be understood as a meeting place between memory and contemporaneity. The collections of the museum reflect the richness of the Church and the importance of royal patronage to which many of its works of art and liturgical implements are most valued. The monochromatic or polychrome sculpture, in wood and stone occupies a high point, illustrating the work of the best Flemish workshops and also the evolution of Portuguese schools from the Middle Ages to the 18th century through numerous masterpieces. Even so, the nuclei of painting, goldsmithing, ceramics and textiles, impose themselves with equal importance and representativeness for the imported art and the national production. Archaeological collections from the city and those of eastern art (resulting from the donations of the Coimbra poet Camilo Pessanha and President Manuel Teixeira Gomes) are also distinguished.

It is not possible to expose the Museum's numerous and diverse assets despite having increased the exhibition area with the recent enlargement. Thus, in the reserve studied in this work, about 900 oil and / or tempera paintings on wood, canvas or copper are stored; and also about 500 large sculptures, or decorative elements in wood, with or without polychrome.

“Casa das Histórias Paula Rego”

The prestigious portuguese artist Paula Rego chose Cascais to build “Casa das Histórias” in 2006. The museum exhibits a great part of her graphic work and some of her husband’s, Victor Willing, who hasalready passed away.The project design was made by the famous and esteemed Portuguese architect Eduardo Souto de Moura (Pritzker Prize 2011).

“Casa das Histórias” has the mission of presenting the knowledge and enjoyment of the work of Paula Rego and her artistic connections. In order to provide a cultural service of excellence, through a rotating exhibition of the collection, an active and dynamic educational service and a diverse parallel program, this cultural facility aims to emphasize not only the Portuguese museums fabric of contemporary art but also, and especially, to fit into the international art circuit and its audiences.

This facility was designed with the care for the surrounding environment, most of all for the trees involvement. It has about 750m^2 of permanent and temporary exhibition areas, a cafeteria, a shop, a bookstore and an auditorium with capacity for 200 people. It is the most international museum space in the county.

Inside the building, the paintings depositary room has a proper climatic monitoring system and holds the pictorial asset of the artist, from which the interventioned artwork was chosen: the painting named “Centauro”.

The thesis general objectives are:

- To determine the importance of fungi as biodeterioration agents in archive and museum environments;

- To analyze different art objects and documents affected by fungal biodeterioration by implementing different methodsfor fungi identification;

- To determine the possible consequences and risks associated to the identified fungal species on the artwork, as on the working personnel of the studied archives and museums, and also on the surrounding environment;

- To contribute to better preserve cultural heritage inside archive and museum indoor environments.

Chapter 1

Diversity of fungal species in ancient parchments collections of the Archive of the University of Coimbra

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Chapter 1

Diversity of fungal species in ancient parchments collections of the Archive of the University of Coimbra

Abstract

In order to study the fungal diversity in different parchment collections from the "Arquivo da Universidade de Coimbra", eighty different documents, belonging to five different collections, were screened for the presence of fungal species. Molecular methods complemented with morphological identification were applied to identify all fungal organisms. In total, 230 isolates, belonging to 22 different genera and 42 different species, were obtained. The most common genera were *Alternaria*, *Cladosporium*, *Epicoccum* and *Penicillium* and the most frequent species were *Alternaria alternata*, *Aureobasidium pullulans*, *Cladosporium cladosporioides*, *Epicoccum nigrum*, *Penicillium citrinum*, *Penicillium glabrum* and *Penicillium spinulosum*. Shannon-Wiener index was calculated for fungal diversity, presenting a low species diversity in all parchments collections. Also a Linear Model regression analysis was calculated between the age of the documents, the number of species and number of isolates, confirming that time is significantly associated with species diversity; older collections generally presented a higher number of fungal isolates.

Keywords - Fungi, Parchment, Collection, Diversity, Opportunistic species.

Chapter 1

1.1- Introduction

Ancient written documents made of parchment were an important communication mean to mankind and have, for that reason, a priceless historical value to our culture heritage. These were the main writing material until the middle ages, but since the advent of paper, parchment has mainly been used for distinguished purposes (Pinzari et al. 2012).

Parchment is made of treated animal skin with collagen as its main component. Collagen is susceptible to deterioration by several microorganisms, serving both as energy and carbon source (Troiano et al. 2014). Fungi have a particularly relevant role in the biodeterioration of parchment as well as other archive materials (Cappitelli and Sorloni 2005; Sterflinger 2010). As result of this biodeterioration, parchment loses some of its original properties, becoming deformed, with possible occurrence of white films, text fading and spots (Tiano 2002). This last phenomenon, usually referred as “foxing”, occurs because of the enzymatic activity of fungi in the substrate digestion process, producing different coloured spots (brown, dark or reddish). Along with this chemical action, fungal hyphae can also penetrate the fibre structures of parchment and induce mechanical damage in the document (Arai 2000; Szczepanowska and Cavaliere 2000; Corte et al. 2003; Sterflinger and Pinzari 2012). For this reason, the identification of fungal species in Archives and Library items (including parchment documents) has been crucial to determine the capability of fungi to damage these items, but also the environmental conditions required for their establishment in the support (Mesquita et al. 2009; Sterflinger and Pinzari 2012), which makes biodeterioration one of the main problems in document preservation (Guiamet et al. 2011).

The Archive of the University of Coimbra (AUC) holds a wide asset of documents either produced or received by the university since its founding by King D. Dinis in 1290, documents that reflect the history of the university in a unique way (Vasconcelos 1991). The AUC is a modern construction, built in the 1940’s, and its staff works to prevent the deterioration of this valuable written heritage (Nunes et al. 2013). The different materials used in these documents enhance the likelihood of different ecological niches

combined with the propitious conditions for the development of fungi (Hyvärinen et al. 2002). The AUC hosts a great collection of parchment documents that is already facing biodeterioration effects (Mesquita el al. 2009).

The use of molecular techniques in the identification of biodeterioration-related fungal species in documents and other culture heritage items is important to the understanding of fungal infection and its further consequences for the material (Sterflinger 2010). The sequencing of the total ITS region is a still a very strong molecular tool for fungal species identification (Martin and Rygiewics 2005; Michaelson 2006; Schoch et al. 2012).

The research on the deterioration of parchment by surface-associated microorganisms is still limited to very few works (Kracová et al. 2012; Pinzari et al. 2012; Troiano et al. 2014; Piñar et al 2015 a,b). Understanding the cultural relevance of parchment, concerning the biodeterioration of most of these documents and taking into account the previous works done by Mesquita et al. (2009), Trovão et al. (2013) and Nunes et al. (2013) in the AUC, our main objective was to study the fungal diversity in different parchment collections stored within this site and to demonstrate that time has an important role in the fungal colonization and deterioration of documents. For this purpose, eighty documents, belonging to five collections from five different sources were object of analysis, regarding the presence of contaminant fungal organisms. The analysed documents belong to the collections of: “Colegiada de Guimarães”, “Mosteiro de Pedroso”, “Cabido da Sé de Coimbra”, “S. João dos Longos Vales” and the other from the University of Coimbra itself. The referred collections date from different centuries (i.e. X to XIX) and in that way represent different timestamps from our history, the oldest belong to the “Colegiada de Guimarães” (century X), prior to the foundation of Portugal. From contracts to diplomas, a really impressive variety can be found within these stored materials. To our knowlege, this is the first study on a broad range of parchment documents, where nearly eighty different documents were screened for fungal species, as biodeterioration promoters.

1.2 - Materials and methods

1.2.1- Sampling

The samples were retrieved from eighty parchment documents, belonging to five different collections, that were deposited in the AUC (Table 1) and some examples are presented in Figure 1. Biodeterioration symptoms on parchment, such as spots or areas with fading text, were selected as targets for analysis.

Figure 1- Examples of sampled parchments: a) "Mosteiro de Pedroso" (document 7); b) "S. João dos Longos Vales" (document 17); c) "Cabido da Sé de Coimbra" (document 58).

Two sampling methods were applied to each document: in the written parts of the documents, sterile cotton swabs were used to collect fungal samples whereas in the borders of the documents, samples were collected with the use of adhesive tape. After this, samples were isolated in Malt Extract Agar (MEA, Difco ™) with streptomycin (0.5 g/L) to prevent bacterial growth. The different colonies were isolated to axenic cultures and incubated for five days at 28±1 °C for further molecular and morphological analysis.

1.2.2 - Molecular and morphological identification

When the colonies reached enough mass for DNA extraction, mycelia were scraped from the agar surfaces using sterile scalpels. Collected material was subjected to total DNA extraction using an ABI Prism™ 6100 Nucleic Acid PrepStation, according to the manufacturer's standards.

After extraction, the ITS region was amplified by PCR, using primers ITS4 and ITS1F (White et al. 1990; Gardes and Bruns 1996). For that purpose, PCR mixes were prepared with 12.5 µl of Jump Start *Taq* DNA Polymerase with $MgCl_2$ (Sigma D9307), 0.5

µl of each primer (10 mM), 10.5 µl of ultra-pure water, and 1 µl of template DNA, for a final reaction volume of 25 µl.

The PCR reactions were performed using an ABI GeneAmp PCR System 9700, with the following conditions: initial denaturation at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 53 °C for 1 min, and extension at 72 °C for 1 min, with a final extension at 72 °C for 5 min. Visual confirmation of the overall amplification of the ITS region was performed using agarose gel electrophoresis (1.2%) stained with Gel Red (Biotium) and photographed in an image capture device (Bio Rad Gel Doc XR^{TM}).

Amplification products were sequenced using an ABI 3730 genetic analyser, with the Big Dye v.3 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Obtained sequences were analysed and ran in NCBI's BLAST (Basic Local Alignment Search Tool) database in order to assess the similarity with published sequences. For similarity values higher than 95%, the molecular identification was considered a valid match, although thoroughly confirmed by morphological traits according to Watanabe (2002) and Seifert et al. (2011). Sequences published in this study were deposited in GenBank under the assession numbers described in Table 1.

1.2.3 - Statistical analysis

Statistical analysis was performed calculating the Shannon-Wiener index ($H' = -\sum Pi \ln (Pi)$) and the species evenness ($E = H' / \ln (S)$), for each collection of documents. The Shannon-Wiener index is used to assess diversity in categorical data. It analyzes entropy, treating the species distribution and the size of a population as a probability, and is used to determine biodiversity values taking into account the number of species, the dominant species, and their distribution (Frosini 2006). The Shannon-Wiener index values have to be within the range of ($0 < H < \ln (S)$) and species evenness between average values of ($0 < E < 1$).

A Linear Model Regression Analysis was also calculated using Past 3.0 software. The goal of the regression model was to determine the relation between the age of each

document and the number of species, but also between the date of the documents and the number of isolates.

1.3 - Results

Table 1 displays the identification results for the isolated fungal species retrieved from the sampled parchment documents from all six collections as well as the corresponding GenBank accession numbers and the similarity values to the sequences deposited in NCBI databases. A total of 230 fungal isolates, corresponding to 22 different genera and 42 different species were obtained, identified and kept in culture (Figure 2). In documents 23 and 29 from "S. João dos Longos Vales" and in documents 63, 66, 67, 68 and 71 of the "Universidade de Coimbra" collection, no fungal isolates were obtained, even after repeated attempts.

Table 1 - Isolated fungi from ancient parchment documents: document number, name of the sampled collection, date of collection (sp. isolates identified using only molecular approach).

Document number	Collection	Century	Isolated fungi	Similarilty (%)	Acession number
1	Mosteiro de Pedroso	XIV	*Epicoccum nigrum*	99	KT898567
1	Mosteiro de Pedroso	XIV	*Phlebiopsis gigantea*	99	KT898568
1	Mosteiro de Pedroso	XIV	*Cladosporium cladosporioides*	99	KT898569
1	Mosteiro de Pedroso	XIV	*Penicillium spinulosum*	99	KT898570
1	Mosteiro de Pedroso	XIV	*Penicillium chrysogenum*	99	KT898571
2	Mosteiro de Pedroso	XIV	*Aureobasidium pullulans*	99	KT898572
2	Mosteiro de Pedroso	XIV	*Epicoccum nigrum*	99	KT898573
2	Mosteiro de Pedroso	XIV	*Alternaria alternata*	99	KT898574
2	Mosteiro de Pedroso	XIV	*Epicoccum* sp.	98	KT898575
3	Mosteiro de Pedroso	XIV	*Cladosporium cladosporioides*	99	KT898576
4	Mosteiro de Pedroso	XIV	*Cladosporium cucumerinum*	99	KT898577
4	Mosteiro de Pedroso	XIV	*Epicoccum nigrum*	99	KT898578
4	Mosteiro de Pedroso	XIV	*Epicoccum nigrum*	99	KT898579
5	Mosteiro de Pedroso	XIV	*Cladosporium cladosporioides*	100	KT898580
5	Mosteiro de Pedroso	XIV	*Chaetomium globosum*	100	KT898581
5	Mosteiro de Pedroso	XIV	*Cladosporium cladosporioides*	99	KT898582
5	Mosteiro de Pedroso	XIV	*Alternaria alternata*	99	KT898583
5	Mosteiro de Pedroso	XIV	*Cladosporium cladosporioides*	99	KT898584
5	Mosteiro de Pedroso	XIV	*Fusarium oxysporum*	99	KT898585
6	Mosteiro de Pedroso	XIV	*Alternaria* sp.	97	KT898586

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6	Mosteiro de Pedroso	XIV	*Nigrospora oryzae*	99	KT898587
7	Mosteiro de Pedroso	XIV	*Epicoccum nigrum*	100	KT898588
7	Mosteiro de Pedroso	XIV	*Penicillium griseofulvum*	99	KT898589
7	Mosteiro de Pedroso	XIV	*Epicoccum nigrum*	99	KT898590
8	Mosteiro de Pedroso	XIV	*Alternaria alternata*	99	KT898591
8	Mosteiro de Pedroso	XIV	*Alternaria alternata*	99	KT898592
8	Mosteiro de Pedroso	XIV	*Cladosporium cladosporioides*	99	KT898593
9	Mosteiro de Pedroso	XIV	*Cladosporium cladosporioides*	100	KT898594
9	Mosteiro de Pedroso	XIV	*Epicoccum nigrum*	99	KT898595
9	Mosteiro de Pedroso	XIV	*Cladosporium cladosporioides*	100	KT898596
9	Mosteiro de Pedroso	XIV	*Alternaria infectoria*	99	KT898597
9	Mosteiro de Pedroso	XIV	*Alternaria alternata*	100	KT898598
10	Mosteiro de Pedroso	XIV	*Penicillium chrysogenum*	100	KT898599
10	Mosteiro de Pedroso	XIV	*Epicoccum nigrum*	99	KT898600
11	Mosteiro de Pedroso	XIV	*Cladosporium cladosporioides*	100	KT898601
11	Mosteiro de Pedroso	XIV	*Epicoccum nigrum*	100	KT898602
12	Mosteiro de Pedroso	XIV	*Nigrospora sphaerica*	99	KT898603
12	Mosteiro de Pedroso	XIV	*Mucor racemosus*	99	KT898604
12	Mosteiro de Pedroso	XIV	*Aspergillus niger*	99	KT898605
12	Mosteiro de Pedroso	XIV	*Aspergillus niger*	99	KT898606
12	Mosteiro de Pedroso	XIV	*Alternaria alternata*	99	KT898607
12	Mosteiro de Pedroso	XIV	*Epicoccum nigrum*	99	KT898608
12	Mosteiro de Pedroso	XIV	*Cladosporium cladosporioides*	99	KT898609
12	Mosteiro de Pedroso	XIV	*Cladosporium cladosporioides*	100	KT898610
12	Mosteiro de Pedroso	XIV	*Epicoccum nigrum*	99	KT898611
13	Mosteiro de Pedroso	XIV	*Alternaria alternata*	99	KT898612
13	Mosteiro de Pedroso	XIV	*Epicoccum nigrum*	99	KT898613
13	Mosteiro de Pedroso	XIV	*Cladosporium cladosporioides*	99	KT898614
13	Mosteiro de Pedroso	XIV	*Epicoccum nigrum*	99	KT898615
14	Mosteiro de Pedroso	XIV	*Phanerochaete sordida*	98	KT898616
14	Mosteiro de Pedroso	XIV	*Alternaria* sp.	98	KT898617
14	Mosteiro de Pedroso	XIV	*Epicoccum nigrum*	99	KT898618
14	Mosteiro de Pedroso	XIV	*Alternaria infectoria*	99	KT898619
14	Mosteiro de Pedroso	XIV	*Epicoccum nigrum*	99	KT898620
14	Mosteiro de Pedroso	XIV	*Alternaria alternata*	99	KT898621
15	Mosteiro de Pedroso	XIV	*Fusarium* sp.	97	KT898622
15	Mosteiro de Pedroso	XIV	*Cladosporium cladosporioides*	99	KT898623
15	Mosteiro de Pedroso	XIV	*Epicoccum nigrum*	99	KT898624
15	Mosteiro de Pedroso	XIV	*Phlebiopsis gigantea*	100	KT898625
16	Mosteiro de Pedroso	XIV	*Epicoccum nigrum*	99	KT898626
16	Mosteiro de Pedroso	XIV	*Alternaria infectoria*	99	KT898627
17	S. João dos Longos Vales	XVI	*Penicillium corylophilum*	100	KT898628
17	S. João dos Longos Vales	XVI	*Aureobasidium pullulans*	99	KT898629
18	S. João dos Longos Vales	XVI	*Epicoccum nigrum*	99	KT898630
18	S. João dos Longos Vales	XVI	*Alternaria alternata*	99	KT898631
18	S. João dos Longos Vales	XVI	*Epicoccum nigrum*	99	KT898632
18	S. João dos Longos Vales	XVI	*Pithomyces chartarum*	99	KT898633

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19	S. João dos Longos Vales	XVI	*Epicoccum nigrum*	99	KT898634
19	S. João dos Longos Vales	XVI	*Mucor racemosus*	99	KT898635
19	S. João dos Longos Vales	XVI	*Mucor racemosus*	99	KT898636
20	S. João dos Longos Vales	XVI	*Chaetomium globosum*	100	KT898637
21	S. João dos Longos Vales	XVI	*Epicoccum nigrum*	99	KT898638
21	S. João dos Longos Vales	XVI	*Cladosporium cucumerinum*	99	KT898639
22	S. João dos Longos Vales	XVI	*Aureobasidium pullulans*	99	KT898640
24	S. João dos Longos Vales	XVI	*Alternaria alternata*	99	KT898641
24	S. João dos Longos Vales	XVI	*Cladosporium cladosporioides*	99	KT898642
25	S. João dos Longos Vales	XVI	*Arthrinium* sp.	99	KT898643
25	S. João dos Longos Vales	XVI	*Epicoccum nigrum*	99	KT898644
26	S. João dos Longos Vales	XVI	*Penicillium citrinum*	99	KT898645
26	S. João dos Longos Vales	XVI	*Penicillium citrinum*	99	KT898646
26	S. João dos Longos Vales	XVI	*Chaetomium globosum*	99	KT898647
26	S. João dos Longos Vales	XVI	*Epicoccum nigrum*	100	KT898648
26	S. João dos Longos Vales	XVI	*Epicoccum nigrum*	99	KT898649
26	S. João dos Longos Vales	XVI	*Hyphodermella rosae*	99	KT898650
27	S. João dos Longos Vales	XVI	*Aureobasidium pullulans*	99	KT898651
27	S. João dos Longos Vales	XVI	*Cladosporium cladosporioides*	99	KT898652
27	S. João dos Longos Vales	XVI	*Trichoderma* sp.	99	KT898653
27	S. João dos Longos Vales	XVI	*Trichoderma* sp.	99	KT898654
28	S. João dos Longos Vales	XVI	*Cladosporium cladosporioides*	99	KT898655
28	S. João dos Longos Vales	XVI	*Aspergillus fumigatus*	99	KT898656
28	S. João dos Longos Vales	XVI	*Trametes* sp.	96	KT898657
30	S. João dos Longos Vales	XVI	*Penicillium purpurogenum*	99	KT898658
30	S. João dos Longos Vales	XVI	*Alternaria alternata*	99	KT898659
31	Colegiada de Guimarães	XIII	*Penicillium* sp.	95	KT898660
31	Colegiada de Guimarães	XIII	*Chaetomium globosum*	99	KT898661
32	Colegiada de Guimarães	XIII	*Alternaria alternata*	99	KT898662
32	Colegiada de Guimarães	XIII	*Cladosporium cladosporioides*	100	KT898663
32	Colegiada de Guimarães	XIII	*Cladosporium cladosporioides*	99	KT898664
33	Colegiada de Guimarães	XIII	*Cladoposrium cladosporioides*	99	KT898665
34	Colegiada de Guimarães	XIII	*Penicillium chrysogenum*	99	KT898666
34	Colegiada de Guimarães	XIII	*Alternaria infectoria*	99	KT898667
34	Colegiada de Guimarães	XIII	*Aureobasidium pullulans*	99	KT898668
34	Colegiada de Guimarães	XIII	*Alternaria infectoria*	99	KT898669
35	Colegiada de Guimarães	XIII	*Penicillium glabrum*	100	KT898670
35	Colegiada de Guimarães	XIII	*Penicillium spinulosum*	99	KT898671
35	Colegiada de Guimarães	XIII	*Alternaria alternata*	99	KT898672
36	Colegiada de Guimarães	XIII	*Penicillium spinulosum*	99	KT898673
36	Colegiada de Guimarães	XIII	*Cladosporium cladosporioides*	99	KT898674
36	Colegiada de Guimarães	XIII	*Penicillium glabrum*	99	KT898675
36	Colegiada de Guimarães	XIII	*Penicillium glabrum*	99	KT898676
36	Colegiada de Guimarães	XIII	*Fusarium oxysporum*	99	KT898677
36	Colegiada de Guimarães	XIII	*Penicillium oxalicum*	99	KT898678
36	Colegiada de Guimarães	XIII	*Penicillium glabrum*	99	KT898679
37	Colegiada de Guimarães	XIII	*Penicillium spinulosum*	99	KT898680

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37	Colegiada de Guimarães	XIII	*Cladosporium cladosporioides*	99	KT898681
37	Colegiada de Guimarães	XIII	*Penicillium glabrum*	99	KT898682
37	Colegiada de Guimarães	XIII	*Penicillium glabrum*	99	KT898683
38	Colegiada de Guimarães	XIII	*Aspergillus versicolor*	99	KT898684
38	Colegiada de Guimarães	XIII	*Cladosporium cladosporioides*	99	KT898685
38	Colegiada de Guimarães	XIII	*Penicillium glabrum*	99	KT898686
38	Colegiada de Guimarães	XIII	*Penicillium spinulosum*	99	KT898687
38	Colegiada de Guimarães	XIII	*Penicillium spinulosum*	99	KT898688
38	Colegiada de Guimarães	XIII	*Penicillium glabrum*	99	KT898689
38	Colegiada de Guimarães	XIII	*Cladosporium cladosporioides*	99	KT898690
38	Colegiada de Guimarães	XIII	*Penicillium spinulosum*	99	KT898691
38	Colegiada de Guimarães	XIII	*Penicillium chrysogenum*	100	KT898692
38	Colegiada de Guimarães	XIII	*Penicillium griseofulvum*	100	KT898693
39	Colegiada de Guimarães	XIII	*Penicillium glabrum*	99	KT898694
39	Colegiada de Guimarães	XIII	*Penicillium spinulosum*	99	KT898695
39	Colegiada de Guimarães	XIII	*Penicillium spinulosum*	99	KT898696
39	Colegiada de Guimarães	XIII	*Pithomyces chartarum*	99	KT898697
39	Colegiada de Guimarães	XIII	*Alternaria infectoria*	100	KT898698
39	Colegiada de Guimarães	XIII	*Penicillium glabrum*	99	KT898699
40	Colegiada de Guimarães	XIV	*Penicillium glabrum*	99	KT898700
40	Colegiada de Guimarães	XIV	*Penicillium spinulosum*	99	KT898701
40	Colegiada de Guimarães	XIV	*Alternaria alternata*	99	KT898702
40	Colegiada de Guimarães	XIV	*Penicillium spinulosum*	99	KT898703
40	Colegiada de Guimarães	XIV	*Epicoccum nigrum*	99	KT898704
41	Colegiada de Guimarães	XIV	*Mucor* sp.	96	KT898705
41	Colegiada de Guimarães	XIV	*Penicillium glabrum*	99	KT898706
41	Colegiada de Guimarães	XIV	*Penicillium spinulosum*	99	KT898707
41	Colegiada de Guimarães	XIV	*Penicillium spinulosum*	99	KT898708
41	Colegiada de Guimarães	XIV	*Penicillium spinulosum*	99	KT898709
42	Colegiada de Guimarães	XIV	*Cladosporium* sp.	99	KT898710
42	Colegiada de Guimarães	XIV	*Penicillium spinulosum*	99	KT898711
42	Colegiada de Guimarães	XIV	*Penicillium corylophilum*	99	KT898712
42	Colegiada de Guimarães	XIV	*Penicillium citrinum*	99	KT898713
42	Colegiada de Guimarães	XIV	*Alternaria alternata*	99	KT898714
42	Colegiada de Guimarães	XIV	*Cladosporium cladosporioides*	100	KT898715
42	Colegiada de Guimarães	XIV	*Alternaria alternata*	99	KT898716
43	Colegiada de Guimarães	XIV	*Penicillium citrinum*	100	KT898717
43	Colegiada de Guimarães	XIV	*Epicoccum nigrum*	99	KT898718
43	Colegiada de Guimarães	XIV	*Alternaria* sp.	99	KT898719
43	Colegiada de Guimarães	XIV	*Arthrinium* sp.	99	KT898720
43	Colegiada de Guimarães	XIV	*Mucor* sp.	96	KT898721
44	Colegiada de Guimarães	XIV	*Aureobasidium pullulans*	99	KT898722
45	Colegiada de Guimarães	X	*Epicoccum nigrum*	99	KT898723
45	Colegiada de Guimarães	X	*Cladosporium sp.*	100	KT898724
46	Cabido da Sé de Coimbra	XIII	*Penicillium citrinum*	99	KT898725
46	Cabido da Sé de Coimbra	XIII	*Penicillium glabrum*	99	KT898726
46	Cabido da Sé de Coimbra	XIII	*Aureobasidium pullulans*	98	KT898727

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47	Cabido da Sé de Coimbra	XIII	*Penicillium chrysogenum*	99	KT898728
47	Cabido da Sé de Coimbra	XIII	*Penicillium chrysogenum*	99	KT898729
47	Cabido da Sé de Coimbra	XIII	*Cladosporium cladosporioides*	99	KT898730
48	Cabido da Sé de Coimbra	XIII	*Phoma* sp.	99	KT898731
48	Cabido da Sé de Coimbra	XIII	*Alternaria alternata*	99	KT898732
48	Cabido da Sé de Coimbra	XIII	*Arthrinium* sp.	99	KT898733
49	Cabido da Sé de Coimbra	XIV	*Epicoccum nigrum*	99	KT898734
49	Cabido da Sé de Coimbra	XIV	*Phoma herbarum*	97	KT898735
49	Cabido da Sé de Coimbra	XIV	*Alternaria alternata*	99	KT898736
50	Cabido da Sé de Coimbra	XIV	*Arthrinium* sp.	99	KT898737
51	Cabido da Sé de Coimbra	XIV	*Alternaria alternata*	99	KT898738
51	Cabido da Sé de Coimbra	XIV	*Aureobasidium pullulans*	100	KT898739
51	Cabido da Sé de Coimbra	XIV	*Penicillium brevicompactum*	100	KT898740
51	Cabido da Sé de Coimbra	XIV	*Aureobasidium pullulans*	99	KT898741
52	Cabido da Sé de Coimbra	XIV	*Cladosporium cucumerinum*	99	KT898742
52	Cabido da Sé de Coimbra	XIV	*Penicillium citrinum*	100	KT898743
53	Cabido da Sé de Coimbra	XIV	*Penicillium griseofulvum*	100	KT898744
53	Cabido da Sé de Coimbra	XIV	*Epicoccum nigrum*	99	KT898745
53	Cabido da Sé de Coimbra	XIV	*Cladosporium cladosporioides*	99	KT898746
53	Cabido da Sé de Coimbra	XIV	*Penicillium citrinum*	99	KT898747
54	Cabido da Sé de Coimbra	XIV	*Penicillium griseofulvum*	99	KT898748
54	Cabido da Sé de Coimbra	XIV	*Epicoccum nigrum*	99	KT898749
54	Cabido da Sé de Coimbra	XIV	*Cladosporium cladosporioides*	99	KT898750
54	Cabido da Sé de Coimbra	XIV	*Penicillium citrinum*	99	KT898751
55	Cabido da Sé de Coimbra	XIV	*Penicillium griseofulvum*	99	KT898752
55	Cabido da Sé de Coimbra	XIV	*Alternaria alternata*	99	KT898753
55	Cabido da Sé de Coimbra	XIV	*Epicoccum nigrum*	99	KT898754
55	Cabido da Sé de Coimbra	XIV	*Penicillium citrinum*	99	KT898755
55	Cabido da Sé de Coimbra	XIV	*Penicillium citrinum*	99	KT898756
56	Cabido da Sé de Coimbra	XIV	*Penicillium citrinum*	99	KT898757
56	Cabido da Sé de Coimbra	XIV	*Alternaria alternata*	99	KT898758
57	Cabido da Sé de Coimbra	XIV	*Penicillium citrinum*	99	KT898759
57	Cabido da Sé de Coimbra	XIV	*Aureobasidium pullulans*	99	KT898760
57	Cabido da Sé de Coimbra	XIV	*Botrytis cinerea*	99	KT898761
58	Cabido da Sé de Coimbra	XIV	*Aureobasidium pullulans*	99	KT898762
58	Cabido da Sé de Coimbra	XIV	*Aureobasidium pullulans*	99	KT898763
59	Cabido da Sé de Coimbra	XIV	*Penicillium citrinum*	100	KT898764
59	Cabido da Sé de Coimbra	XIV	*Penicillium spinulosum*	99	KT898765
59	Cabido da Sé de Coimbra	XIV	*Epicoccum nigrum*	99	KT898766
59	Cabido da Sé de Coimbra	XIV	*Penicillium griseofulvum*	99	KT898767
60	Universidade de Coimbra	XVIII	*Penicillium corylophilum*	99	KT898768
60	Universidade de Coimbra	XVIII	*Penicillium chrysogenum*	100	KT898769
61	Universidade de Coimbra	XVIII	*Aureobasidium pullulans*	99	KT898770
61	Universidade de Coimbra	XVIII	*Penicillium* sp.	100	KT898771
62	Universidade de Coimbra	XVIII	*Epicoccum nigrum*	99	KT898772
62	Universidade de Coimbra	XVIII	*Alternaria alternata*	100	KT898773
64	Universidade de Coimbra	XVIII	*Stagonospora* sp.	98	KT898774

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65	Universidade de Coimbra	XVIII	*Phialocephala dimorphospora*	99	KT898775
65	Universidade de Coimbra	XVIII	*Chaetomium globosum*	99	KT898776
69	Universidade de Coimbra	XVIII	*Penicillium* sp.	98	KT898777
70	Universidade de Coimbra	XVIII	*Eurotium* sp.	100	KT898778
72	Universidade de Coimbra	XVIII	*Penicillium spinulosum*	99	KT898779
73	Universidade de Coimbra	XIX	*Penicillium glabrum*	97	KT898780
74	Universidade de Coimbra	XIX	*Penicillium glabrum*	100	KT898781
74	Universidade de Coimbra	XIX	*Arthrinium* sp.	98	KT898782
74	Universidade de Coimbra	XIX	*Penicillium corylophillum*	99	KT898783
74	Universidade de Coimbra	XIX	*Penicillium spinulosum*	99	KT898784
75	Universidade de Coimbra	XIX	*Penicillium citrinum*	100	KT898785
76	Universidade de Coimbra	XIX	*Penicillium* sp.	97	KT898786
77	Universidade de Coimbra	XIX	*Penicillium corylophilum*	99	KT898787
77	Universidade de Coimbra	XIX	*Penicillium* sp.	96	KT898788
77	Universidade de Coimbra	XIX	*Aspergillus niger*	99	KT898789
78	Universidade de Coimbra	XIX	*Penicillium corylophilum*	100	KT898790
78	Universidade de Coimbra	XIX	*Phoma herbarum*	97	KT898791
78	Universidade de Coimbra	XIX	*Penicillium corylophilum*	100	KT898792
79	Universidade de Coimbra	XIX	*Penicillium corylophilum*	99	KT898793
79	Universidade de Coimbra	XIX	*Aspergillus niger*	100	KT898794
80	Universidade de Coimbra	XIX	*Penicillium chrysogenum*	99	KT898795
80	Universidade de Coimbra	XIX	*Penicillium corylophilum*	100	KT898796

Note: Documents 23, 29,63,66,67,68 and 71 are not included in this table because it was not possible to isolate any fungal samples.

Overall, the most common genera were *Alternaria*, *Cladosporium*, *Epicoccum* and *Penicillium*. The most frequent species overall were *Alternaria alternata*, *Aureobasidium pullulans*, *Cladosporium cladiosporioides*, *Epicoccum nigrum*, *Penicillium citrinum*, *Penicillium glabrum* and *Penicillium spinulosum* (Figure 2).

The most frequent species in the "Mosteiro de Pedroso" collection were *A. alternata*, *C. cladosporioides* and *E. nigrum*. The species *Fusarium oxysporum*, *Nigrospora oryzae*, *Nigrospora sphaerica*, *Phanerochaete sordida* and *Phlebiopsis gigantea* were isolated from this collection exclusively (Figure 2-A). In "Colegiada de Guimarães" the most frequent species were *A. alternata*, *C. cladosporioides*, *P. glabrum* and *P. spinulosum*. *Aspergillus versicolor* and *Penicillium oxalicum* were isolated from this collection only (Figure 2-B).

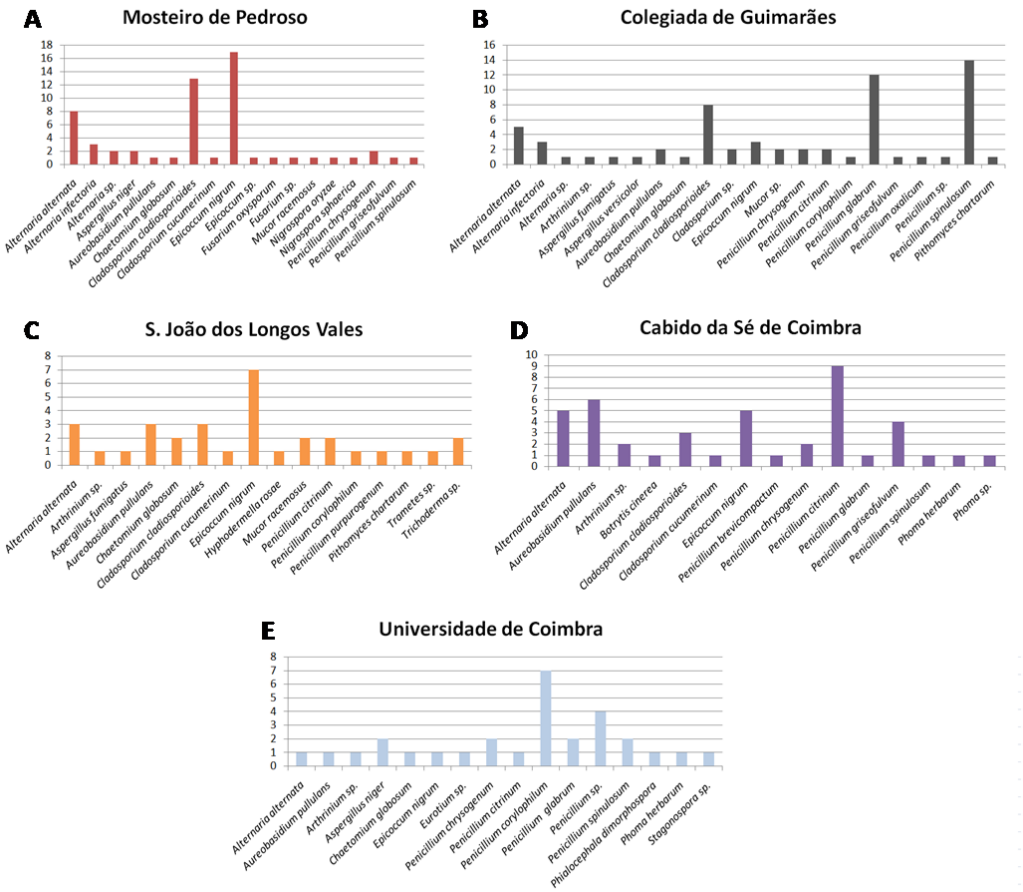

Figure 2 - Number of isolates by fungal species in the different sampled collections (A- "Mosteiro de Pedroso", B- "Colegiada de Guimarães, C- "S. João dos Longos Vales", D- "Cabido da Sé de Coimbra", E- "Universidade de Coimbra").

The most abundant species in the collection "S. João dos Longos Vales" was *E. nigrum,* while the species *Hyphodermella rosae*, *Penicillium purpurogenum*, *Trametes* sp. and *Trichoderma* sp. were exclusive to this collection (Figure 2-C). In "Cabido da Sé de Coimbra" collection the most frequent species were *A. alternata*, *A. pullulans*, *E. nigrum* and *P. citrinum.* The species *Botrytis cinerea*, *Phoma herbarum* and *Phoma* sp. were isolated only from this collection (Figure 2-D). There was a low occurrence of fungal organisms in the "Universidade de Coimbra" collection, however the genus *Penicillium* presented the most isolates and *Penicillium corylophilum* was the most frequent species. *Eurotium* sp. was only observed in this collection (Figure 2-E).

Biodiversity indices (Shannon-Wiener) were calculated for each collection and are displayed in Table 2, and are within average values ($0 < H < \ln(42)$). In general, the communities are dominated by a few abundant species, and many scarcer species (and $0 < E < 1$). The highest values for fungal diversity were found in the collection of "Colegiada de Guimarães", which is the oldest collection studied in this work. The lowest diversity values were found in the collections of "Universidade de Coimbra", which has the most recent documents.

Table 2 - Biodiversity indexes per collection.

Collection	Mosteiro de Pedroso	S. João Longos Vales	Colegiada de Guimarães	Cabido da Sé de Coimbra	Universidade de Coimbra
Nº of isolated fungi	61	32	66	43	29
Nº of species	20	16	21	15	16
Shannon-Wiener index	0.98	0.63	1.08	0.76	0.61
Species eveness	0.26	0.17	0.29	0.20	0.16

The *r2* values for the linear model regression analysis (Table 3) are low but demonstrate significant values for the regression model. Regarding the *p* values, they are significant ($p < 0,05$), which supports that the number of fungal species or the number of isolates, is related with the age of document, as displayed in Figure 3.

Table 3 - Results for the Linear Model Regression analysis.

Data analysis	Date/nº of species	Date/nº of isolates
R2	0.2198	0.2173
p(regr)	1.156E-05	1.311E-05

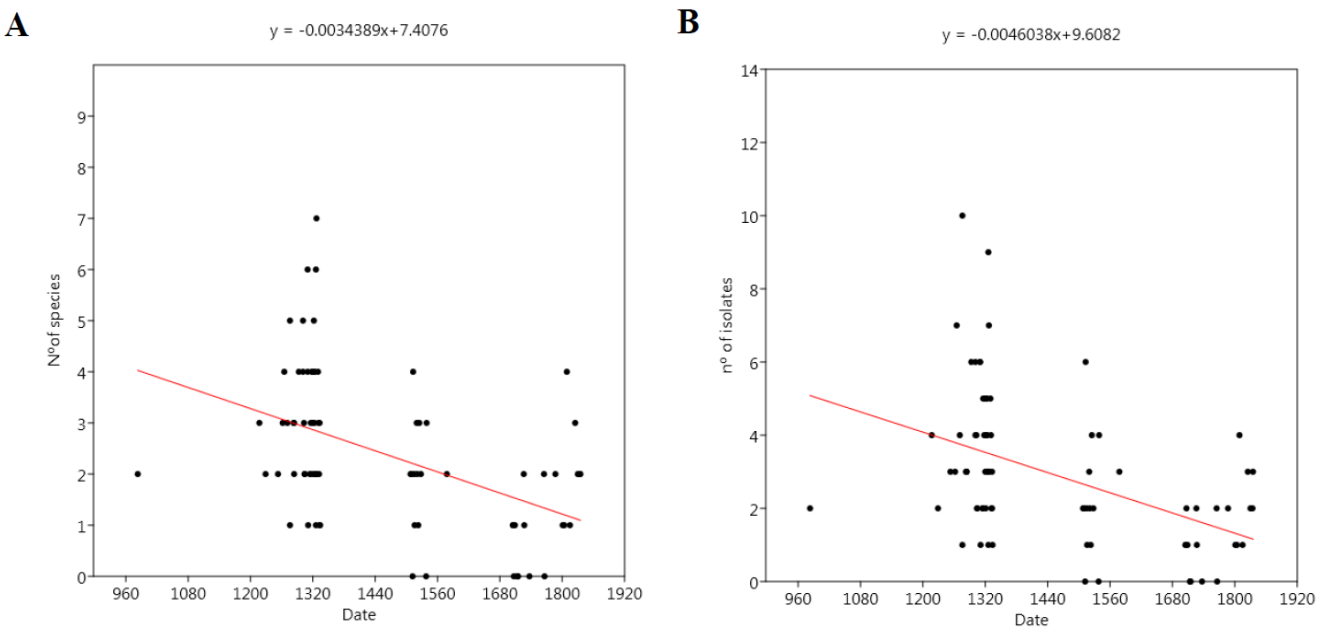

Figure 3 – Graphics for the Linear model regression analysis. **A**- Linear Model Regression between Date of the documents and the number of species; **B**- Linear Model Regression between Date of the documents and the number of isolates.

1.4 - Discussion

Biodeterioration of parchment documents by fungal species is a very important topic in terms of cultural heritage conservation. It is already well known that fungi have a big role in damaging parchment that have such priceless cultural and historical value. In this study, it was clear that the analysed parchment collections stored at the AUC, from different sites and ages, are already struggling with biodeterioration damage. Also, it is worthwhile mentioning that this is the first work done with a high number of parchment documents, in which eighty different documents were screened for fungal species identification.

In all studied collections, most documents presented the typical symptoms of fungal damage such as brown or black spots, text fading or significant structural damage, which allowed a rather directed sampling for specific biodeteriogens.

Regarding the identification of the isolated fungi, the ITS region is still a good marker for the identification of fungal species in documents and objects of art (Michaelson et al. 2006; Cappitelli 2010; Sterflinger 2010; Sterfilinger and Pinzari 2012). In this work, molecular analysis using the total ITS region, complemented by morphological analysis, allowed the identification of most isolates with great precision. It was possible to identify a total of 230 fungal isolates corresponding to 22 genera and 42 species. In general way, most frequent species such as *A. alternata*, *Alternaria* sp., *C. cladosporioides, Cladosporium* sp. and *E. nigrum* had already been isolated from parchment documents, as have other less frequent species, such as *Aspergillus fumigatus*, *Aspergillus niger*, *A. versicolor*, *Chaetomium globosum*, *Eurotium* sp., *Mucor racemosus*, *Penicillium chrysogenum*, *P. oxalicum*, *Penicillium* sp., *Phoma herbarum*, *Phlebiopsis gigantea* and *Trichoderma* sp. (Mesquita et al. 2009; Kraková et al 2012; Pinzari et al. 2012; Troiano et al 2014; Piñar et al 2015 a,b). On the other hand, other isolated species such as *Alternaria infectoria*, *A. pullulans*, *Arthrinum* sp., *B. cinerea*, *Cladosporium cucumerinun*, *F. oxysporum*, *H. rosae*, *N. oryzae*, *N. sphaerica*, *Penicillium brevicompactum*, *P. citrinum*, *P. corylophillum*, *P. glabrum*, *Penicillium griseofulvum*, *P. purpurogenum*, *P. spinulosum*, *Phanerochaete sordida*, *Phoma* sp., *Phialocephala dimorphospora*, *Pithomyces chartarum*, *Stagonospora* sp. and *Trametes* sp., have never been, to our knowledge, isolated from parchment documents. From all of these isolates, species such as *A. pullulans*, *Alternaria* sp., *B. cinerea*, *P. brevicompactum*, *P. citrinum*, *P. glabrum*, *P. griseofulvum*, *P. purpurogenum* and *P. spinulosum* have previously been retrieved from other supports, such as paper (Michaelson et al. 2009,2010; Sterflinger, 2010). Furthermore it can be emphasized that some of the isolated species have confirmed proteolytic activitity (see Table 4), which is likely to cause damage in parchment

Comparing with other works done in the AUC (Trovão et al. 2012; Nunes et al. 2013), species such as *A. alternata*, *A. infectoria*, *Alternaria* sp., *A. niger*, *A.versicolor*, *C. cladiosporioides*, *C. cucumerinum*, *Cladosporium* sp., *E. nigrum*, *P. brevicompactum*, *P. chrysogenum*, *P. citrinum*, *P. griseofulvum*, *Penicillium* sp., *P. spinulosum* and *Phlebiopsis gigantea* were also described and found to be dispersed by arthropods (Trovão et al. 2012). In the study done by Nunes et al. (2013) species such as *Alternaria* sp., *A.*

versicolor, *A. fumigatus*, *C. globosum*, *P. griseofulvum*, *P. oxalicum*, *Penicillium* sp. were also found in the air of this archive, and according to these results, this might explain such fungal presence in the AUC documental asset. Other less frequently isolated species, such as *F. oxysporum*, *H. rosae, N. oryzae*, *N. sphaerica*, *Phialocephala dimorphospora*, *Pithomyces chartarum* and *Stagonospora* sp. are, respectively, plant pathogens in cereal and in fruit (Menkis et al. 2004; Tóth et al. 2007; Pitt and Hocking 2009; Fourie et al. 2011; Babaeizad et al. 2012). Finally, other less common species, such as *Phanerochaete sordida* and *Trametes* sp., are saprophytic fungi responsible for wood decay (Collins and Dobson 1997). All the above mentioned species could also be found in air, soil or water and may be dispersed by different vectors, which might explain their presence in archive environments.

The frequent occurrence of *Epiccocum nigrum* in the collections "Mosteiro de Pedroso", considered as a water-damage related mould, may explain the amount of brown spots found in the documents of this collection, as mentioned by Sterflinger and Pinzari (2012).

The Shannon indices for each collection (Table 2), revealed a low fungal diversity in all sampled collections. Species evenness values are low in all collections, which suggest a heterogenous species distribution. In this way, in the "Colegiada de Guimarães", "Mosteiro de Pedroso", "Cabido da Sé de Coimbra" and "S. João dos Longos Vales" collections, there is a considerable number of isolates belonging to a low number of species, which is reflected in the low index values obtained. In the case of the Universidade de Coimbra" collection, a low number of isolates and species were obtained, which relates with the very low fungal diversity. It is likely that the oldest collections such as "Colegiada de Guimarães", "Mosteiro de Pedroso", "Cabido da Sé de Coimbra" and "S. João dos Longos Vales", have a higher fungal activity because they have been being colonised by fungal species for a longer period of time than more recent ones, such as "Universidade de Coimbra". It should therefore be emphasized the significant biodeterioration effects and fungal damage caused to the eldest collections, in comparison with more recent ones, whose documents were found to be in better, more preserved conditions. Archive environments facilitate the development of different microniches that can easily be occupied by microorganisms capable of

colonizing and rapidly growing with low water activity. This favours the appearance of opportunistic species in these supports as primary colonizers, and then, by succession,

Table 4 - Proteolytic activity of the isolated fungal species.

Isolated Species	Proteolytic activity	Reference
Alternaria alternata	yes	Zaferanloo et al. 2014
Alternaria infectoria	n.r.	-
Aspergillus fumigatus	yes	Kraková et al. 2012
Aspergillus niger	yes	Kraková et al. 2012
Aspergillus versicolor	yes	Michaelson et al. 2010
Aureobasidium pullulans	yes	Banadi et al. 2014
Botrytis cinerea	yes	Marchal et al. 2006
Chaetomium globosum	yes	Pinar et al. 2015ª
Cladosporium cladosporioides	yes	Pinar et al. 2015b
Cladosporium cucumerinum	yes	Robertson 1984
Epicoccum nigrum	yes	Bisht et al. 2004
Fusarium oxysporum	yes	Barata et al. 2002
Hyphodermella rosae	n.r.	-
Mucor racemosus	yes	Kraková et al. 2012
Nigrospora oryzae	yes	Šimonovičová et al. 2015
Nigrospora sphaerica	yes	Neves et al. 2013
Penicillium brevicompactum	yes	Pangallo et al. 2013
Penicillium chrysogenum	yes	Kraková et al. 2012
Penicillium citrinum	yes	Jenitta and Gnanadoss 2014
Penicillium corylophilum	yes	Rodrigues da Silva et al. 2013
Penicillium glabrum	yes	Nevarez et al. 2008
Penicillium griseofulvum	yes	Šimonovičová et al. 2015
Penicillium oxalicum	yes	Pinar et al., 2015b
Penicillium spinulosum	yes	Pangallo et al. 2013
Penicillium purpurogenum	yes	Nwadiaro et al. 2015
Phanerochaete sordida	n.r.	-
Phialocephala dimorphospora	n.r.	-
Phlebiopsis gigantea	n.r.	-
Phoma herbarum	yes	Kraková et al. 2012
Pithomyces chartarum	n.r.	-

Note: "yes": with proteolytic activity ; "n.r.": no proteolytic activity reported.

secondary colonizers establish. These are less frequent species, with high resistance to stress, which are able to grow in less stable environments (Sterflinger 2010; Pinheiro et al. 2011; Sterflinger and Pinzary 2012; Pinzari et al. 2012). Regarding this study,

parchment as a material showed a high water affinity probably because collagen easily binds to water molecules, causing water to be less available for microorganisms and therefore making it more difficult for the first colonization process by fungi to occur, since they have to overcome this stress condition (Piñar et al 2015a). The heterogenous species distribution is, in general, present in all these collections and this may suggest that some species with more evident presence, such as *A. alternata*, *A. pullulans*, *C. cladiosporioides*, *E. nigrum*, *P. citrinum*, *P. glabrum* and *P. spinulosum* should be considered opportunistic species and primer colonizers in the succession process (Pinzari et al. 2012). Moreover, documents belonging to "Colegiada de Guimarães", "Mosteiro de Pedroso", "Cabido da Sé de Coimbra" and "S. João dos Longos Vales" have only recently been stored in the AUC, but they were retrieved from ancient extinct buildings (such as Monasteries and Churches) and it is not possible to know their original storage or exposure conditions across time. These factors may have also influenced and even promoted the structural damaging of these supports.

The differences obtained with regard to fungal species distribution and occurrence between all the sampled collections may partially be due to airborne contamination. Although some species can actually be primary or secondary colonisers of collagen damaged fibres in parchment, indoor environments such as the AUC, have dust deposition that will probably induce fungal contamination in these supports. Moreover, this dust deposition in the AUC may also be influenced by surrounding vegetation and green areas close to its location (Piñar et al. 2015 b).

Obviously, time might influence the fungal diversity in parchment documents, but it is evident that the conditions of storage also have a major impact in fungal contamination and community composition. The results for the calculated linear regression model (Table 3, Figure 3) are conclusive about the influence of time in the number of fungal isolates and species. Time presented a low statistic significance in this relation (r^2) but a high probability ($p<<0.05$) of influencing the fungal colonization and growth in the supports, which suggests that time itself is one important factor in the fungal biodeterioration of parchment.

Documents made of parchment, as happens with other support types, are mostly stored in collections or grouped in proper storage materials. These supports are therefore subjected to the same environmental conditions, and should be studied as a collection and not as isolated cases (Powel 2010). Accordingly, in this work, fungal diversity was calculated per collection and not by support, in order to understand the differences between differently aged collections. It was important to note that the eldest collections were targeted by a larger fungal frequence and diversity than more recent ones. Similarly to the biodegradation of other materials, the material itself can be degraded by the chemical activity promoted by salt components and pH changes, which also propitiate a suitable microenvironment for fungal infection (Piñar et al 2015 a). Moreover, one could speculate that older collections of parchment have a higher probability of being exposed to a larger succession of opportunistic fungi. For that reason, the older a collection is, the more effort in handling and conservation should be considered.

Also, the correct handling of the documents should be advised for both workers and users of the Archive. Some of these isolated species (*Alternaria*, *Aspergillus*, *Aureobasidium*, *Chaetomium*, *Cladosporium* and *Penicillium* spp.) are adverse to human health and might be dispersed by human mechanical activity in the support. Despite the level of contamination obtained in some documents and collections, it is always important to study fungal communities on parchment surfaces because it may lead to the identification of potential biodeteriogenic species, which can grow when favourable conditions are met (Cappitelli et al. 2010; Troiano et al. 2014).

1.5 - Conclusions

Fungi clearly have a significant role in degrading collagen-rich supports such as parchment, and it is therefore very important to proceed with fungal sampling in these important documents, especially when they were stored in different collections, sometimes in very adverse conditions, since it is likely that these factors increase the quantity and diversity of potentially harmful biodeteriogens. Sometimes, the degrading action developed by some early species eventually facilitates the establishment of other species, since the substrate is already somewhat more susceptible and exposed to degradation. In this way, more work should be done in understanding how pioneer and opportunistic species act and relate themselves in degrading collagen, and how they promote the establishment of others, as it has been done for other types of support.

1.6 - Acknowledgements

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Chapter 2

Fungal contamination of paintings and wooden sculptures inside the storage room of a museum: are current norms and reference values adequate?

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Chapter 2

Fungal contamination of paintings and wooden sculptures inside the storage room of a museum: are current norms and reference values adequate?

Abstract

Biodeterioration is a topic of ever-growing concern and is particularly relevant in the context of cultural heritage conservation, since artworks and monuments provide diversified ecological niches for microorganism colonization.

Despite all the gathered knowledge in recent years, current established norms and accepted contamination thresholds have a prominent focus on human health and air quality preservation. Nonetheless they still are not enough or are not adequately applied for cultural heritage preservation.

In the light of this study within a very important Museum from Coimbra (Portugal), the current knowledge and accepted norms are discussed. Despite the meticulous control of environmental parameters inside this art repository, the presence of fungal colonies was unexpectedly detected on wooden sculptures and paintings that were deposited inside a custom-built room.

Contaminated art objects were sampled for fungal isolation and identification, along with seasonal indoor air sampling, for a one-year period. Molecular biology methods complemented with morphological observation were used for the identification of fungal organisms. Direct sampling of 8 contaminated paintings allowed the retrieval of 10 fungal isolates (3 different genera and 4 different species). In addition, 19 fungal isolates (5 different genera and 9 different species) were retrieved from 7 contaminated wooden sculptures. The air sampling process provided a total of 150 isolates (24 different genera and 43 different species), from which the most common genera were *Aspergillus*, *Cladosporium* and *Penicillium,* and the most frequent species were *Aspergillus versicolor*, *Cladosporium cladosporioides*, *Penicillium copticola* and *P. corylophilum*. Although the number of airborne CFU was considerably low in all seasons, some fungal species with known biodeterioration capability and adverse human health effects were found.

The relevance of air contamination monitoring as a single tool for biodeterioration risk assessment is discussed, as are the currently available norms and recommendations. Preventive measures are advised and considerations are made regarding potentially more effective approaches.

Keywords: Fungi; biodeterioration; storage room; art contamination; conservation measures.

Chapter 2

2.1. Introduction and Research Aims

Contamination of artwork in museums is a significant factor in what concerns the biodeterioration of Cultural heritage. In this respect, fungi represent a great nuisance to the work of conservators and restorers, not only because fungal degrading abilities allow these organisms to degrade a wide range of materials, but also because they represent serious risks to human health (Aira et al. 2007; Borrego et al. 2010).

In enclosed areas, such as museum storage rooms, the dispersion of fungal spores and mycelia is effected by the regular activity of workers and by inefficient ventilation systems. Several environmental parameters, such as temperature, relative humidity, or light intensity, have great impacts on fungal development (Guiamet et al. 2011; Krüger and Diniz 2011; Sterflinger and Pinzary 2012). In turn, as these organisms establish themselves and start thriving, hyphal penetration and enzymatic hydrolysis can bring about great damage to art pieces, with great historical and cultural value (Nunes et al. 2013; Sterflinger and Piñar 2013). Furthermore, low temperatures, which are often recommended for museum storage facilities, can promote water condensation on artwork surfaces, even at low relative humidity levels (Sterflinger and Pinzary 2012; Sterflinger and Piñar 2013). Affected materials may then act as nutrient sources, enhancing subsequent fungal growth and colonisation, mainly because microclimate conditions are favourable to sustain microniches (Karbowska-Berent et al. 2011).

The “Museu Nacional de Machado de Castro” (MNMC; Coimbra, Portugal) is a remarkable institution that holds a vast asset of ancient Portuguese religious artwork, such as paintings, sculptures and drawings. It is located in an historical area of Coimbra (Portugal), adjacent to an UNESCO World Heritage Site, where the 13th century-established University of Coimbra is the site’s flagship. With the aim of improving the conservation of its art repository, the MNMC has recently been subjected to an architectural intervention, with the construction of more modern facilities and the acquisition of equipment, mainly HVAC (Heating, Ventilation and Air Conditioning) systems for adequate control of indoor environments. Particularly for the air quality inside the archive and museum repositories, it is substantially affected by the level of

airborne contamination, usually by fungi, which is monitored via seasonal air-sampling assessments (Borrego et al. 2012).

During the renovation of the MNMC, most wooden sculptures and paintings were encased in protective plastic wrapping, having remained this way for over a year. When the architectural intervention was concluded, the artworks were unwrapped. However, a few months later, several items revealed damage consistent with fungal infestation.

The contaminated wood sculptures and paintings (canvas and painted wood boards) were examined for fungal contamination, and the air was sampled. The fungi were cultured, isolated and identified via molecular and morphological approaches, thus allowing for a better understanding of the community structure and its consequence to the infected materials (Sterflinger 2010). The sequencing of the ITS region was used, as it is a useful tool for molecular identification of fungal species and is widely applied to study these communities (Martin and Rygiewicz 2005; Michaelson et al. 2006; Pangallo et al. 2007, 2009).

Although an extensive knowledge has been reported in the literature, currently accepted international recommendations, concerning architectural and HVAC requirements for art conservation purposes, are insufficient or are applied improperly (Krüger and Diniz 2011). Consequently, the ideal conditions for artwork conservation are not always easy to ascertain and implement, often leading to indoor air quality in repositories being assessed only from human health and safety perspectives. Furthermore, existing directives on art repository standards of temperature and humidity are not adequate for everyday decision-making processes of museum conservators, curators and other staff, particularly because the guidelines do not consider the relevance of micro-niches and the fact that most biodeteriogens are microbial extremophiles (Sterflinger 2010; Sterflinger and Pinzary 2012).

Therefore, the aims of this research were to assess the diversity of fungal contaminants at the MNMC repository, to determine the actual culprits for the contamination, to ascertain the potential for cross-contamination between artwork

pieces (such as sculptures and paintings), and finally, to assess potential health-related risks and biodeterioration potential of the involved species.

2.2 - Materials and Methods

2.2.1 - Characterisation of the studied area

The artwork storage room of the Machado de Castro National Museum building is located below ground level and is equipped with a proper HVAC system. Temperature and Relative Humidity (T and RH) values are regularly monitored and stabilised with the Close Control Unit (CCU) (EMERSON - Network Power, Piestanska, Nove Mesto - Slovakia), for the recommended values of 60% and 21ºC, respectively (Bickersteth 2016). This repository has its paintings and wooden sculptures stored in appropriate conservation shelves.

2.2.2 - Biological sampling

2.2.2.1 - Air-sampling

Air-sampling was performed using an SAS Super ISO 100 bio-collector (SAS, Italy), with an air-flow of 100 L min^{-1}during 1 min, impacted in Malt Extract Agar (MEA, Difco™, Le Pont-de-Claix, France) *Petri* dishes, with streptomycin (0.5g/L) to prevent bacterial growth. Two replicates per location were collected in each season for a one-year period, in the different studied areas inside the storage room (Figure 1).

All samples were incubated at 28±1 ºC for 7 days and colony counts were performed. The different colonies observed, were then isolated into axenic cultures for further morphological and molecular analysis.

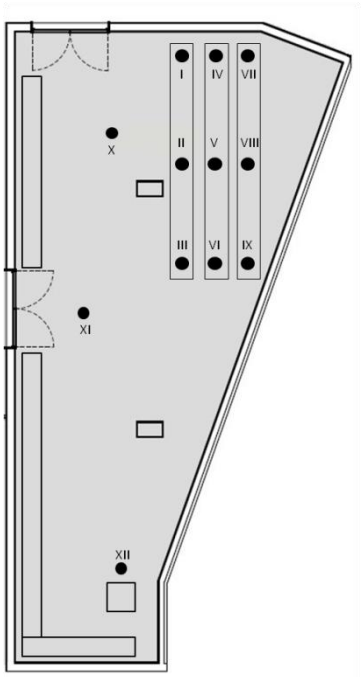

Figure 1 - Painting storage room plant. I to XII are the air sampling points inside this facility.

2.2.2.2 - Art object sampling

Sterile cotton swabs were used to sample visible fungal mycelia or colonies in eight contaminated paintings and seven contaminated wooden sculptures (Figure 2). After sampling, cotton swabs were submerged in 1mL of PBS solution for spore suspension, and dilutions were performed and were respectively plated onto two different media: MEA (Malt Extract Agar, Difco™, Le Pont-de-Claix, France) and PDA (Potato Dextrose Agar, Difco™, Le Pont-de-Claix, France) at 28±1 ºC for 7 days, allowing fungal growth. After this period, colonies were then re-isolated into axenic cultures (using the same culture medium from where they were isolated) for morphological and molecular identification.

Due to technical restrictions, *in situ* microscopic observation of colonies growing on materials was not possible. However, sampling with adhesive tape was performed on the artworks, for subsequent microscopic observation of fungal structures (Figure A Supplementary Material).

Figure 2 - Examples of contaminated paintings a) and b), and wooden sculptures, c) and d).

2.2.3 - Molecular and morphological identification of fungal isolates

DNA extraction from obtained isolates was performed using a custom protocol with REDExtract-N-Amp™ kit (Sigma-Aldrich, Missouri, USA): ± 1 mm^3 of mycelium from a pure culture was put in 10 µL of Extract solution (Marozzi et al. 2017) and submitted to 94°C for 10 min, followed by 60°C for 13 min, and 10°C for 15 min. After this incubation, 10 µL of Dilution solution were added.

After DNA extraction, the total ITS region was amplified by PCR, using the primers ITS4 and ITS1F (White et al. 1994; Gardes and Bruns 1996). For that purpose, PCR mixes were prepared with 12.5 µL of NZY*Taq*Green Master Mix (NZYTech™, Lisbon, Portugal), 0.5 µL of each primer (10 mM), 10.5µL of ultra-pure water, and 1 µL of template DNA, for a final reaction volume of 25 µL. The PCR reactions were performed using an ABI GeneAmpPCRSystem 9700, with the following conditions: initial denaturation at 95ºC for 2 min, followed by 30 cycles of denaturation at 95ºC for 1 min, annealing at 53ºC for 1 min, and extension at 72ºC for 1 min, and a final extension at 72ºC for 5 min. Visual confirmation of the overall amplification of the ITS region was performed using agarose

gel electrophoresis (1.2%) stained with GreenSafe Premium (NZYTech™, Lisbon,Portugal) and photographed in an image capture device (Bio-Rad Gel Doc XR™,California, USA).

Amplification products were sequenced using an ABI 3730 genetic analyser, with the Big Dye v.3 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, California, USA).

Obtained sequences were analysed and ran in NCBI BLAST (Basic Local Alignment Search Tool) database in order to assess the similarity with published sequences. For similarity values higher than 98.4%, the molecular identification were considered as valid matches, although thoroughly confirmed by morphological traits according to Watanabe (2002) and Seifert et al. (2011). Sequences obtained in this study were deposited in GenBank under the accession numbers described in Table A (Supplementary Material).

2.2.4 - Statistical analysis

Statistical analysis was performed calculating the Shannon-Wiener index ($H' = -\sum Pi \ln (Pi)$) and the species evenness ($E = H' / \ln (S)$), for each air-sampling season and for the sculptures and paintings. The Shannon-Wiener index is used to assess diversity in categorical data. It analyses entropy, treating the species distribution and the size of a population as a probability, and is used to determine biodiversity values taking into account the number of species, the dominant species, and their distribution (Frosini 2006). The Shannon-Wiener index values have to be within the range of ($0 < H < \ln (S)$) and species evenness between average values of ($0 < E < 1$).

2.3 - Results

A total of 150 fungal isolates, corresponding to 24 different genera and 43 different species were sampled, identified and kept in culture (Table A Supplementary Material). Figure 3 displays the results of the distribution of the identified fungal species from the seasonal air-sampling. Overall, the most common airborne genera were *Aspergillus*, *Cladosporium* and *Penicillium* and the most frequent species were

Aspergillus versicolor, *Cladosporium cladosporioides*, *Penicillium copticola*, and *Penicillium corylophilum*. The species *A. versicolor*, *C. cladosporioides*, *Penicillium spinulosum* were isolated in all seasons (Figure 3).

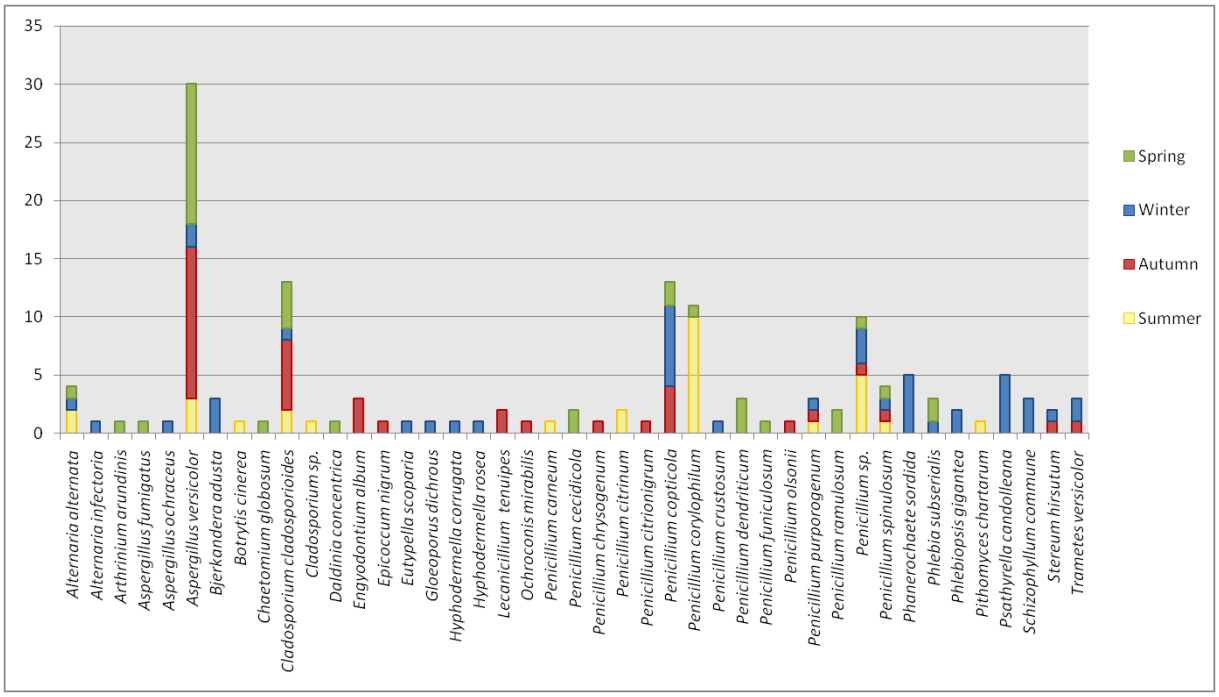

Figure 3 - Results of the isolated fungal species by the seasonal air sampling. X axis- isolated species; Y axis- number of isolates of each species in the different sampling season (according to the column color).

Table1 displays the results for the total CFU in the different sites inside the studied area (Figure 1), for each sampling season. Overall, winter registered the highest CFU counts.

The results of fungal Biodiversity are presented in Table 2. The Shannon-Wiener index (H) and Species Evenness (S) values for the air-sampled species in each season, for the isolated species on the sculptures and for the isolated species on the paintings are within average values ($0 < H < \ln(S)$). In general, the communities are dominated by a few abundant species ($0 < E < 1$). The highest fungal diversity values were detected during the winter season, whereas the lowest occured painting samples.

Table 1 - Average 'Total CFU Counts' for all seasons in all sampled points inside the storage room (results in CFU/m^3 with n=2).

SP*	Summer	Autumn	Winter	Spring
I	20	30	20	50
II	20	30	30	20
III	50	50	45	0
IV	20	20	20	50
V	20	50	10	50
VI	30	70	45	10
VII	30	20	20	10
VIII	45	10	80	10
IX	25	20	40	20
X	20	30	25	50
XI	25	10	45	20
XII	30	45	35	20

* SP – sampling points according to Figure 1.

Table 2 - Biodiversity indexes per season (air-sampling), sculptures and paintings.

	Nº of isolates	Nº of species	H' (Shannon-Wiener)	E (Species Evenness)
Summer	18	9	2.00	0.81
Autumn	38	15	2.21	0.82
Winter	45	22	2.83	0.92
Spring	36	16	2.35	0.85
Sculptures	18	9	1.96	0.89
Paintings	10	4	0.94	0.68

Regarding the wooden sculptures, *Chaetomium globosum* and *P. copticola* were the most frequent fungal species. Moreover, *Aspergillus sclerotiorum* and *Penicillium oxalicum* were exclusively found on wooden sculptures samples, and not in any of the air samples. *Penicillium citrinum, P. copticola, Phlebiopsis gigantea, Pithomyces chartarum* were only retrieved with PDA medium, while *P. oxalicum* was the only isolate obtained using MEA medium (Table B Supplementary Material). Among the fungal species isolated from paintings, *A. versicolor* was the most frequent one, while *Aspergillus ustus* was isolated using PDA, and from a single painting only (Table C Supplementary Material). *A. versicolor* and *C. globosum* were also present in the air samples. No fungal isolates were obtained from paintings 1 and 7, even after repeated

attempts. Interestingly, microscopic observation of the adhesive tape slides directly obtained from the contaminated artworks, revealed the presence of distinctive *Aspergillus* spp. and *Penicillium* spp. reproductive structures (Figure A Supplementary Material). *A. versicolor*, *C. globosum*, *P. copticola*, *P. citrinum*, *P. gigantea* and *P. chartarum* were isolated both from paintings and air samples. *A. versicolor* and *C. globosum* were isolated both from paintings and air samples and were also isolated from paintings and sculptures simultaneously (Figure 4).

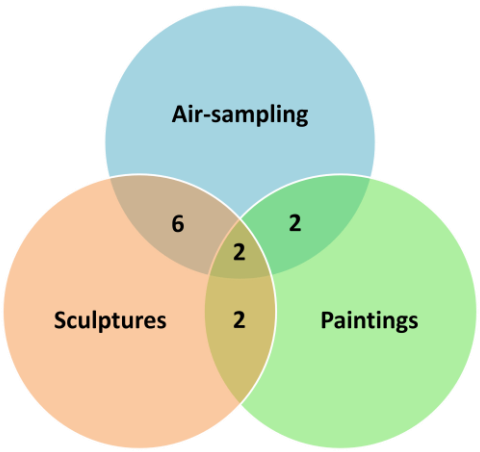

Figure 4 - Venn diagram for the co-occurence of fungal species between air-sampling isolates, sculptures isolates and paintings isolates. 2= {*Aspergillus versicolor* and *Chaetomium globosum*} ; 6= {*Aspergillus versicolor*, *Chaetomium globosum*, *Penicillium copticola*, *P. citrinum*, *Phlebiopsis gigantea* and *Pithomyces chartarum* }.

2.4 - Discussion

The “Museu Nacional de Machado de Castro” is of great relevance for Coimbra and Portugal, since it holds an inestimable collection of ancient artworks and sacral art.

Airborne contamination can be damaging to artwork kept in archives and museums, since fungal spores are mostly airborne and some species present a risk for both human health and art materials. Their biodeterioration ability may potentially cause irreversible damage on art pieces and other cultural heritage objects (Borrego et al. 2010; Sterflinger and Pinzary 2012; Sterflinger and Piñar 2013). Several fungal species which are known to cause damage or decaying symptoms on wood materials have been

reported to have particularly nefarious effects on the preservation of artwork in repositories. Table 3 displays a list of isolated species in this work, which have also been reported elsewhere.

In all sampled areas inside this storage room, air-sampling analyses revealed the presence of fungi that are potentially biodegradative on artworks. Examples of these species are *Alternaria alternata*, *Aspergillus versicolor*, *Chaetomium globosum*, *Cladosporium cladosporioides*, *Penicillium chrysogenum* and *P. citrinum* (Sterflinger 2010). Air-sampling also revealed typical seasonal variation, being winter the season with the highest values for the Shannon-Wiener and for the Species Evenness indices. Moreover, it is curious to note that wood decaying fungal species, such as *P. gigantea* or *P. chartarum* are predominant during winter months (Figure 3 and Table 3). *A. versicolor* is present in all sampled seasons, being dominant in autumn and spring. This species is considered a prime colonizer inside some buildings and for that reason it is frequently characterised as a "storage mould" (Nielsen 2003). Furthermore, *A. versicolor*, *C. globosum*, *Penicillium copticola*, *P. citrinum*, *Phlebiopsis gigantea* and *Pithomyces chartarum*, which were isolated from wooden sculptures and paintings, were also found in air-sampling isolates. These matching results are consistent with airborne contamination being the main vehicle for dissemination, as reported by other authors (Borrego et al. 2012; Sterflinger and Piñar 2013). Moreover, the co-occurrence of the same fungal species in artworks and in air samples is by itself demonstrative of this cross-contamination, as hinted in Figure 4 and corroborated by the microscopic observations of *Aspergillus* spp. and *Penicillium* spp. distinctive reproductive structures (Figure A Supplementary Material)

Institutions such as museums, galleries, libraries, and archives are strongly advised to guarantee optimal thermohygrometric conditions in order to maintain their organic material-based collections safe from mechanical damage and biodeterioration. However, and as seen in this case, even when kept below 60% RH, collections are not safe from fungal contamination and proliferation. The use of HVAC ventilation system with appropriate and regulated RH and T values did not prevent contamination and cross-contamination processes or the growth and establishment of fungi. Water condensation occurs on cold surfaces even at low RH (Sterflinger and Pinzary 2012;

Sterflinger and Piñar 2013), which in turn present growth opportunities to different fungal species, since they are not very nutritionally demanding and are able to thrive in a wide range of substrates.

Table 3 - Bibliographic references on the presence of fungal species in museum environment and/or with reported wood decay ability.

Isolated species	Reported in museum environment	Wood decaying species
Alternaria alternata	Zysca 1997; Sterflinger 2010; Bickersteth 2016	-
Alternaria infectoria	Trovão et al. 2013; Paiva de Carvalho et al. 2016	-
Arthrinium arundinis	-	Kim et al. 2010
Aspergillus fumigatus	Paiva de Carvalho et al. 2016; Kadaifciler 2016	-
Aspergillus ochraceus	Sterflinger 2010; Kadaifciler 2016	-
Aspergillus sclerotiorum	Sterflinger 2010; Carrazana-García et al. 2014	-
Aspergillus ustus	Bickersteth 2016; Kraková et al. 2012b	-
Aspergillus versicolor	Zysca 1997; Sterflinger 2010; Bickersteth 2016	-
Bjerkandera adusta	Trovão et al. 2013; Kraková et al. 2012a	Schmidt 2007
Botrytis cinerea	Zysca 1997; Sterflinger 2010; Kadaifciler 2016	-
Chaetomium globosum	Zysca 1997; Pangallo et al. 2009; Sterflinger 2010; Bickersteth 2016	Popescu et al. 2011
Cladosporium cladosporioides	Pangallo et al. 2009; Sterflinger 2010; Bickersteth 2016	-
Daldinia concentrica	Trovão et al. 2013	Hatakka and Hammel 2011
Engyodontium album	Šimonovičová et al. 2004; Trovão et al. 2013	-
Epicoccum nigrum	Zysca 1997; Sterflinger 2010; Kadaifciler 2016	-
Eutypella scoparia	-	Pildain, Novas and Carmarán 2005
Gloeoporus dichrous	-	Lim et al. 2005
Hyphodermella corrugata	-	Rahimlou et al. 2016
Hyphodermella rosea	Paiva de Carvalho et al. 2016	Rahimlou et al. 2016
Penicillium carneum	-	van der Wal et al. 2016
Penicillium chrysogenum	Zysca 1997; Pangallo et al. 2009; Sterflinger 2010; Bickersteth 2016	-
Penicillium citrinum	Sterflinger 2010	-
Penicillium copticola	Lutterbach et al. 2013	-
Penicillium corylophilum	Kadaifciler 2016	van der Wal et al. 2016
Penicillium crustosum	Trovão et al. 2013	-
Penicillium dendriticum	Trovão et al. 2013	-
Penicillium funiculosum	Zysca 1997	-
Penicillium olsonii	Trovão et al. 2013; Kadaifciler 2016	-
Penicillium oxalicum	Zysca 1997; Paiva de Carvalho et al. 2016	-
Penicillium purporogenum	Sterflinger 2010	-
Penicillium spinulosum	Sterflinger 2010; Trovão et al. 2013; Kadaifciler 2016	-
Phanerochaete sordida	-	Rüttimann, Cullen and Lamar 1994
Phlebia subserialis	Mesquita et al. 2009	Hatakka and Hammel 2011
Phlebiopsis gigantea	Sterflinger 2010; Trovão et al. 2013; Kadaifciler 2016	Schmidt 2007
Pithomyces chartarum	Paiva de Carvalho et al. 2016	Ahonsi et al. 2010
Psathyrella candolleana	-	Fu et al. 2013
Schizophyllum commune	-	Schmidt 2007
Stereum hirsutum	Trovão et al. 2013	Petre and Tănase 2013
Trametes versicolor	-	Schmidt 2007

"-" - not reported

During the renovation of the MNMC, most wooden sculptures and paintings were encased in protective plastic wrapping, which allowed the creation of microenvironments suitable for fungal growth, between the plastic and the artwork surfaces. In fact, the plastic wrapping itself was superficially colonised by the fungi, hampering appropriate air and water vapour exchanges (Sterflinger 2010; Montanari et al. 2012; Micheluz et al. 2015). Furthermore, the paintings were stored in close proximity to each other, facilitating fungal dispersion and cross-contamination. These facts, along with the possibility that inefficient ventilation could have been a vehicle for the dispersion of fungal spores and mycelia, may explain the quick emergence of fungal growth on the artworks, once they were unwrapped, despite climatic conditions being strictly controlled.

Archives and storage rooms are typically less frequently attended by visitors than typical museum rooms, which may lead to infrequent air renovation, and high levels of fungal spores and other particles (Karbowska-Berent et al. 2011; Borrego et al. 2012; Sterflinger and Piñar 2013). Consequently, this may pose serious health hazards for the museum staff, as they are exposed to biological and chemical contaminants for extended periods of time. Inhalation of microorganisms and the handling of mould-infested objects can cause respiratory and dermatologic problems, infections and mycotoxicoses caused by mycotoxin-emitting fungi (e.g. *Aspergillus flavus, Aspergillus fumigates, A. parasiticus*, *A. ustus*, *A. versicolor*, etc.) (Nielsen 2003). All determined fungal load values were low, according to current Portuguese national norms for the indoor air quality in Buildings and Facilities (Technical note NT-SCE-02 2009). Overall, fungal load never reached 500 CFU/m^3 and the values were relatively stable throughout the year, as would be expected for a controlled and closed environment. CFU values were always lower than 80 CFU/m^3. However, some authors have pointed out that, despite low CFU values, the mere presence of certain contaminant fungal species may represent, on its own, a threat for health and for the conservation of cultural heritage items (Sterflinger 2010; Pinheiro 2014). For instance, *Alternaria alternata*, *Aureobasidium pullulans*, *Chaetomium globosum*, *Cladosporium cladosporioides*, *Penicillium corylophilum, P. crustosum, P. chrysogenum*, *P. olsonii* and *P. oxalicum* are considered as potential pathogenic agents according to Directive 2000/54/EC of the

European Parliament and of the Council of 18 September 2000, on the protection of workers from risks related to exposure to biological agents at work by the EU 2000/54/WE Directive, the Regulation of the Minister of Health in Poland (22 April 2005), the European Confederation of Medical Mycology (BSL) and the Institute of Rural Health in Lublin (IMW). The species *Alternaria infectoria*, *Aspergillus fumigatus*, *A. ochraceus*, and *Ochroconis mirabilis,* were all identified in the present study, and are known human pathogens (Nielsen 2003; Shi et al. 2015), which highlights the need for updated and more efficient air quality norms.

Indoor air quality and occupational exposure are strongly regulated worldwide with very distinct quantitative and qualitative guidelines in what fungal contamination is concerned. Portuguese legislation regarding occupational exposure to biologic and chemical contaminants are currently only investigated when complaints arise. Regarding biological (bacterial and fungal) contaminants, previous legislation was stricter, since it warranted studies whenever the ratio between indoor and outdoor contamination levels was unfavourable and/or visible fungal growth was noticed. Based on the 2006 legislation, the 2013 Ordinance attempted to define and quantify which fungal species were potentially more damaging to human health. *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Blastomyces dermatitidis*, *Coccidioides immitis*, *Stachybotrys chartarum*, *Aspergillus versicolor*, *A. flavus*, *A. ochraceus*, *A. terreus*, *A. fumigatus*, *Fusarium moniliforme*, *F. culmorum* and *Trichoderma viride*, were defined as particularly hazardous, as a consequence of their pathogenicity or toxinogenicity. Common and uncommon fungi (all apart from the above mentioned) are evaluated according to their amount per m^3.

Using the classification system on which current legislation is based and considering the characteristics displayed in the sampled locations from a health and safety point of view, several conclusions can be pointed out:

- No pathogenic fungi (such as, *Cryptococcus neoformans, Histoplasma capsulatum, Blastomyces dermatitidis* and *Coccidioides immitis*) were present in the studied storage room;

- There was no visible fungal growth on the premises, walls or shelves, just on the art objects;

- Each considered uncommon species (*Acremonium* sp., *Chrysonilia* sp., *Trichothecium* sp., *Curvularia* sp. and *Nigrospora* sp.) never reached the 50 CFU/m^3. Mixed uncommon species never reached 150 CFU/m^3;

- Mixed common species – such as *Aspergillus* spp. (other than *A. flavus*, *A. fumigatus*, *A. ochraceus*, *A. terreus* and *A. versicolor*), *Alternaria* spp., *Cladosporium* spp., *Eurotium* spp., *Penicillium* spp. and *Wallemia* spp., never went over 500 CFU/m^3;

- Toxinogenic fungi were present. This group includes the *Aspergillus* species excluded from the common contaminants group, as well as *F. moniliforme*, *F. culmorum*, *T. viride* and *S. chartarum*, *A. fumigatus*, *A. ochraceus* and *A. versicolor* were identified in some of the sampled location and their presence may reflect a low air quality inside this art repository (Technical note NT-SCE-02 2009);

- Despite international norms differing in terms of their details, these guidelines are fundamental to ensure appropriate health and safety standards and to assess a given environment, since they ideally gather information from different sources and experiences and combine expert contributions. In this context, the data generated in this study may help designing improved guidelines, given that such thorough analyses help determine which factors are more relevant to minimise contamination.

In terms of conservation, there are no internationally accepted guidelines or admissible limits, either in number or presence of certain species. Some recommendations consider relevant the identification of the fungal species present while others disregard this assessment. The existing recommendations are mainly based on indices (CFU/m^3) and Table 4 presents some examples of these recommendations. The values determined for the studied storage room did not cross any of the established limits but it is easy to conclude that attributing a CFU limit without determining the specific elements present falls short, since it is very different, for a wooden artefact or a

painting, to be stored in a room with 50 CFU/m^3 of *C. cladosporioides* or 50 CFU/m^3 of *Serpula lacrymans*. Even when *S. lacrymans* is present and actively developing on wood, it may not be noticeable on air samples or it may not be easily retrieved on MEA or PDA. Most of the air samples taken in the present study show levels, below 50 CFU/m^3, a number most institutions would find reassuring. Nevertheless, fungal damage was being inflicted without suspicion.

Among the fungi commonly found in environmental studies performed in Archives and Museums some of them display cellulolytic properties such as species from the genera *Trichoderma*, *Penicillium, Botrytis, Trichothecium, Phoma, Chaetomium, Aspergillus, Cladosporium, Stemphylium, Alternaria, Hormodendrum, Aureobasidium*. Among the proteolytic genera, one can find *Aureobasidium*, *Chaetomium, Cladosporium*, *Botrytis*, *Trichoderma*, *Verticillium*, *Mucor, Epicoccum* and *Gymnoascus*. *Paecilomyces* displays lipolytic and proteolytic activity (Pinheiro et al. 2014). *Aureobasidium*, *Botrytis, Chaetomium, Cladosporium*, *Epicoccum* and *Mucor* genera were identified in the present study, in both air and surface samples.

For conservation purposes, and as happens with the occupational exposure and human health guidelines, it is important to complement quantitative limits with qualitative data. Looking at Table 4 and the observed damage in the storage room analysed, it is important that further recommendations also include surface samples, since air samples, when used exclusively, may not display the full fungal contamination scenario. In addition, when facing active fungal growth, it is important to identify contaminant species, not only to determine the best way to prevent their action, but also to enhance our current fundamental knowledge on this subject.

Seasonal sampling and comparison between seasons – from both a qualitative and quantitative point of view – may also bring relevant insights, but only on locations where ventilation is not sufficient to disperse an eventual build-up of fungal colonies. In the present storage room, the registered fluctuations did not reflect the active and ongoing process of biodeterioration taking place, since this room was equipped with a forced ventilation equipment. When possible, surface sampling should also be performed on the HVAC filters and outlets, to ascertain possible contamination or filter

saturation. The use of HVAC and other ventilation systems should be coupled with high-efficiency particulate air (HEPA) filters, to guarantee the lowest contamination possible,

Table 4 - Proposed limits for air fungal contamination (adapted from Pinheiro 2014).

Country	Reference	Limit (CFU/m3)
Poland	Harkawy et al. 2011	150 for a mixture of several species 50 for particular species 500 for common airborne fungal contaminants
	Karbowska-Berent et al. 2011	200
France	Parchas 2009	120
	Flieder and Capderou 2000	100
Italy	MIBAC 2001	150
The Netherlands	Brokerhof, Zanen and den Teuling 2009	0-25, no expected problems 25-100, possible presence of source, further testing needed 100-1000, source present, mould often observed on objects 1000, active mould growth

either from an exterior source or from adjacent rooms. Maintenance of these structures must be performed regularly, or they can easily become the source of the problem, disseminating fungal mycelia and spores after an unexpected fungal outbreak.

Because organic materials are being stored and since the levels of fungal contamination have been low on the studied location, it is important to maintain the recommended thermohygrometic values to assure the best protection possible. Presently, the existing guidelines are under discussion but, for storage of hygroscopic materials, these should not divert much from 40-60% RH (with a 5 to 10% fluctuation over 24h) and 16-25ºC. Care must be taken, however, to not rely solely on the RH and Temperature as these can disregard room features and the activity of staff members, which can be responsible for the creation of microclimates prone to fungal growth.

The existing active biodeterioration and air-contamination by fungi found in supposedly adequate and controlled environment (regarding temperature and relative humidity) reflect the hazardous ability of fungi, not only to the personnel of the institution but also to the collections stored within.

The studied room itself shows no visible fungal growth, water seepage or obvious signs of condensation. Despite the storage room being located below ground level, it is not likely that the observed fungal attacks on the artwork was due to this fact. However, its underground location does make it more difficult to solve problems related to art preservation. Low air renewal and improper airflow rates, and inadequate HVAC design may impede effective implementation of necessary conditions for the preservation of paintings and other artistic works, ultimately frustrating efforts to control contamination and prevent future fungal infestations.

To minimise long lasting effects of the current fungal infestation and to avoid future ones, staff at the MNMC was advised to avoid cross-contamination by keeping contaminated objects apart from the rest of the collection, until appropriate conservation efforts can be employed and no more active fungal growth is noticeable. Extrapolating our observations to a general level, we consider that a quarantine room should be available in every museum, to store uninspected items before these are exhibited or stored.

Given that the potential health risk that fungal contaminations may pose are not always acknowledged perceived by staff members, the use of personal protective equipment must become mandatory and be enforced. This includes disposable gloves, masks and, when necessary, safety goggles. These should be worn during the cleaning and treatment of the contaminated frames and artwork but also to clean the storage room before reintroducing the uncontaminated art objects.

As highlighted by Sterflinger (2010), in most cases, museum staff are not aware of the enormous potential of biodeterioration by fungi. Consequently, training and guidance should be given to staff, so that the gained skills may allow to correctly prevent, or treat biological contamination on artwork. Furthermore, museums should also be instructed on the importance of implementing systems for early fungal growth

detection. Overall, this implies the correct and careful handling, cleaning and supervising of these objects, as well as being able to clearly distinguish between a non-biological and a biological threat.

Once facing a fungal contamination, it is important to rely on the expertise that microbiologists and mycologists can bring, in order to correctly assess the danger posed by these powerful biodeteriorating agents. A multidisciplinary approach is, therefore, always recommended.

2.5 - Conclusions

This study shows the importance that better regulation, regarding preventive artwork conservation, can have on both the protection of objects as well as human health.

The biological damage on the pieces of art are sometimes neglected, or not appropriately treated, given the difficulties of implementing preventive measures. The existence of a real threat, such as the biological contamination of artworks, creates severe and irreversible damage, while putting at risk the cultural and historical value of these pieces.

Referring to scientific microbiological advice is fundamental, since these professionals have the skills and expertise to allow for the correct identification of contaminant microorganisms, and fully acknowledge the risks they may represent, both in terms of the preservation of cultural heritage and to human health. In addition to the correct identification of contaminant organisms, another important step to implement effective contamination deterrent systems is to evaluate dispersion mechanisms and type of damage that certain species may exert in different types of objects in a repository environment. Ultimately, we propose that microbiologists should be called upon to collaborate with art conservators and restorers, and other museum staff, to aid on the design of protocols and other measures to prevent and remediate biological contamination of artwork.

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2.7 - Supplementary Material

Table A - Fungi isolated from air-sampling: sample identification, species name, (isolates identified through molecular approach only).

Season	Sample ID	Species	Similarity (%)	Accession Number
Summer	Su - I - 1	*Penicillium* sp.	99	MF475905
Summer	Su - I - 2	*Penicillium corylophilum*	99	MF475906
Summer	Su - II - 1	*Penicillium corylophilum*	99	MF475907
Summer	Su - II - 2	*Penicillium corylophilum*	99	MF475908
Summer	Su - II - 3	*Penicillium* sp.	99	MF475909
Summer	Su - III - 1	*Penicillium corylophilum*	99	MF475910
Summer	Su - IV - 1	*Penicillium corylophilum*	99	MF475911
Summer	Su - IV - 2	*Pithomyces chartarum*	99	MF475912
Summer	Su - IV - 3	*Penicillium citrinum*	99	MF475913
Summer	Su - V - 1	*Penicilium* sp.	99	MF475914
Summer	Su - V - 2	*Penicillium* sp.	99	MF475915
Summer	Su - VII - 1	*Penicillium purpurogenum*	99	MF475916
Summer	Su - VII - 2	*Alternaria alternata*	99	MF475917
Summer	Su - VII - 3	*Penicillium corylophilum*	99	MF475918
Summer	Su - VII - 4	*Aspergillus versicolor*	99	MF475919
Summer	Su - VIII - 1	*Alternaria alternata*	99	MF475920
Summer	Su - VIII - 2	*Penicillium corylophilum*	99	MF475921
Summer	Su - VIII - 3	*Penicillium corylophilum*	99	MF475922
Summer	Su - VIII - 4	*Aspergillus versicolor*	99	MF475923
Summer	Su - IX - 1	*Penicillium corylophilum*	99	MF475924
Summer	Su - IX - 2	*Cladosporium* sp.	99	MF475925
Summer	Su -X - 1	*Penicillium corylophilum*	99	MF475926
Summer	Su - X - 2	*Penicillium* sp.	93	MF475927
Summer	Su - X - 3	*Aspergillus versicolor*	99	MF475928
Summer	Su - XI -1	*Cladosporium cladosporioides*	99	MF475929
Summer	Su - XII - 1	*Cladosporium cladosporioides*	99	MF475930
Summer	Su - XII - 2	*Penicillium carneum*	100	MF475931
Summer	Su - XII - 3	*Botrytis cinerea*	99	MF475932
Summer	Su - XII - 4	*Penicillium spinulosum*	99	MF475933
Summer	Su - XII - 5	*Penicillium citrinum*	100	MF475934
Autumn	Au - I - 1	*Trametes versicolor*	100	MF475935
Autumn	Au - II -1	*Ochroconis mirabilis*	100	MF475936
Autumn	Au - III - 1	*Aspergillus versicolor*	99	MF475937
Autumn	Au - IV - 1.1	*Aspergillus versicolor*	99	MF475938
Autumn	Au - IV - 2.1	*Penicillium copticola*	99	MF475939
Autumn	Au - IV - 2.2	*Aspergillus versicolor*	99	MF475940
Autumn	Au - IV - 2.3	*Cladosporium cladosporioides*	99	MF475941
Autumn	Au - V - 1.1	*Epicoccum nigrum*	99	MF475942
Autumn	Au - V - 2.2	*Penicillium copticola*	99	MF475943
Autumn	Au - VI - 1.1	*Cladosporium cladosporioides*	99	MF475944
Autumn	Au - VI - 1.2	*Aspergillus versicolor*	99	MF475945

(continued on next page)

Autumn	Au - VI - 2.1	*Aspergillus versicolor*	99	MF475946
Autumn	Au - VII - 1.1	*Cladosporium cladosporioides*	99	MF475947
Autumn	Au - VII - 1.2	*Cladosporium cladosporioides*	99	MF475948
Autumn	Au - VII - 1.3	*Aspergillus versicolor*	99	MF475949
Autumn	Au - VII - 2.1	*Cladosporium cladosporioides*	99	MF475950
Autumn	Au - VII - 2.2	*Aspergillus versicolor*	99	MF475951
Autumn	Au - VIII - 1.1	*Cladosporium cladosporioides*	99	MF475952
Autumn	Au - VIII - 1.2	*Penicillium chrysogenum*	100	MF475953
Autumn	Au - VIII - 1.3	*Penicillium copticola*	99	MF475954
Autumn	Au - VIII - 2.1	*Penicillium spinulosum*	99	MF475955
Autumn	Au - IX - 1.1	*Aspergillus versicolor*	99	MF475956
Autumn	Au - IX - 1.2	*Engyodontium album*	99	MF475957
Autumn	Au - IX - 1.3	*Aspergillus versicolor*	99	MF475958
Autumn	Au - IX - 1.4	*Stereum hirsutum*	99	MF475959
Autumn	Au - IX - 2.1	*Engyodontium album*	99	MF475960
Autumn	Au - IX - 2.2	*Aspergillus versicolor*	99	MF475961
Autumn	Au - X - 1	*Penicillium olsonii*	99	MF475962
Autumn	Au - XI - 1.1	*Penicillium copticola*	99	MF475963
Autumn	Au - XI - 1.2	*Aspegillus versicolor*	99	MF475964
Autumn	Au - XI - 1.3	*Lecanicillium tenuipes*	100	MF475965
Autumn	Au - XII - 1.1	*Penicillium purporogenum*	99	MF475966
Autumn	Au - XII - 1.2	*Penicillium* sp.	99	MF475967
Autumn	Au - XII - 1.3	*Penicillium citrionigrum*	99	MF475968
Autumn	Au - XII - 1.4	*Aspergillus versicolor*	99	MF475969
Autumn	Au - XII - 1.5	*Lecanicillium tenuipes*	99	MF475970
Autumn	Au - XII - 2.1	*Engyodontium album*	99	MF475971
Autumn	Au - XII - 2.2	*Aspergillus versicolor*	99	MF475972
Winter	Wi - I - 1.1	*Gloeoporus dichrous*	99	MF475973
Winter	Wi - I - 1.2	*Bjerkandera adusta*	100	MF475974
Winter	Wi - I - 2.1	*Phanerochaete sordida*	99	MF475975
Winter	Wi - I - 2.2	*Trametes versicolor*	99	MF475976
Winter	Wi - II - 1.1	*Phanerochaete sordida*	99	MF475977
Winter	Wi - II - 1.2	*Aspergillus versicolor*	99	MF475978
Winter	Wi - II - 2.1	*Phlebiopsis gigantea*	100	MF475979
Winter	Wi - II - 2.2	*Penicillium spinulosum*	99	MF475980
Winter	Wi - III - 1.1	*Eutypella scoparia*	99	MF475981
Winter	Wi - III - 1.2	*Penicillium copticola*	99	MF475982
Winter	Wi - III - 1.3	*Hyphodermella rosea*	99	MF475983
Winter	Wi - III - 1.4	*Phanerochaete sordida*	100	MF475984
Winter	Wi - IV - 1.1	*Psatyrella candolleana*	99	MF475985
Winter	Wi - IV - 2.1	*Alternaria alternata*	99	MF475986
Winter	Wi - IV - 2.2	*Penicillium copticola*	99	MF475987
Winter	Wi - IV - 2.3	*Bjerkandera adusta*	99	MF475988
Winter	Wi - V - 1.1	*Trametes versicolor*	100	MF475989
Winter	Wi - V - 1.2	*Penicillium copticola*	99	MF475990
Winter	Wi - VI - 1.1	*Alternaria infectoria*	99	MF475991
Winter	Wi - VI - 1.2	*Hyphodermella corrugata*	100	MF475992
Winter	Wi - VI - 1.3	*Schizophyllum commune*	99	MF475993

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Winter	Wi - VI - 1.4	*Cladosporium cladosporioides*	99	MF475994
Winter	Wi - VI - 2.1	*Penicillium* sp.	100	MF475995
Winter	Wi - VI - 2.2	*Psatyrella candolleana*	99	MF475996
Winter	Wi - VI - 2.3	*Bjerkandera adusta*	99	MF475997
Winter	Wi - VII - 1.1	*Psatyrella candolleana*	99	MF475998
Winter	Wi - VII - 1.2	*Penicillium copticola*	99	MF475999
Winter	Wi - VIII - 1.1	*Penicillium copticola*	99	MF476000
Winter	Wi - VIII - 2.1	*Trametes versicolor*	99	MF476001
Winter	Wi - IX - 1.1	*Phanerochaete sordida*	99	MF476002
Winter	Wi - IX - 1.2	*Penicillium copticola*	99	MF476003
Winter	Wi - IX - 2.1	*Penicillium crustosum*	99	MF476004
Winter	Wi - IX - 2.2	*Aspergillus ochraceus*	99	MF476005
Winter	Wi - IX - 2.3	*Penicillium purpurogenum*	99	MF476006
Winter	Wi - X - 1.1	*Schizophyllum commune*	99	MF476007
Winter	Wi - X - 1.2	*Psathyrella candolleana*	99	MF476008
Winter	Wi - X - 1.3	*Phlebia subserialis*	99	MF476009
Winter	Wi - X - 2.1	*Penicillium* sp.	99	MF476010
Winter	Wi - X - 2.2	*Schizophyllum commune*	99	MF476011
Winter	Wi - XI - 1.1	*Stereum hirsutum*	99	MF476012
Winter	Wi - XI - 1.2	*Penicillium* sp.	99	MF476013
Winter	Wi - XI - 2.1	*Phanerochaete sordida*	99	MF476014
Winter	Wi - XI - 2.2	*Aspergillus versicolor*	99	MF476015
Winter	Wi - XII - 1.1	*Psathyrella candolleana*	99	MF476016
Winter	Wi - XII - 1.2	*Penicillium copticola*	99	MF476017
Winter	Wi - XII - 2.1	*Phlebiopsis gigantea*	100	MF476018
Spring	Sp - I - 1.1	*Penicillium copticola*	99	MF476019
Spring	Sp - I - 1.2	*Daldinia concentrica*	100	MF476020
Spring	Sp - II - 1	*Phlebia subserialis*	100	MF476021
Spring	Sp - II - 2.1	*Penicillium copticola*	99	MF476022
Spring	Sp - II - 2.2	*Penicillium funiculosum*	99	MF476023
Spring	Sp - IV - 1.1	*Aspergillus fumigatus*	99	MF476024
Spring	Sp - IV - 1.2	*Arthrinium arundinis*	99	MF476025
Spring	Sp - V - 1	*Aspergillus versicolor*	99	MF476026
Spring	Sp - VI - 1.1	*Penicillium ramulosum*	99	MF476027
Spring	Sp - VI - 1.2	*Penicillium corylophillum*	99	MF476028
Spring	Sp - VI - 2.1	*Penicillium ramulosum*	99	MF476029
Spring	Sp - VI - 2.2	*Aspergillus versicolor*	99	MF476030
Spring	Sp - VII - 1.1	*Penicillium dendriticum*	99	MF476031
Spring	Sp - VII - 1.2	*Aspergillus versicolor*	99	MF476032
Spring	Sp - VII - 2.1	*Penicillium dendriticum*	99	MF476033
Spring	Sp - VII - 2.2	*Aspergillus versicolor*	99	MF476034
Spring	Sp - VIII - 1.1	*Phlebia subserialis*	99	MF476035
Spring	Sp - VIII - 1.2	*Aspergillus versicolor*	99	MF476036
Spring	Sp - VIII - 2.1	*Aspergillus versicolor*	99	MF476037
Spring	Sp - VIII - 2.2	*Penicillium dendriticum*	99	MF476038
Spring	Sp - IX -1.1	*Aspergillus versicolor*	99	MF476039
Spring	Sp - IX - 1.2	*Chaetomium globosum*	99	MF476040
Spring	Sp - IX - 2.1	*Aspergillus versicolor*	99	MF476041

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Spring	Sp - IX - 2.2	*Cladosporium cladosporioides*	99	MF476042
Spring	Sp - X - 1	*Aspergillus versicolor*	100	MF476043
Spring	Sp - XI - 1.1	*Cladosporium cladosporioides*	99	MF476044
Spring	Sp - XI - 1.2	*Aspergillus versicolor*	99	MF476045
Spring	Sp - XI -1.3	*Penicillium* sp.	99	MF476046
Spring	Sp - XI - 2.1	*Penicillium spinulosum*	99	MF476047
Spring	Sp - XI - 2.2	*Alternaria alternata*	99	MF476048
Spring	Sp - XI - 2.3	*Cladosporium cladosporioides*	99	MF476049
Spring	Sp - XII - 1.1	*Aspergillus versicolor*	99	MF476050
Spring	Sp - XII - 2.1	*Cladosporium cladosporioides*	99	MF476051
Spring	Sp - XII - 2.2	*Penicillium cecidicola*	99	MF476052
Spring	Sp - XII - 2.3	*Penicillium cecidicola*	99	MF476053
Spring	Sp - XII - 2.4	*Aspergillus versicolor*	99	MF476054

Table B - Fungal species isolated from wooden sculptures.

Sample	Species	Similarity (%)	Accession Number
A1**	*Aspergillus sclerotiorum*	99	MF476055
A2*	*Penicillium copticola*	99	MF476056
A3*	*Chaetomium globosum*	99	MF476057
B1*	*Penicillium copticola*	99	MF476058
B2*	*Chaetomium globosum*	99	MF476059
C1*	*Penicillium copticola*	99	MF476060
D1**	*Chaetomium globosum*	99	MF476061
D2**	*Aspergillus sclerotiorum*	99	MF476062
D3*	*Penicillium* sp.	98	MF476063
E1**	*Chaetomium globosum*	99	MF476064
E2*	*Pithomyces chartarum*	99	MF476065
E3*	*Penicillium citrinum*	99	MF476066
E4*	*Phlebiopsis gigantea*	100	MF476067
F1**	*Aspergillus versicolor*	99	MF476068
F2*	*Aspergillus versicolor*	99	MF476069
G1**	*Penicillium oxalicum*	99	MF476070
G2**	*Chaetomium globosum*	99	MF476071
G3*	*Chaetomium globosum*	99	MF476072

Note: *- isolated in PDA; **- isolated in MEA; A-H: Sculpture ID followed by the isolate number (1,2,3 or 4).

Table C - Fungal species isolated from paintings.

Sample	Species	Similarity (%)	Accession Number
2b*	*Aspergillus ustus*	99	MF476073
3a**	*Aspergillus versicolor*	99	MF476074
3b *	*Aspergillus versicolor*	99	MF476075
4a**	*Aspergillus versicolor*	99	MF476076
4b*	*Aspergillus versicolor*	99	MF476077
5a**	*Daldinia* sp.	98	MF476078
6a**	*Aspergillus versicolor*	99	MF476079
6b*	*Aspergillus versicolor*	99	MF476080
8a**	*Aspergillus versicolor*	100	MF476081
8b*	*Chaetomium globosum*	99	MF476082

Note: 2-6 and 8: number attributed for each sampled painting; * - isolated in PDA; ** - isolated in MEA.

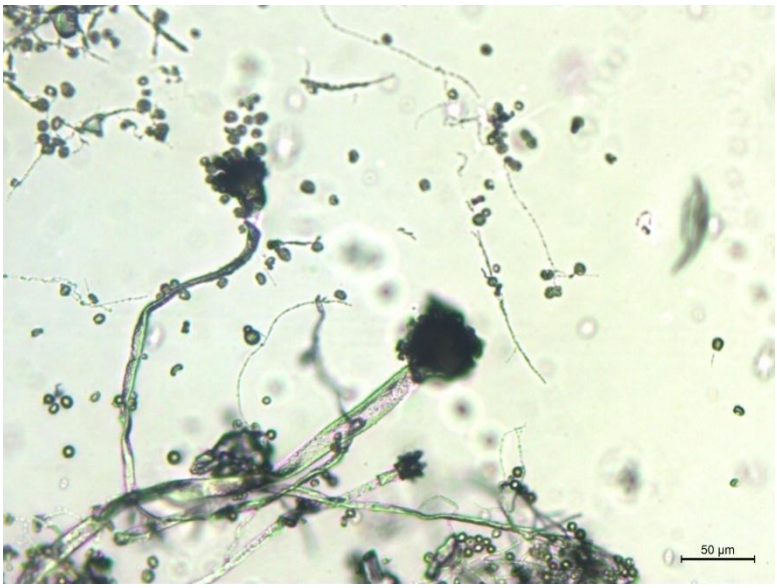

Figure A - Optical microscopy image of an adhesive tape slide with examples of *Aspergillus* spp. and *Penicillium* spp. structures, retrieved from the contaminated artworks.

Chapter 3

Fungal Stains on Paper: Is What You See What You Get?

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Chapter 3

Fungal Stains on Paper: Is What You See What You Get?

Abstract:

Effectively cleaning fungal stains from paper documents and artworks represents a challenge to paper conservators worldwide. To better target cleaning treatments, the identification of the causative fungal species and respective colorants is paramount. The present work was aimed at obtaining an association between specific fungal stains and fungal species. Twenty-three stains from three paper documents were sampled.*In situ* observation of fungal structures with optical and scanning electron microscopy was compared with the identification of collected samples by molecular biology tools. Correlations between the observed fungal structures *in situ* and the identified fungal isolates were achieved, varying from 13% to 64% of the samples within the three studied documents. Black and dark brown stains were associated with *Chaetomium globosum*, *C. murorum*, *Penicillium chrysogenum*, *P. commune*, *Myxotrichum deflexum* and *Stachybotrys chartarum*. *Eurotium rubrum* was isolated from a foxing stain and *Penicillium citrinum* was identified on light orange stains.

Keywords: Fungal stains; Paper conservation; Identification of fungi; SEM; DNA.

Chapter 3

3.1 - Introduction

Biodeterioration caused by moulds is a major problem affecting paper-based collections in museums, archives and libraries all over the world (Sequeira et al. 2014). Fungi have the ability to decompose paper materials, namely by the production of a variety of metabolites and enzymes. Excreted substances and fungal structures themselves are often coloured and interfere with the readability of the artefacts, diminishing their artistic and monetary value. In order to better target cleaning methods in the field of paper conservation, the fungal species and respective colourants responsible for the stains need to be assessed.

Most studies covering fungal identification in the field of cultural heritage are still mainly based on classic culturing methods (Pinzari et al. 2011). Even when fungal DNA is directly extracted from collected samples, what is identified may be contaminant species and not the actual colonization/degradation culprit (Piñar et al. 2015 c) A few recent studies are already examining microbial cells on biodeteriorated areas directly, to confirm the biodeterioration cause (Micheluz et al. 2015; Piñar et al. 2015 a,c), but there is still a general lack of knowledge of the association of particular types of biodeterioration with specific microorganisms.

The aim of the present work was to correlate fungal stains observed on paper objects with the fungal species actually responsible for them. To do so, fungal structures directly observed in sampled stains were compared with isolated fungal species identified by morphologic and molecular biology methods.

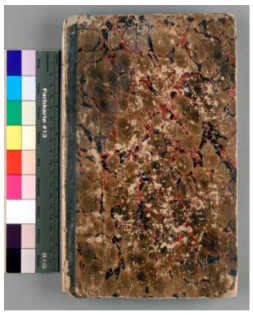
3.2 - Materials and Methods

3.2.1 - Studied documents

Two books and one print on paper (Figure 1), showing fungal stains, were selected. Document 1 (D1), a paperback wood pulp printed book dated from 1982, exhibited coloured stains mainly on the back cover and endleaves. Document 2 (D2), a

quarter leather binding book dated from 1853, was composed of rag paper (endleaves) and printed woodpulp paper (text block). Document 3 (D3), a painted print on paper, was profusely stained, with severe loss of mechanical strength. In all three documents, the stains were located within or nearby areas delimited by tide lines, where direct contact with water took place.

Document 1
Printed book, published in 1982, with the title "O que é a energia", by Franco Seleri, Editorial News, Basic Knowledge Library. Binding of individual folios glued on the spine, with a soft cover of cardboard coated with plastic. Text body composed of black mechanical paper.



Document 2
Printed book, 1853, entitled "Jornal do Associação Industrial Portuense, TOMO I", published in Porto, at “Typographia de Faria Guimarães”. Binding, with cardboard folders and covering in leather stockings with panels in marbled paper.

Document 3
Painted print on paper with representation of dog and bird, [s.d.], author signature not readable, 73x48cm.

Figure 1 - Documents (D1, D2 and D3) used for fungal sampling.

3.2.2 - Microscopic examination of stains

Fungal stains with distinct appearances under the stereomicroscope(Leica MZ16) were selected and sampled using small squares (2.25mm^2) of adhesive tape (Scotch® Magic),or scalpel and tweezers (Pinzari et al. 2010). Fungal structures existent on the collected samples were observed by optical microscopy (ZeissAxioplan 2 Imaging system) and scanning electron microscopy (Field Emission Gun Scanning Electron Microscope (FEG-SEM, JEOL 7001F)).

3.2.3 - Identification of fungal species

Sterile cotton swabs were used to collect samples from the selected stains. After a brief shaking in sterile water, for propagule dispersion, the solution was inoculated in two Petri dishes, one with potato dextrose agar (PDA, Difco™) and another with malt extract agar (MEA, Difco™), followed by incubation at ± 28°C. The different colonies were isolated into axenic cultures and incubated for subsequent morphologic and molecular identification. DNA was extracted from the colonies using the Extract-N-AMP (Sigma-Aldrich) kit, according to the manufacturer's instructions. After extraction, the ITS region was amplified by PCR, using ITS4 and ITS1F primers. For that purpose, PCR mixes were prepared with 12.5 µl of Green Master MIX (NZYtech) with MgCl2, 0.5 µl of each primer (10 mM), 10.5 µl of ultra-pure water, and 1 µl of DNA extract, for a final reaction volume of 25 µl. PCR reactions were performed using an ABI GeneAmp PCR System 9700 (Applied Biosystems), with the following conditions: initial denaturation at 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 53°C for 1 min, and extension at 72°C for 1 min, with a final extension at 72°C for 5 min. Visual confirmation of the overall amplification of the ITS region was performed using agarose gel electrophoresis (1.2%) stained with Greensafe Premium (NZYTech) and photographed in an image capture device (Bio Rad Gel Doc XR™). Amplification products were sequenced using an ABI 3730 genetic analyser, with the Big Dye v.3 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Obtained sequences were analysed and ran in NCBI BLAST (Basic Local Alignment Search Tool) database in order to assess the similarity with published sequences. For similarity values higher than 99%, the

molecular identification was considered a valid match, although thoroughly confirmed by morphological traits according to Watanabe (2002) and Seifert et al. (2011).

3.3 - Results and Discussion

Sampled stains of which observed fungal structures did not correspond to the identified fungal species, were classified as a negative correlation (Table 1). On the other hand, when the observed fungal structures could have been produced by the identified fungal isolates we could not ascertain an unequivocal match since there are numerous species from each genus producing similar cells. In those cases,a possible correlation was assigned (Table 1).

Although eight stains were sampled from book D1 (all showing fungal structures under the microscope), only two yielded fungal isolates (Table 1). Unlike documents D2 and D3, document D1 had already been mechanically cleaned. This procedure, by removing the aerial fungal structures, diminished the probability of collecting viable spores with the sampling swabs. Only in D1-H sample, a possible match between the identified *Chaetomium globosum* and the observed structures (black hairy perithecia) was obtained.

On D3, on the other hand, the stains were profusely covered by aerial fungal structures, which resulted in a higher percentage of samples with retrievable isolates (Figure 2). In fact, most samples yielded multiple isolates (Table 1).

Document D2 showed *Penicillium*-like conidiophores on the light orange spots on stain D2-A, where *P. citrinum* was identified. Accordingly, this fungus is known to produce yellow-orange soluble colourants (Houbraken et al. 2011). Even though, on the purple area of the stain (Figure 3A), several 2-3 µm spherical conidia with roughened walls were observed within the fibres (Figure 3B).These remained unidentified since no other isolates were obtained.

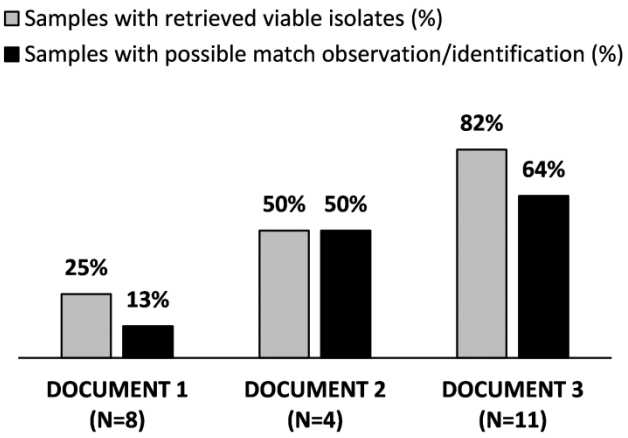


Figure 2 - Percentage of samples yielding viable isolates, and samples with possible correspondence between observed fungal structures on the stains and identified isolates.

Samples D2-C and D2-D were both collected from foxing spots, but only from sample D (Figure 3C), an isolate was obtained: *Eurotium rubrum* (Table 1). Fungal spores compatible with *Eurotium* species were also observed on the stain (Figure 3D). This xerophilic fungus (Pitt and Hocking 2009) has been previously identified on foxing stains and is able to produce brown colourants (Florian and Manning 2000; Karbowska-Berent et al. 2014).

Document D3 exhibited mostly dark brown or black stains. Sample D3-A (Figure 4A) revealed *Stachybotrys chartarum* characteristic conidiophores and conidia (Figure 4B), in a well-developed colony. However, the obtained isolates did not match this species (Table 1). On sample D3-E, on the other hand, an isolate from *S.chartarum*was identified, which could result from cross-contamination, since D3-A and D3-E were closely located. *S. chartarum*, a known paper colonizer (Das et al. 1997; Ricelli et al. 1999), is a producer of high toxicity mycotoxins (Foladi et al. 2013). The identification of such a developed colony on this document alerts to potential health hazards related to the handling of fungal contaminated objects.

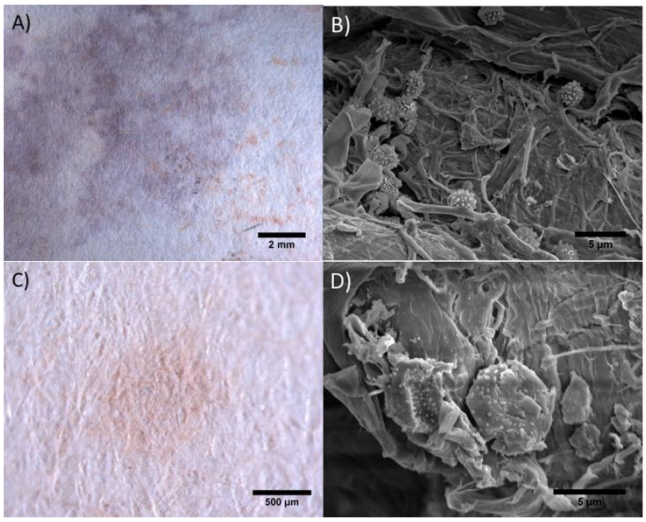


Figure 3 - Stereo microscopy images of stains and SEM images of the respective fungal structures observed on document D2. A) Stain D2-A under the stereo microscope. B) SEM image of conidia observed on the purple area of sample D2-A. C) Stain D2-Dunder the stereo microscope. D) SEM image of spores observed on sample D2-D.

The high frequency of *Chaetomium murorum* on document D3 is noteworthy, since this species is rarely identified on paper (Zysca 1997), whilst *C. globosum* is much more frequent (Rakotonirainy et al. 2007; Mesquita et al. 2009). *Myxotrichum deflexum*, observed (Figure 4D) and identified (Table 1) on stain D3-D (Figure 4C), with its large dark brown balls of branching hyphae, is apaper colonizer and producer of red colourants (Campbell et al. 2013; Sato et al. 2014).

On both D3-G and D3-H samples, analogous agglomerates of spherical to ellipsoidal smooth walled conidia (2.5-3.7µm) were observed, with some conidial chains. These cells are consistent with the identified *Penicillium* species (*P. chrysogenum* and *P. commune)*. Although no conidiophores were observed on the samples, which would help sustain the visual correlation with the obtained identification, getting multiple isolates of the same species in each sample supports a possible correlation. Fresh colonies of these *Penicillium* species have green hues but get darker with age (Samson et al. 2000), which could justify the dark brown colour observed on the stains.

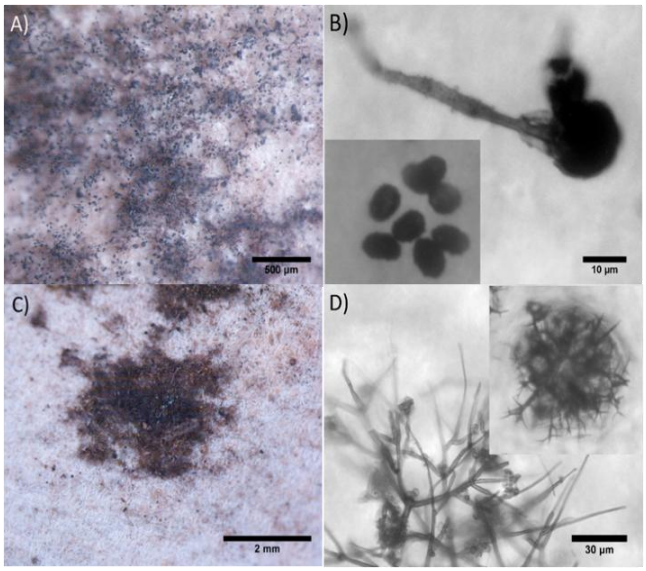


Figure 4 - Stereo microscopy images of stains and optical microscopy images of the respective fungal structures observed on document D3. A) Stain D3-A under the stereo microscope. B) Optical microscopy image of conidiophore and conidia (lower left corner detail) of *Stachybotrys chartarum* observed on sample D3- A. C) Stain D3-D under the stereo microscope. D) Optical microscopy image of peridial hyphae and cleistothecium (upper right corner detail) of *Myxotricum deflexum*, observed on sample D3-D.

On document D3, multiple isolates were retrieved from most stains. This can be the result of cross-contamination within the document: several fungal species were isolated from stains other than the ones where they were microscopically observed (Table 1). Besides, a stain caused by microorganisms can result from sequential or simultaneous colonization by different species. According to Figure 1, the percentage of samples with a possible match between observed fungal structures and identified fungi varied from 13% to 64%, within the three studied documents. Besides fungi, bacteria, which were not targeted on the present work, can also be responsible for stains on paper and can coexist with fungal species (Piñar et al. 2015a). Since the identification of fungi was preceded by culture, only the species still viable and able to develop on the tested growth media could be analysed. The use of different culture media may allow the retrieval of other species that can be difficult to grow in the tested media. Also, the lack of sampling material on previously cleaned stains (D1) was a limiting factor. In order to improve the correlation rates between identified microorganisms and

biodeterioration indicators, we are currently developing and testing an innovative sample collection method, which will be complemented by direct extraction of DNA from samples and analysis by Next Generation Sequencing.

Table 1 - Fungi isolated from stained areas in Document 1 (D1), Document 2 (D2) and Document 3 (D3) and respective accordance with fungal structures directly observed in the stains.

Document	Stain	Colour	Isolated fungi (similarity %)	Accession number	Observed fungal structures in accordance with isolates*
D1	A	Dark brown/black	N/A	N/A	N/A
D1	B	Dark brown/black	*Penicillium citrinum* (99)	KT898637.1	–
			Chaetomium globosum (99)	EU128633.1	–
D1	C	Redish brown	N/A	N/A	N/A
D1	D	Reddish/ dark orange	N/A	N/A	N/A
D1	E	Greyish brown	N/A	N/A	N/A
D1	F	Purple/brown	N/A	N/A	N/A
D1	G	Brown, black, olive green	N/A	N/A	N/A
D1	H	Dark brown	*Chaetomium globosum* (99)	EU330625.2	+
			Chaetomium globosum 99)	AB449671.1	+
D2	A	Purple with light orange spots	*Penicillium citrinum* (99)	KP942904.1	+
D2	B	Brown/orange, grey spots	N/A	N/A	N/A
D2	C	Brown/orange	N/A	N/A	N/A
D2	D	Brown/orange	*Eurotium rubrum* (99)	U18357.1	+
D3	A	Black	*Chaetomium murorum* (99)	JQ946413.1	–
			Chaetomium nigricolor (99)	JF439467.1	–
			Penicillium chrysogenum (100)	KT898599.1	–
D3	B	Orange stain w/ black fungal structures	*Chaetomium murorum* (99)	JQ946413.1	+
			Penicillium chrysogenum (99)	KT898599.2	–
			Chaetomium murorum (100)	JQ946413.1	+
			Penicillium chrysogenum (99)	KT898599.1	–
			Chaetomium murorum (100)	JQ946413.1	+
D3	C	Dark Brown/black	*Chaetomium murorum* (99)	JQ946413.1	+
			Chaetomium sp. (99)	KC427007.1	+
			Penicillium chrysogenum (99)	KT898599.1	–
			Penicillium chrysogenum (99)	KT898599.1	–
			Chaetomium murorum (100)	JQ946413.1	+
			Chaetomium murorum (100)	JQ946413.2	+

(*continued on next page*)

D3	D	Dark Brown/black	*Myxotrichum deflexum* (99)	JQ781738.1	+
			Penicillium sp. (99)	JQ781832.1	–
			Myxotricum deflexum (99)	JQ781738.1	+
			Myxotricum deflexum (99)	JQ781738.1	+
D3	E	Orange stain w/ black fungal structures	*Chaetomium murorum* (99)	JQ946413.1	+
			Penicillium chrysogenum (99)	KT898599.1	–
			Chaetomium murorum (99)	JQ946413.1	+
			Chaetomium murorum (99)	JQ946413.2	+
			Stachybotrys chartarum (99)	AF081468.2	–
D3	F	Dark Brown/black	*Penicillium chrysogenum* (99)	KT898599.1	–
D3	G	Dark Brown/black	*Penicillium chrysogenum* (99)	LN809047.1	+
			Penicillium chrysogenum (99)	KT898599.3	+
			Penicillium chrysogenum (99)	LN809047.1	+
D3	H	Dark Brown/black	*Penicillium commune* (99)	GQ458026.1	+
			Penicillium commune (99)	GQ458026.1	+
			Penicillium chrysogenum (99)	JQ781835.1	+
			Penicillium commune (99)	GQ458026.1	+
D3	I	Brown/olive green	N/A	N/A	N/A
D3	J	Brown/olive green	N/A	N/A	N/A
D3	K	Black	*Chaetomium murorum* (99)	JQ946413.2	+
			Chaetomium murorum (100)	JQ946413.2	+
			Chaetomium globosum (99)	EU301639.1	+
			Chaetomium murorum (99)	JQ946413.1	+
			Chaetomium globosum (99)	AB449671.1	+

* (–: negative correlation; +: possible correlation) N/A: not applicable, no isolates were obtained

3.4 - Acknowledgements

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Chapter 4

First application of high-throughput sequencing to identify fungal communities on a canvas painting.

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Chapter 4

First application of high-throughput sequencing to identify fungal communities on a canvas painting**.**

Abstract:

The present study describes the biodiversity of fungal communities in a 1960's canvas painting from the iconic Portuguese artist Paula Rego, using high-throughput sequencing on the *Illumina* MiSeq platform. Optical and Scan Electron Microscopy were employed to visualize fungal growth on the painting, with the purpose of choosing suitable sampling points. In order to prevent any damage to the artwork, a non-invasive sampling method was used to collect mycobiota, consisting of soft aspiration of the different sampling points. Typically, high values of fungal diversity were obtained in the points with greater fungal contamination. Fungal sequences from the classes *Dothideomycetes*, *Eurotiomycetes, Sordariomycetes, Agaricomycetes, Microbotryomycetes* and *Tremellomycetes* were identified in all of the 7 sampled points of the painting. The most representative orders were *Capnodiales*, *Eurotiales*, *Hypocreales*, *Russulales* and *Sporidiobolalles*. Genera such as *Aspergillus*, *Candida*, *Cladosporium*, *Penicillium*, *Tolypocladium*, *Rhodotorula*, *Stereum* or *Tricholoma* were also identified in the samples. The combination of a non-invasive sampling and next-generation sequencing by *Illumina* platform revealed relevant information about the diversity of fungal communities dwelling on this artwork. To our knowledge, this is the first study applying this modern molecular sequencing method for fungal diversity identification on a canvas painting.

Keywords: Fungi; Biodeterioration; Painting Canvas; Next-Generation Sequencing.

Chapter 4

4.1 - Introduction

Biodeterioration of cultural heritage artifacts by fungi is a concerning issue. Fungi are capable of establishing themselves, grow and thrive on a wide range of art objects, including canvas paintings (Lopez-Miras et al. 2013; Poyatos et al. 2017). Environmental conditions at exhibition rooms, museums and in artwork repositories must be correctly controlled and monitored, otherwise, fungi may severely affect the objects. When propitious light, temperature and relative humidity conditions are met, fungal growth and colonization starts and may irreversibly affect the materials, if no countermeasures are taken (Sterflinger and Pinzari 2012; Grabpek-Lejko et al. 2017).

Paintings, composed of different kinds of organic materials, like linen canvas, wooden frames, glues, pigment media or varnishes, are a rich medium for fungal colonization, mainly in conditions of high water activity (Grabpek-Lejko et al. 2017). Also, dirt, dust and environmental surface contaminants are other important nutritive sources (Santos et al. 2009; Capodicasa et al. 2010; Lopéz-Miras et al. 2012, 2013). By contrast, the presence of heavy metals, such as lead, zinc or chromium, in certain pigments can increase the resistance of the paint layer to microbial deterioration (Ciferri 1999; Tiano 2002).

Fungal species can thrive in canvas paintings and their growth results in aesthetical (pigment production, stains), mechanical or physical (by hyphal penetration leading to cracking and disintegration of the painted surface, formation of blisters and degradation of the support) and biochemical decay (enzymes and organic acids production) (Capodicasa et al. 2010; Sterflinger and Piñar 2013).

Fungi can degrade cellulose (main component of canvas and paper) by producing cellulolytic enzymes that can hydrolyze it. Glucose and other metabolites are consequently released, which can be used by other fungi or other community microorganisms as a carbon source for growth and energy. This cellulolytic action explains the depolymerization and loss of fiber structural integrity (Doménech-Carbó et al. 2007; Okpalanozie et al. 2016). The most frequent fungal species which can colonize

canvas paintings belong to the genera *Alternaria*, *Aureobasidium*, *Aspergillus*, *Chaetomium*, *Cladosporium*, *Penicillium* and *Stachybotrys* (Santos et al. 2009; Sterflinger 2010; Vukjević and Grbić 2010; Grabpek-Lejko et al. 2017).

Paula Rego, who is one of the most relevant contemporary Portuguese artists, is particularly known for her paintings and prints based on storybooks. One of such paintings, "Centauro", was the focus of the present study, as it is has been aesthetically altered by fungal staining. Composed by a canvas support covered with paper collages and painted with acrylic paint, "Centauro" was a fertile substrate for fungal colonization.

Molecular biology techniques for the metagenomics study of fungal communities are crucial to identify the diversity of fungal species present on an object. Therefore, it is important to choose the appropriate method. Until now, the existing few studies which report on the identification of fungal communities on canvas paintings make use of the Denaturing Gradient Gel Electrophoresis (DGGE) method, involving the Sanger sequencing of the community DNA samples, with adequate designed primers, and rDNA library construction for clone profiles comparison (Capodicasa et al. 2010; Lopéz-Miras et al. 2012, 2013; Okpalanozie et al. 2016). DGGE has been applied to the characterization of fungal communities in cultural heritage because of its advantage of allowing to directly profile microbial populations present in specific artifact materials (Schabereiter-Gurtner et al. 2001; Michaelson et al. 2009, 2010). However, despite being one of the most frequently used culture-independent method for the typing of microbial communities in artworks, few sequences are obtained with the clone libraries analysis, thus giving little information about the community structure. Moreover, it has a high economic cost per sequencing unit (Lupan and Popescu, 2012; Rosado et al. 2014).

In Cultural Heritage management, it is fundamental to accurately identify and characterize in detail the diversity of microbial communities present in the art pieces, given that this allows the planning and development of improved and more effective conservation and damage mitigation strategies (Rastogi and Sani, 2011; Rosado et al. 2014). Next generation sequencing (NGS) technology represents a high-throughput tool, which allows investigating deeper layers of the microbial communities. This type of detailed studies are essential to yield reliable and reproducible datasets about the

phylogenetic composition and functional diversity of the microbial communities being studied. Furthermore, it can quickly generate a great number of reads at lower costs, which can be useful for many applications (Rastogi and Sani, 2011, Kraková et al. 2017). In this way, culture-independent methods employing NGS technology are now a remarkable molecular tool to describe the communities dwelling in cultural heritage materials and objects, and several high-throughput sequencing platforms are available for this matter (Cutler et al. 2013; Rosado et al. 2014; Adamiak et al. 2017). *Illumina* MiSeq is one of such platforms, which has already given relevant results about microorganism diversity on different cultural heritage materials such as: stone (Chiementi et al. 2016), wood (Liu et al. 2017), brick (Adamiak et al. 2017), paper (Szulc et al. 2018), books (Kraková et al. 2017) or facades (Gaylard et al. 2017). This technology has also been useful to collect data regarding fungal communities colonizing cultural heritage materials (Gutarowska et al. 2015; Kraková et al. 2017; Szulc et al. 2018). To our knowledge, this is the first study reporting the diversity of fungal communities on a canvas painting by means of NGS.

In previous studies, the approach for microorganism sampling involved the use of scalpels to scrape the object surface (Santos et al. 2009; Capodicasa et al. 2010; Lopéz-Miras et al. 2012, 2013). In the present study, such sampling approach was not deemed appropriate, due to the high risk of causing irreversible damage to the object of the current study, the “Centauro” painting, which is of great artistic and cultural value. The use of non-invasive sampling methods through the use of cotton swabs or nitrocellulose membranes are other possible approaches (Okpalanozie et al. 2016). In this work, it would not be efficient enough as the sampling points were too small (3-4mm), thus risking the loss of biological material or of cross-contamination during the process. Therefore, in order to prevent any damage, a non-invasive sampling method was developed to collect mycobiota, by soft aspiration of the different sampling points.

The main objectives of this study were to: 1) develop and evaluate a new non-invasive sampling method based on soft aspiration; 2) evaluate the fungal diversity on the studied art-piece; 3) and to employ for the first time NGS methods to study biodeteriogen fungal species on a canvas painting.

4.2-Materials and Methods

4.2.1 – Art-piece sampling

The studied painting is an acrylic and graphite painting on canvas with paper collages (140x139cm), named “Centauro” (Figure 1). Inspired by her husband´s reading of ancient Greek storybooks, Paula Rego became interested in the Greek centaurs and everything involved with them, creating this artwork in 1964. “Centauro” represents a warrior standing on top of a female centaur. Paula Rego was not a pop artist but her love by comic strips and her fascination by Pop Art (that was becoming very popular at the time) brought in the flatness of the color and the more linear and designed look to this piece, which in fact ended up changing her work style.

Figure 1 - “Centauro” painting by Paula Rego (1964). Sampling points are indicated on the figure (A-G). (Reprinted with permission of “Casa das Histórias Paula Rego”)

This painting is accommodated in an artwork repository of this museum, equipped with a proper HVAC system. Temperature and Relative Humidity (T and RH) are regularly monitored and stabilised with the console CWO 14 RCAT, for the values of 19ºC and 65%, respectively. This artwork was selected from the Paula Rego’s vast repertoire, due to the presence of fungal stains on its surface. “Centauro” has been

stored under proper conditions since it has integrated the "Casa das Histórias Paula Rego" collection (Cascais, Portugal) in 2009. It is not possible though, to retrieve the history of the environmental conditions this painting had been previously subjected to, which at some point enabled the development of fungal colonies.

Sampling points were selected through visual screening using a stereoscopic microscope (OM; Leica MZ16; Figure 2) and the fungal mycelia or reproductive structures were observed with higher magnification, using a scanning electron microscope (SEM-FIB; Zeiss Auriga CrossBeam; Figure 3). Seven sampling points were selected, from point A to G (Figure 1). For comparison with points of higher contamination, sample G was selected as an unstained sample, showing no fungal structures under SEM observation.

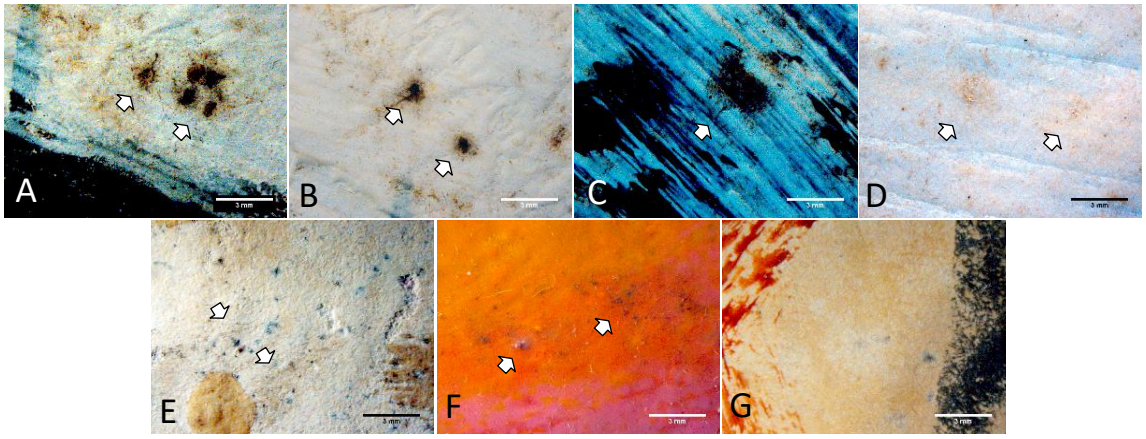

Figure 2- OM images of the painting sampled points: (A-F) arrows indicating stained areas; (G) unstained surface. Scale bar= 3mm.

Given the cultural value of the art piece, invasive sampling was not permitted on the "Centauro" painting. Therefore, an alternative non-invasive sampling procedure was developed, based on local soft aspiration for 10s, with a small vacuum pump, connected to a sterilized filtered pipette tip (20-200µl, Gilson ™, USA; Figure S1 of Supplementary Material). All parts composing the final sampling equipment (i.e. vacuum pump and tube) were sterilized by UV radiation for 30 minutes and packed in proper sterilized bags until usage. This sampling procedure was made inside the painting's repository room with extreme caution, and all the personnel were properly equipped to avoid any kind of cross contamination during this process.

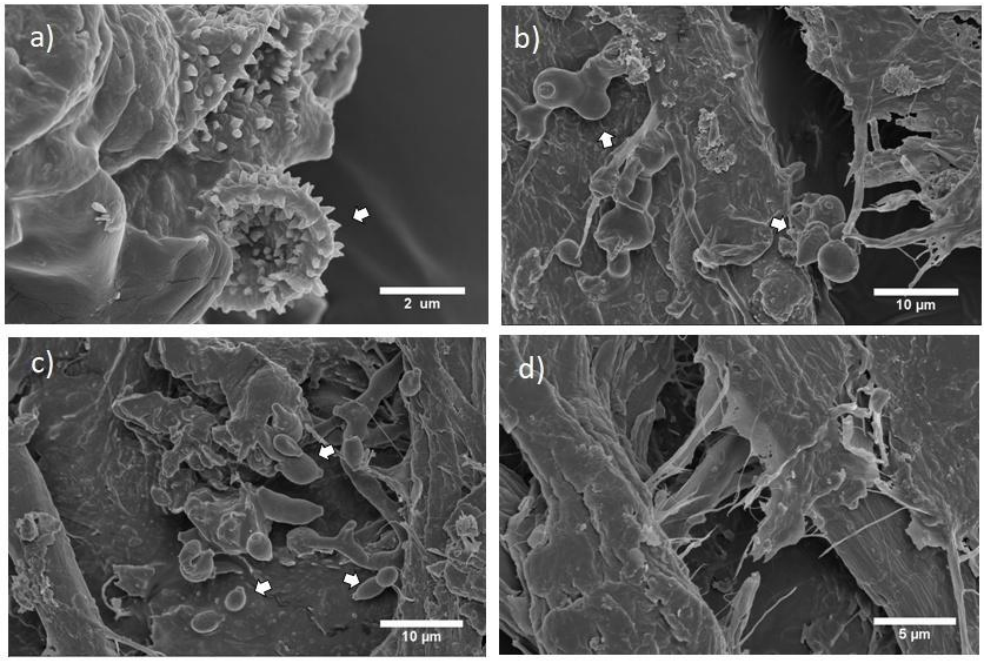

Figure 3 - SEM images of the painting sampled points: a), b) and c) example of fungal spores on samples A, D and E, respectively, pointed out with white arrows; d) sample G with no evident fungal colonization.

After the sampling procedures, the used filtered pipette tips were transported in previously sterilized falcon tubes to the laboratory. Each filter (Ø=4.5mm) used in each sample collection was removed from the respective pipette tip under sterile conditions in a Class II Biological Safety Cabinet, model MSC 1.2-Advantage™ (Thermo-Fisher Scientific™, USA), and suspended in 0.4ml of PBS buffer in 2ml sterilized microtubes, for subsequent DNA extraction and sequencing analysis.

4.2.2- Sample Preparation, DNA extraction and Sequencing

DNA was extracted from the retrieved samples using Nucleospin Soil Kit (Macherey Nagel, Düren, Germany) and Buffer SL1 in combination with Enhancer SX, according to manufacturer's instructions. For internal control, a non-sampled sterile pipette filter was also submitted to DNA extraction.

Samples were prepared for *Illumina* MiSeq Sequencing, targeting the Internal Transcribed Spacer 2 (ITS2) region. The DNA was amplified for the ITS2 region with specific primers and further reamplified in a limited-cycle PCR reaction to add sequencing adapters and dual indices. First PCR reactions were performed for each

sample using KAPA HiFiHotStart PCR Kit according to manufacturer suggestions, 0.3 µM of each PCR primer: a pool of forward primers: ITS3NGS1_F 5'-CATCGATGAAGAACGCAG-3',ITS3NGS2_F 5'-CAACGATGAAGAACGCAG-3', ITS3NGS3_F 5'-CACCGATGAAGAACGCAG-3', ITS3NGS4_F 5'-CATCGATGAAGAACGTAG-3', ITS3NGS5_F 5'-CATCGATGAAGAACGTGG-3', and ITS3NGS10_F 5'-CATCGATGAAGAACGCTG-3', and reverse primer ITS4NGS001_R 5'-TCCTSCGCTTATTGATATGC-3' (Tedersoo et al. 2014) and 50 ng of template DNA in a total volume of 25 µL. The PCR conditions involved a 3 min denaturation at 95 ºC, followed by 30 cycles of 98°C for 20 s, 60°C for 30 s and 72°C for 30 s and a final extension at 72°C for 5 min. Second PCR reactions added indices and sequencing adapters to both ends of the amplified target region according to manufacturer's recommendations (Illumina, 2013). Negative PCR controls were included for all amplification procedures. PCR products were then one-step purified and normalized using SequalPrep 96-well plate kit (ThermoFisher Scientific, Waltham, USA) (Comeau, Douglas and Langille 2017), pooled and pair-end sequenced in the Illumina MiSeq® sequencer with the V3 chemistry, according to manufacturer's instructions (Illumina, San Diego, CA, USA) at Genoinseq (Cantanhede, Portugal).

4.2.3- Data Analysis

Sequence data processing was performed at Genoinseq (Cantanhede, Portugal). Raw reads were extracted from *Illumina* MiSeq® System in FASTQ format and quality-filtered with PRINSEQ version 0.20.4 (Schmieder and Edwards 2011) to remove sequencing adapters, reads with less than 100 bases and to trim bases with an average quality lower than Q25 in a window of 5 bases. The forward and reverse reads were merged by overlapping paired-end reads with Adapter Removal version 2.1.5 (Schubert, Lindgreen and Orlando 2016) using default parameters. Raw data files in FASTQ format were deposited in NCBI Sequence Read Archive (SRA) with the study accession number SRP149788 under Bioproject number PRJNA474596.

The QIIME package version 1.8.0 (Caporaso et al, 2010) was used for Operational Taxonomic Unit (OTU) generation, taxonomic identification, sample diversity and richness indices calculation. Sample IDs were assigned to the merged reads and converted to fasta format. Chimeric merged reads were detected and removed using

UCHIME (Edgar et al. 2011) against UNITE/QIIME ITS database version 12.11 (Abarenkov et al. 2010). ITSx version 1.0.11 (Bengtsson-Palme et al. 2013) was used to extract the highly variable fungal ITS2 subregion from the merged reads. OTUs were selected at 97% similarity threshold using the open reference strategy. Merged reads were pre-filtered by removing sequences with a similarity lower than 60% against UNITE version 7.1 (Kõljalg et al. 2013) and the remaining merged reads were clustered at 97% similarity against the same database. Merged reads that did not cluster in the previous step were de novo clustered into OTUs at 97% similarity. OTUs with less than two reads were removed from the OTU table. A representative sequence of each OTU was then selected for taxonomy assignment. Alpha diversity indices chao1, dominance, equitability, goods coverage, observed species, Shannon and Simpson were calculated to reflect the diversity and richness of the fungal communities in the different samples. Chao1 rarefaction curves were also calculated.

4.3- Results

SEM revealed the presence of fungal structures in most sampling points of the painting (Figure 3). Fungal spores and mycelia were more readily detectable in samplings A, B, D and E. For samplings C, F and G, fungal structures were not detected under SEM analyses (Figure 3).

DNA extraction of the internal control filter gave negative results for DNA presence, thus confirming it as a negative control. In the case of the 7 sampled points, positive results of extracted DNA were obtained.

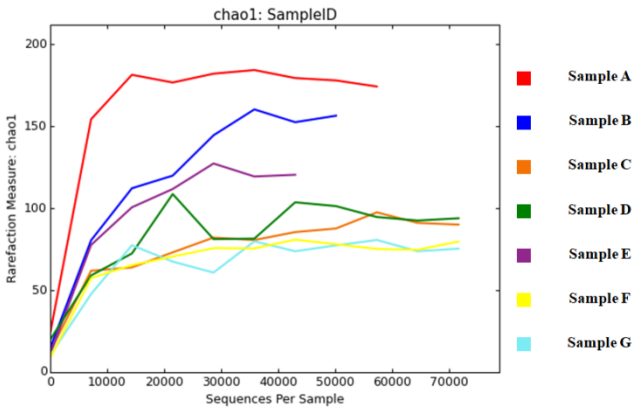

Figure 4 - Rarefaction curves (chao1) plot for each sample point.

High-throughput sequencing by *Illumina* technologies revealed the presence of considerably different fungal communities between the 7 sampling points. The results of the α-diversity (Table1) demonstrate that samples A, B and E were the ones with highest values of retrieved sequences, while samples F and G have the lowest values. The graphic for Chao1 rarefaction curves parameter clearly demonstrates this (Figure 4).

Table 1- Statistical data analysis derived from the seven studied samples.

Sample	chao1	dominance	equitability	goods coverage	observed species	shannon	simpson
A	173.0	0.055	0.725	0.999	168.0	5.360	0.944
B	155.769	0.099	0.655	0.999	110.0	4.445	0.900
C	91.833	0.081	0.6674	0.999	76.0	4.171	0.918
D	94.571	0.088	0.662	0.999	81.0	4.1995	0.911
E	121.545	0.239	0.513	0.999	92.0	3.348	0.760
F	73.791	0.139	0.530	0.999	70.0	3.254	0.860
G	75.3	0.0716	0.714	0.999	60.0	4.217	0.928

In general, the diversity values were high (Shannon and Simpson) for all samples, and the differences between Chao 1 values and the observed species are low. Samples B, C and E registered the highest difference between the expected number of sequences and the observed ones (Table 1). Nevertheless, the species distribution in the community (dominance and equitability, Table1) is according with the respective species diversity values. Moreover, the high value for the goods coverage in all samples reflects that the used molecular technique was highly sensitive in identifying a great and different amount of total fungal sequences.

Illumina MiSeq NGS of the samples taken from the seven points (A-G) revealed the presence of 117 genera belonging to 13 different classes of fungi. Sequences of *Ascomycota* were dominant, where the class of *Dothideomycetes* was the most representative with 23.1% of the total amount; followed by the class of *Agaricomycetes* (*Basidiomycota*) with 14.5% (Figure 5). *Eurotiomycetes* (*Ascomycota*) also had a significant presence of 10.5% (Figure 5). Fungal sequences from the classes *Dothideomycetes*, *Eurotiomycetes, Sordariomycetes, Agaricomycetes, Microbotryomycetes* and *Tremellomycetes* could be identified in all of the 7 sample

points of the painting. The most representative orders were *Capnodiales* (19.9%), *Eurotiales* (9.9%), *Saccharomycetales* (4.0%), *Hypocreales* (7.5%), *Russulales* (6.8%) and *Sporidiobolalles* (6.8%). From these, only *Hypocreales*, *Eurotiales* and *Capnodiales* were present in all the 7 samples of the studied artwork (Figure 6). The most dominant genera were *Cladosporium*, *Aspergillus*, *Penicillium*, *Candida, Talaromyces* and *Tolypocladium* (*Ascomycota* Phylum) and *Stereum*, *Rhodotorula*, *Trechispora*, *Trichosporon, Tricholoma* and *Malassezia* (*Basidiomycota* Phylum). The genus *Aspergillus* was present in all of the 7 sample points. (Table S1, Supplementary Material).

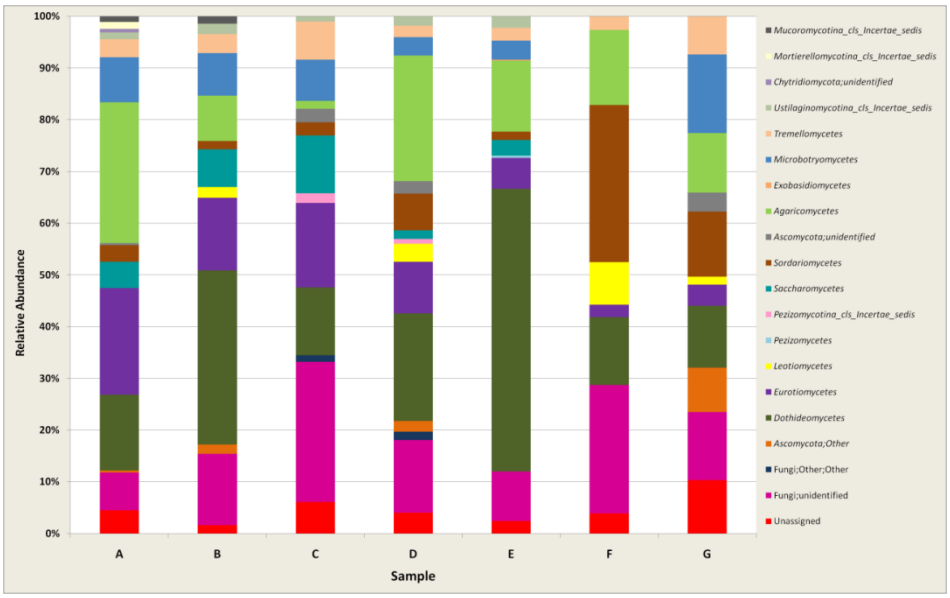

Figure 5 - Relative abundances (%) for each identified fungal classes in terms of sequences and OTUs, for each studied sample.

The fungal diversity observed in the different sampling points was also reflected at the level of relative OTU proportions. Generally, samples A, B and E had the highest values of different fungal taxa, whereas the sample F and G were the ones with the lowest fungal presence and diversity (Figure 5 and 6, Table S1 Supplementary Material). The highest abundance values were registered within the orders: *Capnodiales* in sample E (52%), followed by *Hypocreales* in sample F (30.4%), *Russulales* in sample A (25.7%) and *Agaricales* in sample D (20.8%)(Figure 6). The order *Capnodiales* also had a significant presence in samples B (29.1%), C (11%), D (17.6%) and G (12%). The same was

observed for the order *Eurotiales* in samples A (17.6%), B (13.10%), C (16.3%) and D (10%). Other significant percentage abundances occur with the orders *Saccharomycetales* in sample C (11.20%), *Hypocreales* in sample G (10.8%), *Russulales* in sample E (13.2%), *Trechisporales* in sample F (14.5%) and *Sporidiobolales* in sample G (15.2%)(Figure 5). Sequences belonging to organisms from the order *Eurotiales* are mostly represented by the genus *Aspergillus* in sample C (10.8%) and D (8.9%), while the order *Hypocreales* is primarily represented by the genus *Tolypocladium* in sample F (18.8%).The order *Agaricales* is mostly dominated by sequences of the genus *Tricholoma* in sample D (20.8%). The order *Russulales* is significantly represented by the genus *Stereum* in samples A (18.7%) and E (12.1%). The order *Trechisporales* is dominated by the genus *Trechispora* in sample F (14.5%), and *Sporidiobolales* has a high percentage of OTUs with the genus *Rhodotorula* in samples A (8.7%) and G (15.2%)(Figure 6, Table S1 Supplementary Material).

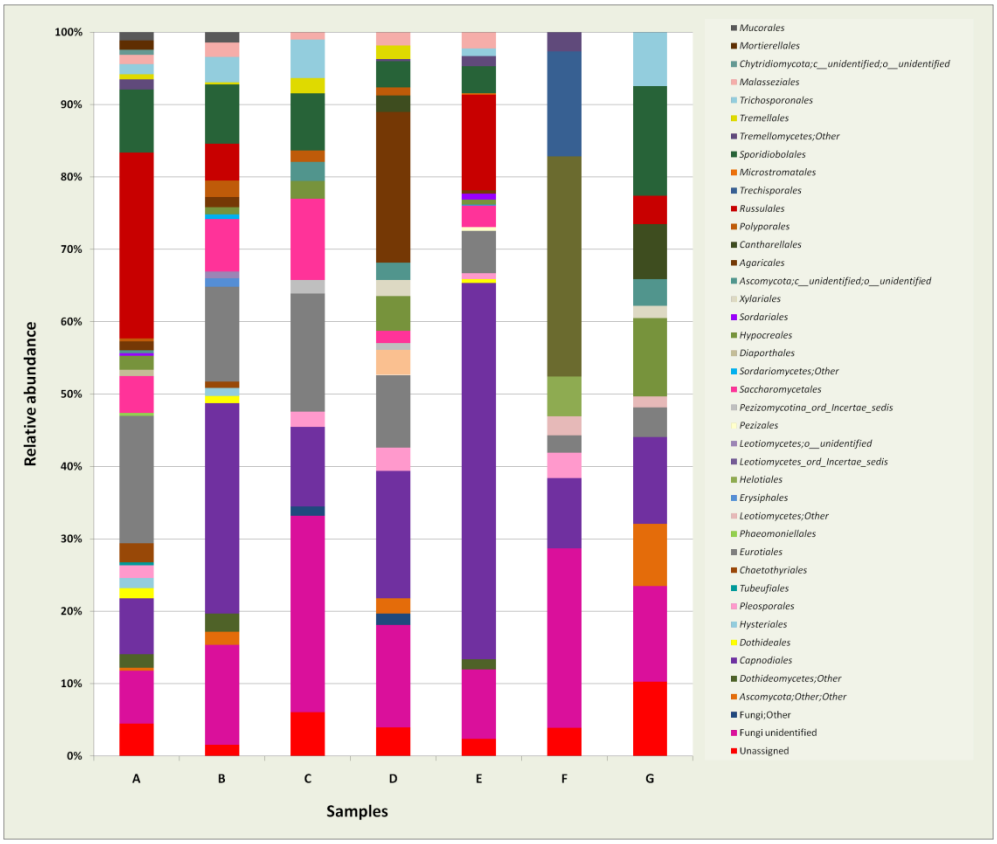

Figure 6 - Relative abundances (%) for each identified fungal orders in terms of sequences and OTUs, for each studied sample.

Remarkably, each of the 7 sampling points (A-G) contained unique genera. *Pyrenochaeta* (0.8%)*, Roussoella* (0.4%)*, Exophiala* (0.7%), *Neophaeomoniella* (0.4%), *Kluyveromyces* (0.6%), *Chaetomium* (0.4%), *Inocybe* (0.4%), *Lactarius* (0.3%), *Mortierella* (1.3%), *Mucor* (0.8%), were exclusively detected in Sample A. The same occurred for genera *Cercospora* (0.4%), *Cladophialophora* (0.9%), *Sawadaea* (1.2%), *Cyberlindnera* (0.4%), *Metacordyceps* (1.0%), *Lopharia* (1.2%), *Rigidoporus* (0.8%),in Sample B. Also the genera *Oidiodendron* (1.4%), *Lunulospora* (1.9%), *Aquanectria* (1.5%), *Cryptococcus* (0.6%) were only identified in Sample C, while the genera *Campylospora* (0.9%), *Craterellus* (1.5%), *Trametes* (1.1 %), *Tricholoma* (20.8%) were only identified in Sample D. In the case of sample E, the genera *Hanseniaspora* (0.8%), *Purpureocillium* (0.7%), *Amanita* (0.5%) were only present in this sampling point, whereas *Aquasubmersa* (3.5 %), *Tolypocladium* (18.8%) and *Trechispora* (14.5%) were only identified in Sample F. The genus *Stachybotrys* (2.1%) was only present in sample G (Table S1, Supplementary Material).

4.4- Discussion

Fungal attack frequently degrade important pieces of our Cultural Heritage assets. Therefore, in order to implement effective conservation strategies, it is essential to perform detailed studies for the identification of the fungal perpetrators of the damage caused in the artwork. Modern, ultra-sensitive and high-throughput molecular techniques are excellent tools for accurate metagenomic studies. One of such tools, the *Illumina* MiSeq NGS system, as used in this study, allowed the identification of a great amount of fungal sequences (more than 380 different OTUs). To our knowledge, this is the first study using NGS techniques for studying fungal communities on a canvas painting.

OM and SEM screening images from the sampling points allowed to confirm the presence of fungal structures in some of them (Figure 2 and 3), which were those responsible for the most conspicuous stains of biological origin on the art object. Although, at this time, the fungal species could not be identified via this approach, these

preliminary microscopic studies were very relevant for the choice of the NGS analysis sampling points.

Our novel non-invasive sampling method, by soft aspiration (Figure S1, Supplementary Material), enabled us to collect a higher amount of sample on a smaller area, in comparison with other non-invasive sampling methods, such as cotton swabs or nitrocellulose membranes, by accumulating the vacuumed material in the pipette filter. The collected samples retained on the small filter were subsequently used for DNA extraction followed by NGS. This non-invasive sampling technique proved to be successful and therefore can be adapted to sampling other sensible cultural heritage materials.

Previous studies of the fungal communities growing on canvas paintings, have used the DGGE methodology. In these reports, species of the identified genera, such as *Alternaria*, *Aspergillus*, *Aureobasidium*, *Candida, Chaetomium*, *Cladosporium*, *Penicillium* and *Pichia* were also associated to the canvas supports (Santos et al. 2009; Capodicasa et al. 2010; Lopéz-Miras et al. 2012, 2013; Okpalanozie et al. 2016). In general, DGGE brought better results for mycobiota communities' identification, comparatively to culture-dependent approaches, regarding the analysis of fungal community structure on painting canvas. However, molecular techniques applying clone library methodology, such as DGGE, are less efficient in highlighting the microbial diversity on heritage materials, when compared to NGS technology (Gutarowska et al. 2015). Here, we have shown that this modern, culture-independent method is a very valuable tool, which allows easily identification of considerable amounts of fungal genera, unlike DGGE.

Fungal organisms are known for their catalytic versatility, which allows them to colonize and degrade cellulosic materials and a wide range of compounds, namely canvas and paper (Capitelli et al. 2010; Sterflinger 2010), which were used as substrates in the studied artwork (canvas with paper collages). Canvas, as a cellulosic material, is often affected by spots and stains of fungal origin (Tiano 2002; Sterfinger and Pinzary 2012; Poyatus et al. 2017). The *Illumina* MiSeq NGS system has already provided information on fungal diversity of foxing-related species in paper cellulosic materials (Szulc et al. 2018). As had been suggested by the SEM screening of the sampling points,

samples A, B, D and E were those with the highest fungal presence, followed by C, F and G, which had a lower fungal presence, as revealed by the different percentage abundance of obtained OTUs (Figure 4, 5 and 6). Samples A, B and E had higher abundance of different fungal genera, such as *Aspergillus*, *Penicillium, Talaromyces*, *Stereum* and *Rhodotorula*, belonging to the orders *Eurotiales*, *Russulales* and *Sporidiobolales,* respectively. Sample E had the lowest diversity values among these 3 points because the species distribution is not as heterogeneous as in samples A and B (Table 1). This is mainly due to the presence of 50.4% unidentified genera of *Capnodiales* order (Figure 6). *Capnodiales* is a relevant order for biodeterioration, as it includes certain genera of moulds responsible for damaging artworks and other cultural heritage items (Sterfinger and Pinzary 2012; Sterfinger and Piñar 2013; Poyatus et al. 2017). Samples C and D are dominated by *Eurotiales*, with the genera *Aspergillus*, *Penicillium* and *Sporidiobolales*, with the genus *Rhodoturula*. *Candida* (*Sacharomycetale*s) has also a significant presence in sample C as well as the unique genus *Tricholoma* (*Agaricales*) in sample D. Samples F and G, which did not show any fungal structures on the SEM analysis, have the lowest values of fungal OTUs amount and are dominated by a few number of fungal genera (Table S1, Supplementary Material).

"Centauro" is an acrylic and graphite painting with paper collages, fulfilled with story and color. It is possible that, besides the raw material of the canvas support, the use of collages may have added other chemical and physical microniche layers which facilitated the establishment of such different fungal communities. The presence of these additional materials (paper and glue) may have had a major influence on the fungal bioreceptivity of the painting, by turning available other potential nutrients sources (Doménech-Carbó et al. 2007; Santos et al. 2009; Lopez-Miraz et al. 2012, 2013). Our results showed the presence of some fungal genera such as *Aspergillus* (*Eurotiales*), *Cladosporium* (*Capnodiales*), *Chaetomium* (*Sordariales*) and *Penicillium* (*Eurotiales*) which were identified in most of the sampling points of the studied artwork. These genera were already reported as xerophilic fungal organisms in paper cellulosic materials (Corte et al. 2003; Karbowska-Berentent et al. 2014; Szulc et al. 2018). In fact, different fungal strains may produce a variety of colorants (e.g., black, green, blue, purple, violet) from different chemical classes, such as carotenoids, melanins or

quinones (Melo et al. 2018). Excluding sample D, all stains observed on the sampled points are black coloured. Black fungal stains are frequently associated with the presence of fungal melanins. Melanins are biosynthesized by various paper colonizing fungi, such as *Alternaria alternata*, *Aspergillus fumigatus, Aspergillus nidulans, Aspergillus niger, Aspergillus penicilloides, Aureobasidium pullulans, Chaetomium globosum, Cladosporium cladosporioides, Eurotium herbariorum* (Melo et al. 2018). These same taxa and also some species from the identified genera *Alternaria* (*Pleosporales*), *Aureobasidium* (*Dothideales*) and *Mucor* (*Mucorales*) are considered the most frequent colonizing fungi on paintings (Sterflinger 2010; Vukjević and Grbić 2010; Grabpek-Lejko et al. 2017) and were again detected in our study (Figure 6, Table S1 Supplementary Material).

The above mentioned taxa are also considered as common genera in museum environment and exhibition rooms (Lupan and Popescu 2012; Poyatus et al. 2017). One particular case of a type of fungi which is common in these museum environments is the genus *Rhodotorula* (Lupan and Popescu 2012), which we have detected as the only representative of the order *Sporidiobolales* in sample G. This yeast was particularly abundant in 6 of our sampling points (Table S1, Supplementary Material). Dermatophyte fungi such as *Candida* (*Sacharomycetales*) and *Malassezia* (*Malasseziales*) were detected on the artwork and are generally associated to human skin as opportunistic pathogen species (White et al. 2014). Their presence is possibly related to the author hands use to create the painting, which is a common used technique by Paula Rego, or even might be a cause of inappropriate handling during the last 50 years.

As previously reported, NGS is a very useful system for the metagenomic study of microbial communities occurring in cultural heritage items (Cutler et al. 2013; Chiementi et al. 2016; Adamiak et al. 2017). High throughput *Illumina* MiSeq NGS has already demonstrated valid results in retrieving and identifying various DNA fungal sequences from different cultural heritage materials. In fact, it is possible to confirm some common fungal genera obtained in several works, such as *Cladosporium* (*Capnodiales*), *Alternaria* (*Pleosporales*), *Aureobasidium* (*Dothideales*), *Aspergillus* and *Penicillium* (*Eurotiales*) (Gutarowska et al. 2015; Kraková et al. 2017; Szulc et al.,2018). Interestingly, Kraková et al. (2017) pointed out in their *Illumina* MiSeq NGS study that despite a marked

predominance of the *Ascomycota* phyllum, there is also a significant presence of the *Basidiomycota* group in archive items. Also, in our work, orders such as *Agaricales*, *Cantharellale*s, *Russulales*, *Polyporales* and *Trechisporales* from the *Agaricomycetes* class, as well as the orders *Tremellales* and *Trichosporonales* from the class *Tremellomycetes* (Figures 5 and 6), which are not common as cultural items contaminants, were identified and are a significant part of the fungal diversity present in the sampled painting.

In our study, the *Illumina* MiSeq NGS platform has revealed that the diversity results still showed a 15.7% unidentified and 4.7% unassigned OTUs from the total amount of sequences. Nevertheless, it allowed a more accurate analysis of the fungal communities, detecting in all samples many fungal genera that were present, even within a lower range (0.4-4%).

Therefore, we consider that this modern molecular methodology represents an important tool to identify and describe the structure of fungal communities occurring in artwork items, or less generally, in canvas paintings. Consequently, it allowed a deep understanding of the colonizing fungal organisms dwelling on the object, which is a crucial step for future preventive and conservation

4.5- Conclusions

High-throughput *Illumina* MiSeq NGS was revealed to be a powerful molecular tool to describe the diversity in the fungal communities growing on the studied painting. In fact, the small size of the sampling points was challenging, not only for the sampling itself, but also for inquiring the existing fungal diversity.

The used non-invasive sampling technique proved to be effective on samples collection and about 380 of total fungal OTUs were obtained with this deep sequencing technique. The sequencing process was done with a combination of five forward primers and one reverse primer, which considerably raised the total amount of obtained sequences.

Paintings canvas supports are indeed suitable microniches for microbial contamination, where fungi are able to establish and develop. With the knowledge of

the fungal population diversity thriving on the artwork, it is still possible to retrieve those which have a greater deterioration capability.

4.6 - Acknowledgments

The authors want to thank to Catarina Alfaro and Paula Aparício from "Casa das Histórias" museum for their contribution to this work. The authors also want to thank to Igor Tiago for is valuable help in submitting the sequencing data obtained in this study to the SRA database.

4.7- Supplementary Material

Table S1- Fungal diversity on the painting samples.

Phyllum	**Classes**	**Orders**	**Genera**	**Abundance in fungal genera in samples** [%]*						
				A	**B**	**C**	**D**	**E**	**F**	**G**
Unassigned	other	other	unassigned	4.5%	1.6%	6.1%	4.0%	2.4%	3.9%	10.3%
Other	other	other	other	0.0%	0.0%	1.3%	1.6%	0.0%	0.0%	0.0%
Ascomycota	other	other	other	0.4%	1.8%	0.0%	2.1%	0.0%	0.0%	8.6%
Ascomycota	*Dothideomycetes*	other	other	1.9%	2.5%	0.0%	0.0%	1.4%	0.0%	0.0%
Ascomycota	*Dothideomycetes*	*Pleosporales*	*Alternaria*	0.0%	0.0%	0.0%	1.6%	0.8%	0.0%	0.0%
Ascomycota	*Dothideomycetes*	*Pleosporales*	*Anguillospora*	0.0%	0.0%	2.1%	1.6%	0.0%	0.0%	0.0%
Ascomycota	*Dothideomycetes*	*Pleosporales*	*Aquasubmersa*	0.0%	0.0%	0.0%	0.0%	0.0%	3.5%	0.0%
Ascomycota	*Dothideomycetes*	*Dothideales*	*Aureobasidium*	1.4%	1.0%	0.0%	0.0%	0.5%	0.0%	0.0%
Ascomycota	*Dothideomycetes*	*Capnodiales*	Other	0.4%	1.6%	2.9%	2.5%	1.1%	0.0%	3.8%
Ascomycota	*Dothideomycetes*	*Capnodiales*	*Cladosporium*	1.0%	0.1%	0.0%	3.1%	0.6%	0.0%	1.7%
Ascomycota	*Dothideomycetes*	*Hysteriales*	*Cenococcum*	1.4%	1.1%	0.0%	0.0%	0.0%	0.0%	0.0%
Ascomycota	*Dothideomycetes*	*Capnodiales*	*Cercospora*	0.0%	0.4%	0.0%	0.0%	0.0%	0.0%	0.0%
Ascomycota	*Dothideomycetes*	*Capnodiales*	Other	0.4%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Ascomycota	*Dothideomycetes*	*Pleosporales*	Other	0.6%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Ascomycota	*Dothideomycetes*	*Pleosporales*	*Pyrenochaeta*	0.8%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Ascomycota	*Dothideomycetes*	*Pleosporales*	*Roussoella*	0.4%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Ascomycota	*Dothideomycetes*	*Capnodiales*	unidentified	6.4%	26.9%	8.1%	12.0%	50.4%	9.7%	6.4%
Ascomycota	*Eurotiomycetes*	*Eurotiales*	Other	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Ascomycota	*Eurotiomycetes*	*Eurotiales*	*Aspergillus*	6.2%	3.9%	10.3%	8.9%	4.7%	1.4%	0.0%
Ascomycota	*Eurotiomycetes*	*Chaetothyriales*	*Cladophialophora*	0.0%	0.9%	0.0%	0.0%	0.0%	0.0%	0.0%
Ascomycota	*Eurotiomycetes*	*Chaetothyriales*	*Exophiala*	0.7%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Ascomycota	*Eurotiomycetes*	*Phaeomoniellales*	*Neophaeomoniella*	0.4%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Ascomycota	*Eurotiomycetes*	*Eurotiales*	*Penicillium*	5.8%	4.3%	6.0%	1.1%	0.1%	0.0%	2.0%
Ascomycota	*Eurotiomycetes*	*Eurotiales*	*Talaromyces*	3.2%	3.6%	0.0%	0.0%	0.8%	0.0%	2.1%
Ascomycota	*Eurotiomycetes*	*Chaetothyriales*	Other	1.2%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%

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Ascomycota	*Eurotiomycetes*	*Eurotiales*	Other	0.0%	0.5%	0.0%	0.0%	0.0%	0.0%	0.0%
Ascomycota	*Eurotiomycetes*	*Chaetothyriales*	unidentified	0.7%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Ascomycota	*Eurotiomycetes*	*Eurotiales*	unidentified	0.7%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Ascomycota	*Eurotiomycetes*	*Eurotiales*	unidentified	1.8%	0.9%	0.0%	0.0%	0.4%	1.0%	0.0%
Ascomycota	*Leotiomycetes*	other	Other	0.0%	0.0%	0.0%	0.0%	0.0%	2.6%	1.5%
Ascomycota	*Leotiomycetes*	*Helotiales*	Other	0.0%	0.0%	0.0%	1.0%	0.0%	0.0%	0.0%
Ascomycota	*Leotiomycetes*	*Helotiales*	*Lemonniera*	0.0%	0.0%	0.0%	2.5%	0.0%	5.5%	0.0%
Ascomycota	*Leotiomycetes*	*Incertae_sedis*	*Oidiodendron*	0.0%	0.0%	1.4%	0.0%	0.0%	0.0%	0.0%
Ascomycota	*Leotiomycetes*	*Erysiphales*	*Sawadaea*	0.0%	1.2%	0.0%	0.0%	0.0%	0.0%	0.0%
Ascomycota	*Leotiomycetes*	unidentified	unidentified	0.0%	0.9%	0.0%	0.0%	0.0%	0.0%	0.0%
Ascomycota	*Pezizomycetes*	*Pezizales*	*Byssonectria*	0.0%	0.0%	0.0%	0.0%	0.5%	0.0%	0.0%
Ascomycota	*Incertae sedis*	*Incertae sedis*	*Campylospora*	0.0%	0.0%	0.0%	0.9%	0.0%	0.0%	0.0%
Ascomycota	*Incertae sedis*	*Incertae sedis*	*Lunulospora*	0.0%	0.0%	1.9%	0.0%	0.0%	0.0%	0.0%
Ascomycota	*Saccharomycetes*	*Sacharomycetales*	Other	0.0%	1.2%	0.0%	1.1%	0.0%	0.0%	0.0%
Ascomycota	*Saccharomycetes*	*Sacharomycetales*	*Candida*	1.6%	3.7%	7.2%	0.0%	1.6%	0.0%	0.0%
Ascomycota	*Saccharomycetes*	*Sacharomycetales*	*Cyberlindnera*	0.0%	0.4%	0.0%	0.0%	0.0%	0.0%	0.0%
Ascomycota	*Saccharomycetes*	*Sacharomycetales*	*Debaryomyces*	2.5%	1.4%	0.0%	0.0%	0.0%	0.0%	0.0%
Ascomycota	*Saccharomycetes*	*Sacharomycetales*	*Hanseniaspora*	0.0%	0.0%	0.0%	0.0%	0.8%	0.0%	0.0%
Ascomycota	*Saccharomycetes*	*Sacharomycetales*	Other	0.4%	0.0%	0.0%	0.0%	0.4%	0.0%	0.0%
Ascomycota	*Saccharomycetes*	*Sacharomycetales*	*Kluyveromyces*	0.6%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Ascomycota	*Saccharomycetes*	*Sacharomycetales*	*Pichia*	0.0%	0.5%	0.0%	0.0%	0.2%	0.0%	0.0%
Ascomycota	*Saccharomycetes*	*Sacharomycetales*	*Saccharomyces*	0.0%	0.0%	3.9%	0.6%	0.0%	0.0%	0.0%
Ascomycota	*Sordariomycetes*	Other	Other	0.0%	0.6%	0.0%	0.0%	0.1%	0.0%	0.0%
Ascomycota	*Sordariomycetes*	*Hypocreales*	Other	0.0%	0.0%	1.1%	2.3%	0.0%	7.1%	5.1%
Ascomycota	*Sordariomycetes*	*Bionectriaceae*	Other	0.7%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Ascomycota	*Sordariomycetes*	*Sordariales*	Other	0.0%	0.0%	0.0%	0.0%	0.8%	0.0%	0.0%
Ascomycota	*Sordariomycetes*	*Diaporthales*	Other	0.9%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Ascomycota	*Sordariomycetes*	*Hypocreales*	Other	0.4%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Ascomycota	*Sordariomycetes*	*Hypocreales*	*Aquanectria*	0.0%	0.0%	1.5%	0.0%	0.0%	0.0%	0.0%
Ascomycota	*Sordariomycetes*	*Sordariales*	*Chaetomium*	0.4%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Ascomycota	*Sordariomycetes*	*Hypocreales*	*Flagellospora*	0.0%	0.0%	0.0%	1.6%	0.0%	4.5%	1.8%
Ascomycota	*Sordariomycetes*	*Hypocreales*	*Metacordyceps*	0.0%	1.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Ascomycota	*Sordariomycetes*	*Hypocreales*	*Purpureocillium*	0.0%	0.0%	0.0%	0.0%	0.7%	0.0%	0.0%
Ascomycota	*Sordariomycetes*	*Hypocreales*	*Stachybotrys*	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%	2.1%
Ascomycota	*Sordariomycetes*	*Hypocreales*	*Tolypocladium*	0.0%	0.0%	0.0%	0.0%	0.0%	18.8%	0.0%
Ascomycota	*Sordariomycetes*	*Hypocreales*	unidentified	0.8%	0.0%	0.0%	0.9%	0.0%	0.0%	1.8%
Ascomycota	*Sordariomycetes*	*Xylariales*	unidentified	0.0%	0.0%	0.0%	2.2%	0.0%	0.0%	1.7%
Ascomycota	unidentified	unidentified	unidentified	0.4%	0.0%	2.6%	2.4%	0.0%	0.0%	3.7%
Basidiomycota	*Agaricomycetes*	*Agaricales*	*Amanita*	0.0%	0.0%	0.0%	0.0%	0.5%	0.0%	0.0%
Basidiomycota	*Agaricomycetes*	*Cantharellales*	*Craterellus*	0.0%	0.0%	0.0%	1.5%	0.0%	0.0%	0.0%
Basidiomycota	*Agaricomycetes*	*Agaricales*	*Inocybe*	0.4%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Basidiomycota	*Agaricomycetes*	*Russulales*	*Lactarius*	0.3%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Basidiomycota	*Agaricomycetes*	*Polyporales*	*Lopharia*	0.0%	1.2%	0.0%	0.0%	0.0%	0.0%	0.0%
Basidiomycota	*Agaricomycetes*	*Polyporales*	*Phlebia*	0.4%	0.3%	1.6%	0.0%	0.0%	0.0%	0.0%
Basidiomycota	*Agaricomycetes*	*Agaricales*	*Pleurotus*	0.8%	1.4%	0.0%	0.0%	0.0%	0.0%	0.0%
Basidiomycota	*Agaricomycetes*	*Polyporales*	*Rigidoporus*	0.0%	0.8%	0.0%	0.0%	0.0%	0.0%	0.0%

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Basidiomycota	*Agaricomycetes*	*Russulales*	*Russula*	5.9%	3.8%	0.0%	0.0%	1.1%	0.0%	0.0%
Basidiomycota	*Agaricomycetes*	*Russulales*	*Stereum*	18.7%	1.3%	0.0%	0.0%	12.1%	0.0%	3.9%
Basidiomycota	*Agaricomycetes*	*Polyporales*	*Trametes*	0.0%	0.0%	0.0%	1.1%	0.0%	0.0%	0.0%
Basidiomycota	*Agaricomycetes*	*Trechisporales*	*Trechispora*	0.0%	0.0%	0.0%	0.0%	0.0%	14.5%	0.0%
Basidiomycota	*Agaricomycetes*	*Agaricales*	*Tricholoma*	0.0%	0.0%	0.0%	20.8%	0.0%	0.0%	0.0%
Basidiomycota	*Agaricomycetes*	*Cantharellales*	unidentified	0.0%	0.0%	0.0%	2.3%	0.0%	0.0%	7.6%
Basidiomycota	*Agaricomycetes*	*Russulales*	unidentified	0.8%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Basidiomycota	*Exobasidiomycetes*	*Microstromatales*	unidentified	0.0%	0.0%	0.0%	0.0%	0.2%	0.0%	0.0%
Basidiomycota	*Microbotryomycetes*	*Sporidiobolales*	*Rhodotorula*	8.7%	8.2%	7.9%	3.6%	3.7%	0.0%	15.2%
Basidiomycota	*Tremellomycetes*	other	Other	1.4%	0.0%	0.0%	0.3%	1.4%	2.6%	0.0%
Basidiomycota	*Tremellomycetes*	*Tremellales*	Other	0.0%	0.3%	0.0%	1.9%	0.0%	0.0%	0.0%
Basidiomycota	*Tremellomycetes*	*Tremellales*	Other	0.7%	0.0%	1.5%	0.0%	0.0%	0.0%	0.0%
Basidiomycota	*Tremellomycetes*	*Tremellales*	*Cryptococcus*	0.0%	0.0%	0.6%	0.0%	0.0%	0.0%	0.0%
Basidiomycota	*Tremellomycetes*	*Trichosporonales*	*Trichosporon*	1.4%	3.5%	5.3%	0.0%	1.1%	0.0%	7.4%
Basidiomycota	*Incertae sedis*	*Malasseziales*	*Malassezia*	1.3%	2.0%	1.0%	1.8%	2.2%	0.0%	0.0%
Chytridiomycota	unidentified	unidentified	unidentified	0.7%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Zygomycota	*Incertae sedis*	*Mortierellales*	*Mortierella*	1.3%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Zygomycota	*Incertae sedis*	*Mucorales*	*Mucor*	0.8%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
Zygomycota	*Incertae sedis*	*Mucorales*	*Umbelopsis*	0.3%	1.4%	0.0%	0.0%	0.0%	0.0%	0.0%
unidentified	unidentified	unidentified	unidentified	7.3%	13.8%	27.1%	14.1%	9.6%	24.8%	13.2%

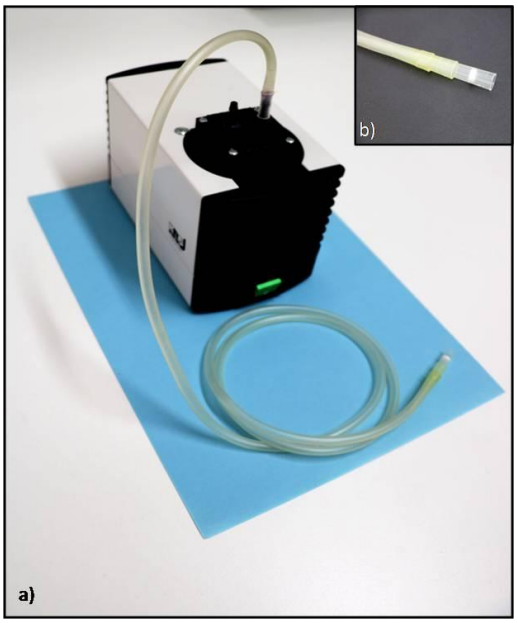

Figure S1- Aspiration device (vacuum pump) used for sampling the contaminated studied painting. a) general view of the equipment; b) detail of the filtered pipete tip coupled at the end of the tube.

General Discussion

General Discussion

The research that fulfills this thesis demonstrates, once more, that fungi are definitely biodeterioration agents in different and various cultural heritage objects. In fact, several fungal species can be found growing and colonizing over different types of supports. In the different chapters of this investigation, examples of species such as *Aspergillus versicolor*, *Chaetomium globosum*, *Penicillium corylophilum* or *Phlebiopsis gigantea* were isolated from different types of cultural heritage materials such as parchments, paper documents, paintings and wooden sculptures, and are often found as biodeterioration agents in these different sorts of supports. In addition, to assess important information regarding fungal contaminant species in the affected objects, it is essential to choose appropriate methodologies. As such, in our opinion, the employed methodologies allowed us to understand the action of these organisms concerning the colonization and further biodeterioration of our cultural heritage, and will allow developing future mitigation and conservation measures, with the aim of eradicating the contamination and damage posed by fungi as much as possible.

In chapter 1, the biodeterioration of different aged parchment collections was evidenced by the isolation of different fungal species. Some of them have proved biodeterioration capability and could be related to the emergence of some foxing symptoms, as these are proteolytic fungi, such as *Aspergilus versicolor*, *Chaetomium globosum*, *Cladosporium cladosporioides*, *Penicillium chrysogenum* or *P. spinulosum* isolated in this work. It was demonstrated that time is statistically significant here and, in this context, it has an important role on the biodeterioration of parchment documents. This was observed by the different amount of sampled isolates and identified species within the different sampled documents: the more ancient a parchment document is, the more damaged it is by biodeterioration, especially by the consequent damage after fungal growing and colonization. The storage and environment conditions have direct implications in this matter, because climate factors (relative humidity, temperature and light) influence the materials surface bioreceptivity for microorganisms' deterioration and could, most of the time, enhance the creation of

suitable microniches for them (Hyvärinen et al. 2002; Pinzary et al. 2012; Micheluz et al. 2015; Polo et al. 2017). As a final remark, one cannot ignore that parchment is an appropriate material for biodeterioration, since it results from treated animal skin, in such a way that the treatment with strong alkaline solutions induce alterations to the raw material, thus turning it in a good and available nutrient source for potential damaging fungi (Troiano et al. 2014; Piñar et al. 2015a). The handling and care of these historical artifacts is another issue to be mentioned, since it could be another contamination vehicle for parchment documents. The respective archives and museums' personnel should be advised to use appropriate gloves and other protective clothing during the transport and restoration process of the referred artifacts (Sterflinger 2010; Sterflinger and Piñar 2013).

In chapter 2, the art repository room for paintings and wooden sculptures in the "Museum Nacional de Machado de Castro" was a target of evident fungal contamination, even in controlled climate conditions. In spite of the repository's humidity and monitored temperature conditions, once this facility was equipped with a modern HVAC system, it was possible to observe fungal contamination in some of the stored artwork. In fact, it has already been emphasized that, in such indoor environments, many fungal species have airborne origin (Guiamet et al. 2011; Sterflinger and Piñar 2013; Šimonovičova et al. 2015). Also, some of the isolated species can have an internal object origin, i.e., they are already present in the raw material, such as the wood-based objects (Pangallo et al. 2007, 2009; Kraková et al. 2012a). For example, species of wood-rot fungi such as *Chaetomium globosum*, *Phlebiopsis gigantea* and *Pithomyces chartarum* were isolated from the affected art pieces and were responsible for damaging wood materials. In addition, the seasonal air-sampling approach allowed us to isolate several fungal species and thus to characterize the inside air contamination and evaluate the ventilation system efficiency. The results did not show any kind of dangerous air contamination values (in terms of CFU counts) but some health-related and material damaging species were identified. Nevertheless, their values were not considered dangerous for human health but it was curious to note a co-occurrence of species between the air-samples and the art-pieces. In fact, it is possible to assume that this room indoor ventilation system may have positively influenced the fungal spores'

dispersal inside the room. The presence of such fungal species could also be related to the fact that different types of supports and art pieces were stored in the same room, i.e., paintings and wooden sculptures were kept in the same repository, which is not advised at all, as more kinds of suitable growing "media" are available for fungal colonization (Grabek-Lejko et al. 2017). In regard to the main issue of this study, it is important to establish new quarantine measures for the arriving pieces before entering in this type of storage rooms, as for example, implementing a mandatory quarantine room for the artworks, as well as to ascertain a periodically rigorous cleaning and disinfection of the repositories and their ventilation systems (filters, pipelines, etc). Another relevant issue is the application of protective cultural heritage measures when repositories have renovation periods, which will surely affect the art pieces' preservation during this phase. This must be carefully provided for the objects, as the current procedures make use of some protective plastic wrapping, which was the case during a long renovation period that, in this way, allowed for an emerging fungal colonization of the art objects that were wrapped by plastic foils, creating optimal microniches for fungal growth (Sterflinger 2010; Sterflinger and Pinzary 2012).

In chapter 3, three different paper objects (two books and one print on paper) were subject to fungal sampling by classical methods, such as the cotton swab sampling and further isolation on culture media. The current used methodology for artwork sampling makes use of invasive and non-invasive approaches. It is very important to make use of non-invasive and non-destructive methodologies but in some cases it is still inevitable to take a small portion of the damaged object so that it would be possible to isolate fungal species (Cappitelli et al. 2010; Gutarowska et al. 2015; Sanmartin et al. 2016; Kraková et al. 2017; Szulc et al. 2018). This way, non-invasive methods such as the cotton swab are often used although it is well known that it only allows for the recovery of a small fraction of the available fungal species. Nevertheless, a spore suspension of these swabs is an advantageous technique over direct incubation on growing media, as the suspension turns the fungal spores more available for further platting onto the referred media (Borrego et al. 2012; Piñar et al. 2015a). The use of nitrocellulose membranes are also a suitable solution as a non-invasive sampling procedure and it has been performed in some studies (Michaelson et al. 2010; Piñar et al. 2015b,c;

Okpalanozie et al. 2016). After sampling, cultivation methods are a common used methodology for isolation and growth of fungal species with the aim of their identification and characterization. Nevertheless, most of the fungal species involved in the community of the sampled support are not isolated in total by this method (just about 70%), and it cannot be unsurely confirmed that a certain biological damage is associated to a specific fungal organism (Capitelli et al. 2010; Rosado et al. 2014; Gutarowska et al. 2015). Indeed, this methodology allows the isolation of some currently active species, or just mere opportunist ones or even, in some cases, "specialized" species from the support (Sterflinger and Piñar 2013). To overcome this, different types of media can still be used in this culture-dependent approach and the diversity of fungal organisms depends on the type of used media (Mesquita et al. 2009; Kraková et al. 2012a,b, 2017). This is an important issue because richer media (like PDA or MEA) provides the isolation of generalist species, and in the case of some particular species, such as xerophilic or halophilic fungi, need appropriate media for their isolation (for example DG-18 and MEA with NaCl addition, respectively). The xerophilic and halophilic fungi may have a negative impact on damaging the studied object and for that reason they are very important to isolate and to identify. In chapter 3, it was well demonstrated that these culture methods are not conclusive. The obtained correlations by the applied methodologies varied between 13% and 64% of the samples within the three studied documents. In this way another methodology was followed for this purpose, as was demonstrated in Chapter 4.

In Chapter 4, we used a new sampling method as a non-invasive approach. The sampling of fungal structures was performed by soft aspiration of the sampling points on the studied painting. The use of a small vacuum-pump with an attached filtered tip allowed to retrieving valuable information about the fungal communities on this artwork. The filters retained the aspirated fungal structures and, after employing proper DNA extraction protocols, they were further analyzed by Next Generation Sequencing (NGS) through *Illumina* technology. This modern and fast deep-sequencing tool was performed in all of the extracted DNA sampled points of the painting and gave positive results in characterizing the fungal communities thriving in the object. As such, NGS methods proved to be effective in assessing the fungal diversity present in the

contaminated artwork, allowing the identification of putative contaminants and damaging species for the object. Moreover, this improved molecular technique, when identifying a great amount of different partial genome sequences (OTUs), allows this way the identification of such a variety of fungi that possibly participate in different stages of the supports' biodeterioration process, especially in the case of non-active species at that moment (Rosado et al. 2014; Kraková et al. 2017). It is then demonstrated that this high-throughput sequencing methodology is really useful to deeply assess the fungal diversity of the communities that were present in the artwork.

Culture methods permit the isolation of a considerable fraction of the community's organisms (in some cases close to 70%) and also some xerophilic and halofilic fungi, that have a particular role in the biodeterioration process (Sterflinger 2010; Sterflinger and Piñar 2013). Culture-independent methods, for example through the use of modern NGS platforms, allow a deep knowledge of the community structure but only give wide information on the presence of organisms and nothing on their activity in the support. This way, the use of a single approach only, i.e., using only a culture-dependent approach or solely a culture-independent methodology is discouraging because it does not give enough information about the real culprits in the fungal communities that are somehow responsible for the biodeterioration of the studied object. Indeed, the use of culture-dependent and culture-independent strategies together is always more useful to correctly and accurately identify the fungal deterioragens. As such, it is important to emphasize that the identification of fungal communities dwelling on a support must be achieved through the combination of different approaches, at least when possible, as it will be more accurate in actually discriminating the organisms that participate actively in the biodeterioration process (Rosado et al. 2014; Sanmartin et al. 2016; Kraková et al. 2017). The addition and contribution of other techniques, such as microscopy screening and also metabolic activity detection methods, are also encouraging to use and are proved to be important tools in discriminating the biodeterioration agents of the affected supports (Gutarowska et al. 2015; Piñar et al. 2015 a,b,c; Szulc et al. 2018).

Cultural heritage objects' conservation and their future preservation makes it essential to gather efforts of all the responsible professionals, in figuring out the best

solutions for fighting and delaying the biodeterioration process. In spite of their different functions within the problem of the affected supports, conservators, restaurateurs and scientists must join forces, combining their experience and knowledge, for the artwork's preservation. So, when a biological contamination is noticed, an expert scientist, such as a microbiologist, who must analyze the organism or organisms responsible for the object damaging, should observe it. After achieving the identification and the type of action of the contaminants, conservators and restaurateurs could then apply their intervention techniques in a way that no further contamination should be able to occur.

Conclusions and Future Perspectives

Conclusions

The research developed in this thesis visibly demonstrates that fungi are real and adverse biodeterioration agents of different and various cultural heritage objects. This could be noticed by the fact that several fungal species were identified growing and colonizing over different types of supports of different proveniences. Several fungal species were found on different materials such as parchment documents, paintings and wooden sculptures as also as air contaminants. It is worth considering that fungi are definitely a biological threat to our cultural heritage assets.

Parchment is made from treated animal skin, which is transformed in order to strengthen it and to make it more suitable as a writing material. Besides the influence of environmental factors (temperature, relative humidity, light), the applied treatments (leaching/soaking) for its transformation also induces alterations to the raw material, making parchment documents an appropriate material for biodeterioration. We have noticed that time was relevant for the parchments current state, as the older documents had more evidence of biodeterioration symptoms, and because more species were isolated and identified comparing to the recent documents. So, in spite of being a treated material in order to be stronger and durable, it was demonstrated that the most attacked documents were the ones belonging to older collections. Moreover, it is always important to emphasize that documents made of parchment, are mostly stored in collections or grouped in proper storage materials. These supports are therefore subjected to the same environmental conditions, and should be studied as a collection and not as isolated items.

Art repositories are custom-built rooms for artwork preservation and despite all the gathered knowledge in recent years, current established norms and accepted contamination thresholds have a prominent focus on human health and air quality preservation. This study shows the importance that a better regulation, regarding preventive artwork conservation, can have on both the protection of objects as well as human health. The biological damage on the pieces of art are sometimes neglected, or not appropriately treated, given the difficulties of implementing preventive measures,

because they are still not enough or are not adequately applied for cultural heritage preservation. Also, we propose that microbiologists should be called upon to collaborate with art conservators and restorers, and other museum staff, to aid in the design of protocols and other measures in preventing and remediating biological contamination of artwork.

Cultural heritage artwork and objects are not easy targets for biological sampling due to its cultural and historical value and to its current state of preservation. It is very important to make use of non-invasive and non-destructive methodologies but in some works it is still inevitable to take a small portion of the damaged object. Our non-invasive sampling method, by soft aspiration enabled us to collect a higher amount of sample on a smaller area, in comparison with other non-invasive sampling methods, such as cotton swabs or nitrocellulose membranes, by accumulating the vacuumed material in the pipette filter. Taken samples were further analyzed by NGS, which retrieved positive results about the community structure colonizing in the sampled painting. The developed non-invasive sampling technique proved to be successful and therefore can be adopted in sampling other sensible cultural heritage materials.

As a final remark, it is important to refer that the identification of fungal communities dwelling on a support must be achieved through the combination of different approaches, as it will be more accurate in discriminating the organisms that participate actively in the biodeterioration process. The cultivable approach does not retrieve a sufficient fraction of the total community of the biodeteriorogens, while the culture-independent approach gives a deep knowledge about the community structure but nothing on their activity in the support. Nevertheless, the combination of these two methodologies with the addition of other techniques, such as microscopy screening and also metabolic activity detection methods, seems to be more efficient in studying the biodeterioration agents in the affected supports. The more methodologies we combine the more accurate information we will gather on the real culprits for the biodeterioration process.

Future perspectives

In the present research, in spite of the different used approaches for fungal contaminants' isolation and identification, information about the real culprits for the damaging material is still hard to accomplish. In this research field, it is still difficult to ascertain the initial fungal growth species, which are considered the triggering strains for fungal biodeterioration. Accordingly, classical cultivation approaches do not give the entire information of the fungal community nor its function on the biodeterioration processes. In addition, the application of next-generation sequencing techniques, such as the used through the *Illumina* MiSeq platform, even allowing the identification of non-active fungi at the moment, only give a broad spectrum of the fungal diversity and does not allow establishing the real cause for fungal biodeterioration. In this extent, future studies must combine microscopy screening and genomic approaches with an available deterioration-detection by metabolomic approach, as already triedout by some researchers. A metabolomic methodology makes it possible to measure the amount of excreted components by the active fungal species, resulting from their production of primary and secondary metabolites, as a consequence of their activity in deteriorating the affected support. This combination of microscopy imaging with genomic and metabolomic techniques allows to connect the phylogenetic information and metabolic profiles of fungal the communities involved in the biodeterioration process. Actually, to our concern, it will strengthen the cause/effect relationship between the identified fungal species and the damages in the support.

Cultural heritage items are very numerous and diverse and represent a very important legacy of many countries worldwide. Some of these artifacts, such as paper, parchments, paintings or sculptures have been deeply studied as targets of biodeterioration damage. Nevertheless, other important cultural and historical objects, such as the ones related to our musical heritage assets, have been almost forgotten or less studied, in terms of their preservation. Musical heritage objects comprise various ancient and former several music-related objects, such as music sheet books and instruments, and are indeed important artifacts of our musical history. These objects are

composed by a variety of organic origin materials, such as wood, paper and leather, which are suitable for fungal biodeterioration. As such, it is intended to continue this research line in studying the biodeterioration of the Portuguese Cultural Heritage musical assets, which will be, to our knowledge, the first type of research of this type in Portugal. It is important to remember that UNESCO recently considered Fado a world heritage with regard to all its tradition, singing and for the instruments used in it, such as the Portuguese guitar. In addition, Portugal is a very rich country in stringed instruments asset. In each of the different regions in the country, there is a typical chordophone, whose effort has been to maintain and continue the construction and use of these musical instruments. In order to prolong their preservation more than expected, biodeterioration research should also have a relevant role here.

References

Abarenkov, K., Nilsson, R.H., Larsson, K., Alexander, I.J., Eberhardt, U., Erland, S., Høiland, K., Kjøller, R., Larsson, E., Pennanen, T., Sen, R., Taylor, A.F.S., Tedersoo, L., Ursing, B.M., Vrålstad, T., Liimatainen, K., Peintner, U., Kõljalg, U., 2010. The UNITE database for molecular identification of fungi – recent updates and future perspectives. New Phytologist 186, 281-285.

Adamiak, J., Otlewska, A., Tafer, H., Lopandic, K., Gutarowska, B., Sterflinger, K., Pinar,G., 2016. First evaluation of the microbiome of built cultural heritage by using the Ion Torrent next generation sequencing platform. International Biodeteriortion and Biodegradation, 1-8. article in press. doi: 10.1016/j.ibiod.2017.01.040.

Ahonsi, M.O., Agindotan, B.O., Williams, D.W., Arundale, R., Gray, M.E., Voigt, T.B., Bradley, C.A., 2010. First Report of *Pithomyces chartarum* causing a Leaf Blight of Miscanthus × giganteus in Kentucky Plant Disease 94 (4), 480-480.

Aira, M.J., Jato, V., Stchigel, A.M., Rodríguez-Rajob, F.J., Piontel, E., 2007. Aeromycological study in the Cathedral of Santiago de Compostela (Spain). International Biodeterioration and Biodegradation 60, 231–237. doi:10.1016/j.ibiod.2007.02.007

Allsopp, D., Seal, K.J., Gaylarde, C.C., 2004, Introduction to biodeterioration, chapter 1 (2nd ed.), Cambridge University Press, USA, pp. 1-5.

Anaya, M., Borrego S., Gámez, E., Castro, M., Molina, A., Valdés, O., 2016. Viable fungi in the air of indoor environments of the National Archive of the Republic of Cuba. Aerobiologia 32,513–527. doi: 10.1007/s10453-016-9429-3

Arai, H., 2000. Foxing caused by fungi: twenty-five years of study. International Biodeterioration and Biodegradation 46, 181-188.

Axelsson, M., Larsen, R., Sommer, D., 2012. Dimensional studies of specific microscopic fibre structures in deteriorated parchment before and during shrinkage. Journal of Cultural Heritage 13, 128-136. doi: 10.1016/j.culher.2011.08.001.

Babaeizad, V., Sayari, M., Rahimian, H., 2012. Occurrences of a new fruit rot of peach caused by *Hyphodermella rosae* in northern Iran. New Disease Reports 26, 1.

Banani, H., Spadaro, D., Zhang, D., Matic, S., Garibaldi, A., Gullino, M.L., 2014. Biocontrol activity of an alkaline serine protease from *Aureobasidium pullulans* expressed in *Pichia pastoris* against four postharvest pathogens on apple. International Journal of Food Microbiology 182–183, 1-8.

Barata, R.A., Andrade, M.H., Rodrigues, R.D., Castro, I.M., 2002. Purification and Characterization of an Extracellular Trypsin-Like Protease of *Fusarium oxysporum* var. *mini*. Journal of Bioscience and Bioengineering 94 (4),304-308.

Behjati, S., Tarpey, P.S., 2013. What is next generation sequencing? Archives of Disease in Childhood Education and Practic Edition 98,236–238. doi:10.1136/archdischild-2013-304340

Behnsen, J., Lessing F., Schindler, S., Wartenberg, D., Jacobsen, I., Thoen, M., Zipfel, P., Brakhage, A., 2010. Secreted *Aspergillus fumigatus* Protease Alp1 Degrades Human Complement Proteins C3, C4, and C5. Infection and Immunity 78(8), 3585-3594.

Bengtsson-Palme, J., Ryberg, M., Hartmann, M., Branco, S., Wang, Z., Godhe, A., De Wit, P., Sánchez-García, M., Ebersberger, I., de Sousa, F., Amend, A., Jumpponen, A., Unterseher, M., Kristiansson, E., Abarenkov, K., Bertrand, Y.J.K., Sanli, K., Eriksson, K.M., Vik, U., Veldre, V., Nilsson, R.H., 2013. Improved software detection and extraction of ITS1 and ITS2 from ribosomal ITS sequences of fungi and other eukaryotes for analysis of environmental sequencing data. MethodsEcolEvol 4, 914-919.

Bickersteth, J., 2016. IIC and ICOM-CC Declaration on environmental guidelines. Studies in Conservation 61 (s1), 12-17 doi: 10.1080/00393630.2016.1166018

Bisht, V., Arora, N., Singh, B.P., Pasha, S., Gaur, S.N., Sridhara, S., 2004. Epi p 1, an allergenic glycoprotein of *Epicoccum purpurascens* is a serine protease. FEMS Immunology and Medical Microbiology 42 (2), 205-211.

Borrego, S., Guiamet, P., Gómez de Saravia, S., Batistini, P., Garcia, M., Lavin, P., Perdomo, I., 2010. The quality of air at archives and the biodeterioration of photographs. International Biodeterioration and Biodegradation, 64, 139-145. doi:10.1016/j.ibiod.2009.12.005

Borrego, S., Lavin, P., Perdomo, I., Gómez de Saravia, S.,. Guiamet, P., 2012. Determination of Indoor Air Quality in Archives and Biodeterioration of the Documentary Heritage, ISRN Microbiology Article ID 680598, 10 pages. doi:10.5402/2012/680598

Bower, M.A., Campana, M.G., Scott, C.C., Knight, B., Howe, C.J., 2010. The potential for extraction and exploitation of DNA from parchment: a review of the opportunities and hurdles. Journal of the Institute of Conservation 33(1), 1-11. doi: 10.1080/19455220903509937

Brokerhof, A.W., van Zanen, B., den Teuling, A., 2007. Fluffy Stuff - Integrated control of mould in archives, 39.

Campbell, C.K., Johnson, E.M., Warnock, D.W., 2013. Identification of Pathogenic Fungi (2nd ed.), Wiley-Blackwell, Chichester.pp.. 107-135.

Capodicasa, S., Fedi S., Porcelli, A.M., Zannoni, D., 2010. The microbial community dwelling on a biodeteriorated 16th century painting, International Biodeterioration& Biodegradation, 64, 727-733. doi:10.1016/j.ibiod.2010.08.006

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nature Methods 7, 335-336.

Cappitelli, F., Pasquariello, G., Tarsitani, G., Sorlini, C., 2010. Scripta management? Assessing microbial risk to paper heritage. Trends in Microbiology 18, 538-542. doi: 10.1016/j.tim.2010.09.004.

Cappitelli, F., Sorlini, C., 2005. From papyrus to compact disc: the microbial deterioration of documentary heritage. Critical Reviews in Microbiology 31, 1-10.

Carrazana-García, D., González-Álvarez, D., Díaz-Álvarez, E., Mesa-Garriga, L., Banguela-Castillo, A., Chea-González, A., Cupull-Santana, R., 2014. *Aspergillus sclerotiorum*: a risk to cultural heritage and health. Univ. Sci, 19 (3), 323-332. doi: 10.11144/Javeriana.SC19-3.asr

Chimienti, G., Piredda, R., Pepe, G., van der Werf, I., Sabbatini, L., Crecchio, C., Ricciuti P., D'Erchia, A., Manzar,i C. , Pesole, G., 2016. Profile of microbial communities on carbonate stones of the medieval church of San Leonardo di Siponto (Italy) by Illumina-based deep sequencing. Applied Microbiology Biotechnology 100, 8537-8547. doi: 10.1007/s00253-016-7656-8

Ciferri O., 1999. Microbial Degradation of Paintings. Applied and Environmental Microbiology 65 (3), 879-885.

Collins, P.J., Dobson, A.D.W., 1997. Regulation of Laccase Gene Transcription in *Trametes versicolor*. Applied and Environmental Microbiology 63 (9), 3444–3450.

Comeau, A. M., Douglas, G. M., Langille, M. G. I., 2017. Microbiome Helper: a Custom and Streamlined Workflow for Microbiome Research. *mSystems*, 2(1). doi: 10.1128/mSystems.00127-16

Corte, A.M., Ferroni, A., Salvo V.S., 2003. Isolation of fungal species from test samples and maps damaged by foxing, and correlation between these species and the environment. International Biodeterioration and Biodegradation 51(3), 167-173. doi:10.1016/S0964-8305(02)00137-3.

Cutler, N.A., Oliver, A.E., Viles, H.A., Ahmad, S., Whiteley, A.S., 2013. The characterisation of eukaryotic microbial communities on sandstone buildings in Belfast, UK, using TRFLP and 454 pyrosequencing. International Biodeterioration and Biodegradation 82, 124-133. doi: 10.1016/j.ibiod.2013.03.010.

Das, M.K.L., Prasad, J.S., Ahmad, S.K., 1997. Endoglucanase production by paper-degrading mycoflora. Letters in Applied Microbiology 25(5), 313-315. doi: 10.1046/j.1472-765X.1997.00217.x

De Santis, T.Z, Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T., Dalevi, D., Hu, P., Andersen, G.L. , 2006. Greengenes, a chimera-checked 16S rRNA gene data base and work bench compatible with ARB. ApplEnvironMicrobiol 72, 5069–5072.

Deacon, 2006. Fungal Mycology, Blackwell Publishing, USA, chapter 1 (4th ed.), pp.1-6; chapter 11 pp.229-235.

Doménech-Carbó, M. T., Bitossi, G., Osete-Cortina, L., Yusá-Marco, D. J., Bolívar-Galiano, F., López-Miras, M., Fernández-Vivas, M. A., Martín-Sánchez, I., 2007. Introduction to the study on the biodeterioration of the poly(vinyl) acetate resin mowilith 50 using FTIR spectroscopy and pyrolysis-gas chromatography-mass spectrometry. Arché. Publicación del instituto universitario de restauración del patrimonio de la UPV 2, 109-114.

Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27, 2 194-2200.

Fischer, G., Dott, W., 2003. Relevance of airborne fungi and their secondary metabolites, for environmental, occupational and indoor hygiene. Archival Microbiology 179 , 75–82. doi: 10.1007/s00203-002-0495-2

Flieder, F., Capderou C., 2000. Sauvegarde des collections du patrimoine la luttecontre les détériorations biologiques. Bulletin Des Bibliothèques de France 4, 1–2. http://bbf.enssib.fr/consulter/bbf-2000-04-0139-005

Florian, M.L.E. and Manning, L., 2000. SEM analysis of irregular fungal fox spots in an 1854 book: population dynamics and species identification. International Biodeterioration and Biodegradation 46, 205-220. doi: 10.1016/S0964-8305(00)00062-7

Foladi, S., Hedayati, M.T., Shokohi, T., Mayahi, S., 2013. Study on fungi in archives of offices, with a particular focus on Stachybotrys chartaru., Journal de Mycologie Medicale 23(4), 242-246. doi:10.1016/j.mycmed.2013.09.003

Fourie, G., Steenkamp, E.T., Ploetz, R.C., Gordon, T.R., Viljoen, A., 2011. Current status of the taxonomic position of *Fusarium oxysporum* formae specialis cubense within the *Fusarium oxysporum* complex. Infection, Genetics and Evolution 11(3), 533-42. doi: 10.1016/j.meegid.2011.01.012.

Frosini, B.V., , 2006. Environmetrics. In: Jureckova, J. (Ed.) Descriptive Measures of Ecological Diversity. In: El-Shaarawi, A.H. (Ed.), Encyclopedia of Life 622 Support Systems (EOLSS), EOLSS Publishers, Oxford, UK, pp. 29-57.

Fu K., Fu S., Zhan H., Zhou P., Liu M., Liu H., 2013. A newly isolated wood-rot fungus for laccase production in submerged cultures. BioResources 8 (1), 1385-1397.

Gardes, M., Bruns, T.D., 1996. Community structure of ectomycorrhizal fungi in a Pinusmuricata forest: above- and below- ground views. Canadian Journal of Botany 74, 1572-1583.

Gaylard, C., Baptista-neto, J.A., Ogawa, A., Kowalski, M., Celikkol-Aydin, S., Beech, I., 2017. Epilithic and endolithic microorganisms and deterioration on stone church facades subject to urban pollution in a sub-tropical climate. Biofouling 33(2), 113-127. doi: 10.1080/08927014.2016.1269893

Grabek-Lejko, D., Tekiela, A., Kaspryk, I., 2017. Risk of Biodeterioration of cultural heritage objects, stored in the historical and modern repositories in the Regional Museum in Rzeszow (Poland). A case study. International Biodeterioration and Biodegradation 123, 46-55. doi: 10.1016/j.ibiod.2017.05.028

Guiamet, P., Borrego, S., Lavin, P., Perdomo, I., Gómez de Saravia, S., 2011. Biofouling and biodeterioration in materials stored at the Historical Archive of the Museum of La Plata, Argentine and at the National Archive of the Republic of Cuba. Colloids and Surfaces B: Biointerfaces 85, 229-234. doi: 10.1016/j.colsurfb.2011.02.031

Gutarowska, B., Celikkol-Aydin, S., Bonifay, V., Otlewska, A., Aydin, E., Oldham, A.L., Brauer, J.I., Duncan, K. E., Adamiak, J., Sunner, J.A., Beech, I.B. , 2015. Metabolomic and high-throughput sequencing analysis - modern approach for the assessment of biodeterioration of materials from historic buildings. Frontiers in Microbiology 6, article 979. doi: 10.3389/fmicb.2015.00979

Harkawy, A., Górny, R.L., Ogierman, L., Wlazło, A., Ławniczek-Wałczyk, A., Niesler, A., 2011. Bioaerosol assessment in naturally ventilated historical library building with restricted personnel access. Annals of Agricultural and Environmental Medicine 18 (2), 323–329.

Hatakka, A., Hammel, K.E, 2011. Fungal Biodegradation of Lignocelluloses. In: Hofrichter M. (eds) Industrial Applications. The Mycota (A Comprehensive Treatise on Fungi as Experimental Systems for Basic and Applied Research), vol 10. Springer, Berlin, Heidelberg. doi:10.1007/978-3-642-11458-8_1

Houbraken, J., Frisvad, J.C., Samson, R.A., 2011. Taxonomy of Penicillium section Citrina, Studies in Mycology 70, 53-138. doi:10.3114/sim.2011.70.02

Hueck, H.J., 2001, The Biodeterioration of the materials - an appraisal, International Biodeterioration and Biodegradation 48, 5-11. Doi: 10.1016/S0964-8305(01)00061-0

Hyvärinen, A., Meklin, T., Vepsäläinen, A., Nevalainen, A., 2002. Fungi and actinobacterianmoisture-damaged building materials – concentrations and diversity. International Biodeterioration and Biodegradation 40, 27-37.

Illumina. 2013. 16S Metagenomic Sequencing Library Preparation Preparing 16S Ribosomal RNA Gene Amplicons for the Illumina MiSeq System.

Jennita, J.X., Gnadoss, J.J., 2014. Effect of carbon, nitrogen sources and inducers on protease production by *Penicillium citrinum* LCJ 222. International Journal of Current Research 6 (11), 9544-9549.

Jurado, V., Porca E., Pastrana M.P., Cuezva, S., Fernandez-Cortes, A., Saiz-Jimenez, C., 2010. Microbiological study of bulls of indulgence of the 15th–16th centuriesScience of the Total Environment 408, 3711-3715. doi: 10.1016/j.scitotenv.2010.05.008

Kadaifciler, D.G., 2016. Bioaerosol assessment in the library of Istanbul University and fungal flora associated with paper biodeterioration. Aerobiologia 33 (1), 151-166. doi: 10.1007/s10453-016-9457-z

Karbowska-Berent, J., Górny, R.L., Strzelczyk, A.B., Wlazło, A., 2011. Airborne and dust borne microorganisms in selected Polish libraries and archives. Building and Environment 46, (10) 1872–1879. doi: 10.1016/j.buildenv.2011.03.007

Karbowska-Berent, J., Jarmiłko, J., Czuczko, J., 2014. Fungi in Fox Spots of a Drawing by Leon Wyczółkowski .Restaurator. International Journal for the Preservation of Library and Archival Material 35(2), 159-179. doi: 10.1515/rest-2014-1000

Kim, J., Lee, S., Ra, J., Lee, A., Huh, N., Kim, G., 2010. Fungi associated with bamboo and their decay capabilities. Holzforschung 65, 271-275. doi:10.1515/hf.2011.004

Kõljalg, U., Nilsson, R.H., Abarenkov, K., Tedersoo, L., Taylor, A. F. S., Bahram, M., Bates, S. T., Bruns, T. D., Bengtsson-Palme, J., Callaghan, T. M., Douglas, B., Drenkhan, T., Eberhardt, U., Dueñas, M., Grebenc, T., Griffith, G. W., Hartmann, M., Kirk, P.M., Kohout, P., Larsson, E., Lindahl, B.D., Lücking, R., Martín, M.P., Matheny, P. B., Nguyen, N.H., Niskanen, T., Oja, J., Peay, K.G., Peintner, U., Peterson, M., Põldmaa, K., Saag, L., Saar, I., Schüßler, A., Scott, J.A., Senés, C., Smith, M.E., Suija, A. ,Taylor, D.L., Telleria, M.T., Weiss, M., Larsson , K.H. 2013. Towards a unified paradigm for sequence-based identification of fungi. Molecular Ecology, 22(21), 5271–5277. doi:10.1111/mec.12481

Krakova, L. ,Soltys, K. ,Otlewska, A. , Pietrzak, K. ,Purkrtová, S. , Savická, D. , Puškárová, A. , Bučková, M. , Szemes, T. , Budis, J. , Demnerova, K. , Gutarowska, B. , Pangallo, D., 2017. Comparison of methods for identification of microbial communities in book collections: Culture-dependent (sequencing and MALDI-TOF MS) and culture-independent (Illumina MiSeq). International Biodeterioration and Biodegradation 1-9, article in press. doi: 10.1016/j.ibiod.2017.02.015.

Kraková, L., Chovanová, K., Selim, S.A., Šimonovičová, A., Puškarová, A., Maková, A., Pangallo, D., 2012a. A multiphasic approach for investigation of the microbial diversity and its biodegradative abilities in historical paper and parchment documents. International Biodeterioration and Biodegradation 70, 117-125. doi: 10.1016/j.ibiod.2012.01.011

Kraková, L., Chovanová, K., Puškarová, A., Bučková, M., Pangallo, D., 2012b. A novel PCR-based approach for the detection and classification of potential cellulolytic fungal strains isolated from museum items and surrounding indoor environment Letters in applied microbiology 54 (5), 433-440. doi: 10.1111/j.1472-765X.2012.03227.x

Krüger, E.L., Diniz, W., 2011. Relationship between indoor thermal comfort conditions and the Time Weighted Preservation Index (TWPI) in three Brazilian archives. Applied Energy 88, 712-723. doi:10.1016/j.apenergy.2010.09.011

Lim, Y.W., Lee, J.S., Kim, K.M., Jung, H.S., 2005. Wood-rotting Fungal Flora of Kanghwa Island. Mycobiology 33(4), 167-172. doi:10.4489/MYCO.2005.33.4.167.

Liu, Z., Wang, Y., Pan, X., Ge, Q., Ma, Q., Li, Q., Fu T., Hu C., Zhu X. Pan, J. 2017 Identification of Fungal Communities Associated with the Biodeterioration of Waterlogged Archeological Wood in a Han Dynasty Tomb in China. Frontiers in Microbiology 8, 1633. doi: 10.3389/fmicb.2017.01633

López-Miras, M., Piñar, G., Romero-Noguera, J., Bolívar-Galiano, F.C., Ettenauer, J., Sterflinger, K., Martín-Sánchez, I., 2012. Microbial communities adhering to the obverse and reverse sides of an oil painting on canvas: identification and evaluation of their biodegradative potential. Aerobiologia 29, 301-314. doi: 10.1007/s10453-012-9281-z

López-Miras M., Martín-Sánchez I., Yebra-Rodríguez Á., Romero-Noguera J., Bolívar-Galiano F., Ettenauer J., Sterflinger, K., Piñar, G. 2013. Contribution of the Microbial Communities Detected on an Oil Painting on Canvas to Its Biodeterioration. PLoS ONE 8(11), 1-13. doi: 10.1371/journal.pone.00801982013

Lupan, I., Popescu, O., 2012. Metagenomics and future perspectives for biodeterioration and biodegradation studies. Annals of the Romanian Society for Cell Biology 17, 37-42.

Lutterbach, M.T.S., Oliveira, A.L.C., Zanatta, E.M., Costa, A. C. A., 2013. A berlinda de aparato do imperador D. Pedro II: identificação de fungos em partes selecionadas e sua relação com biodeterioração e aerobiologia. Conservar Património 17, 59-72. doi: 10.14568/cp2013003

Marchal, R., Warchol, M., Cilindre, C. Jeandet, P., 2006. Evidence for protein degradation by *Botrytis cinerea* and relationships with alteration of synthetic wine foaming properties. Journal of Agriculture and Food Chemistry 54, 5157-5165.

Marozzi, G., Sánchez, S., Benucci, G.M.N., Bonito, G., Falini, L.B., Albertini, E., Donnini, D., 2017. Mycorrhization of pecan (*Caryaillinoinensis*) with black truffles: *Tuber melanosporum* and *Tuber brumal*. Mycorrhiza 27(3), 303-309. doi: 10.1007/s00572-016-0743-y

Martin, K.J., Rygiewicz, P.T., 2005. Fungal-specific PCR primers developed for analysis of the ITS region of environmental DNA extracts. BMC Microbiology 5, 28. doi: 10.1186/1471-2180-5-28

McCrimmon B., 2017. A brief history of art: from antiquity to impressionism. Available at https://www.theodysseyonline.com/history-art.

Menkis, A., Allmer, J., Vasiliauskas, R., Lygis, V., Stenlid, J., Finlay, R., 2004. Ecology and molecular characterization of dark septate fungi from roots, living stems, coarse and fine woody debris. Mycological Research 108(8), 965-73.

Mesquita, N., Portugal, A., Videira S., Rodriguez-Echeverria, S., Bandeira, A.M.L., Santos, M.J.A., Freitas, H., 2009. Fungal diversity in ancient documents. A case study on the Archive of the University of Coimbra. International Biodeterioration and Biodegradation 63, 626-629. doi: 10.1016/j.ibiod.2009.03.010

Melo, D., Sequeira, S.O., Lopes, J.A., Macedo, M.F., 2018. Stains *versus* colourants produced by fungi colonizing paper cultural heritage: A review. Journal of Cultural Heritage. Article in press. doi:10.1016/j.culher.2018.05.013

MIBAC (Italian Ministry of Cultural Heritage) Atto di indirizzosuicriteritecnico-scientifi ci e sugli standard di funzionamento e sviluppodeimusei, Ambito VI. D.Lgs. 112/1998 (art. 150, comma 6) (2001) 81-94.

Michaelsen, A., Piñar, G., Montanari, M., Pinzari, F., 2009. Biodeterioration and restoration of a 16thcentury book using a combination of conventional and molecular techniques: a case study. International Biodeterioration and Biodegradation 63, 161-168.

Michaelsen, A., Piñar, G., Pinzari, F., 2010. Molecular and microscopical investigation of the microflora inhabiting a deteriorated Italian manuscript dated from the thirteenth century. Microbial Ecology 60, 69-80.

Michaelsen, A., Pinzari, F., Ripka, K., Lubitz, W., Pinar, G., 2006. Application of molecular techniques for identification of fungal communities colonizing papermaterial. International Biodeterioration and Biodegradation 58, 133-14.

Micheluz, A., Manente, S., Tigini, V., Prigione, V., Pinzari, F., Ravagnan, G., Varese, G.C., 2015. The extreme environment of a library: Xerophilic fungi inhabiting indoor niches. International Biodeterioration and Biodegradation 99, 1-7. doi: 10.1016/j.ibiod.2014.12.012

Montanari, M., Melloni, V., Pinzari, F., Innocenti, G., 2012. Fungal biodeterioration of historical library materials stored in Compactus movable shelves. International Biodeterioration and Biodegradation 75, 83-88. doi: 10.1016/j.ibiod.2012.03.011

Nevarez, L., Vasseur, V., Le Dréan, G., Tanguy, A., Guisle-Marsollier I., Houlgatte, R. Barbier G., 2008. Isolation and analysis of differentially expressed genes in Penicillium glabrum subjected to thermal stress. Microbiology, Microbiology Society, pp.3752-3765. doi: 10.1099/mic.0.2008/021386-0

Nielsen, K., 2003. Mycotoxin production by indoor moulds. Fungal Genetics and Biology 39, 103–117. doi: 10.1016/S1087-1845(03)00026-4

Nunes, I., Mesquita, N., Cabo Verde, S., Bandeira, A.M.L., Carolino, M.M., Portugal, A., Botelho, M.L., 2013. Characterization of an airborne microbial community: A case study in the archive of the University of Coimbra, Portugal. International Biodeterioration and Biodegradation 79, 36-41.

Nwadiaro, P.O., Chuku, A., Onyimba, I. A., Ogbonna, A.I., Nwaukwul, I.A., Adekojo, D.A., 2015. Keratin Degradation by *Penicillium purpurogenum* isolated from Tannery Soils in Jos, Nigeria. British Microbiology Research Journal 8(1), 358-366.

O'Donnell, D., Wang, L., Xua, J., Ridgway, D., Gua, T., Moo-Young, M., 2001. Enhanced heterologous protein production in *Aspergillus niger* through pH control of extracellular protease activity. Biochemical Engineering Journal 8, 187–193.

Okpalanozie, O. E., Adebusoye, S. A., Troiano, F., Polo, A., Cappitell,i F., Ilori, M. O., 2016. Evaluating the microbiological risk to a contemporary Nigerian painting: Molecular and biodegradative studies. International Biodeterioration and Biodegradation 114, 184-192. doi: 10.1016/j.ibiod.2016.06.017

Paiva de Carvalho, H., Mesquita, N., Trovão, J., Peixoto da Silva, J., Rosa, B., Martins, R., Bandeira, A.M.L., Portugal, A., 2016. Diversity of fungal species in ancient parchments collections of the Archive of University of Coimbra, International Biodeterioration and Biodegradation 108, 57-66. doi: 10.1016/j.ibiod.2015.12.001

Pangallo, D., Šimonovičová, A., Chovanová, K., Ferianc, P., 2007. Wooden art objects and the museum environment: identification and biodegradative characteristics of isolated microflora. Letters in Applied Microbiology 45, 87-94. doi: 10.1111/j.1472-765X.2007.02138.x

Pangallo, D., Chovanová, K., Šimonovičová, A., Ferianc, P., 2009. Investigation of microbial community isolated from indoor artworks and air environment: identification, biodegradative abilities, and DNA typing. Canadian Journal of Microbiology 55, 277-287. doi: 10.1139/w08-136.

Pangallo, D., Chovanova, K., Makova, A., 2010. Identification of animal skin of historical parchments by polymerase chain reaction (PCR)-based methods. Journal of Archaeological Science 37, 1202–1206. doi: 10.1016/j.jas.2009.12.018

Pangallo, D., Kraková, L., Chovanová, K., Bučkova, M., Puškarová, A., Šimonovičová, A., 2013 Disclosing a crypt: Microbial diversity and degradation activity of the microflora isolated from funeral clothes of Cardinal Peter Pazmany. Microbiological Research 168, 289– 299.

Parchas, M.D., 2009. Comment Faire Face aux Risques Biologiques? Direction des archives de France, Paris. http://www.archivesdefrance.culture.gouv.fr/static/2922

Petre, C. V., Tănase, C., 2013. Culture characteristics of 20 lignicolous basidiomycetes species that synthesize volatile organic compounds. An. Stiint. Univ. Al. I. Cuza Iasi, Sect. II a. Biol. veget., 59, 37-51.

Piecková, E., 2012. Adverse health effects of indoor moulds, Arhiv Za Higijenu Rada I Toksikologiju 63, 545-549. doi: 10.2478/10004-1254-63-2012-2221

Pildain, M.B., Novas, M.V., Carmarán, C.C., 2005. Evaluation of anamorphic state, wood decay and production of lignin-modifying enzymes for diatrypaceous fungi from Argentina. Journal of Agricultural Technology 1 (1), 81-9.

Piñar, G., Sterflinger, K, Pinzari, F., 2015a. Unmasking the measles-like parchment discoloration: molecular and microanalytical approach. Environmental Microbiology 17(2), 427-443.

Piñar, G., Sterflinger, K, Ettenauer, J., Quandt, A., Pinzari, F., 2015b. A combined approach to assess the microbial contamination of the Archimedes Palimpsest. Environmental Microbiology 69: 118-134.

Piñar, G., Tafer, H., Sterflinger, K., Pinzari, F., 2015c. Amid the possible causes of a very famous foxing: Molecular and microscopic insight into Leonardo da Vinci's self-portrait. Environmental Microbiology Reports 7(6), 849-859. doi:10.1111/1758-2229.12313

Pinheiro, A.C., Macedo, M.F, Jurado, V., Saiz-Jimenez, C., Viegas, C., Brandão, J., Rosado, L., 2011. Mould and yeast identification in archival settings: Preliminary results on the use of traditional methods and molecular biology options in Portuguese archives. International Biodeterioration and Biodegradation 65, 619-627.

Pinheiro, A.C.M.S., 2014. Fungal communities in archives: assessment strategies and impact on paper conservation and human health. PhD Thesis, Universidade Nova de Lisboa, Lisboa, Portugal.

Pinzari, F., Montanari, M., Michaelsen, A., Piñar, G., 2010. Analytical protocols for the assessment of biological damage in historical documents. COALITION 19, 6-13.

Pinzari, F., Troiano, F., Piñar, G., Sterflinger, K., Montanari, M., 2011. The Contribution of Microbiological Research in the Field of Book, Paper and Parchment Conservation', in: New Approaches to Book and Paper Conservation Restoration, eds. P. Engel, J. Schirò, R. Larsen, E. Moussakova and I. Kecskeméti, Verlag Berger, Wien/Horn pp. 575-594.

Pinzari, F., Colaizzi , P., Maggi , O., Persiani, A. M., Schütz R., Rabin, I., 2012. Fungal bioleaching of mineral components in a twentieth-century illuminated parchment. Analytical and Bioanalytical Chemistry 402, 1541–1550. doi:10.1007/s00216-011-5263-1

Pitt, J.I. and Hocking, A.D., 2009. Fungi and Food Spoilage (3rd ed.), Springer, New York, USA.

Polo, A., Cappitelli, F., Villa, F., Pinzari, F., Piecková, E., 2017. Biological invasion in the indoor environment: the spread of *Eurotium halophilicum* on library materials. International Biodeterioration and Biodegradation 118, 34-44. doi: 10.1016/j.ibiod.2016.12.010

Popescu, C., Tibirna, C. M., Manoliu, A.L., Gradinariu, P., Vasile, C., 2011. Microscopic Study OF Lime Wood Decayed by *Chaetomium Globosum*. Cellulose Chemistry and Technology 45 (9-10), 565-569.

Poulakakis, N., Tselikas, A., Bitsakis, I., Mylonas, M., Lymberakis, P., 2007. Ancient DNA and the genetic signature of ancient Greek manuscripts. Journal of Archaeological Science 34, 675-680. doi:10.1016/j.jas.2006.06.013

Powell, C., 2010. Analysis and consolidation of lifting ink in a twelfth-century manuscript. Journal of the Institute of Conservation 33(1), 13-27. doi: 10.1080/19455220903509945

Poyatos, F., Morales, F., Nicholson, A.W., Giordano, A., 2017. Physiology of biodeterioration on canvas paintings. Journal of Cellular Physiology 1–11. doi: 10.1002/jcp.26088

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., Glöckner, F.O. 2013. The SILVA ribosomal RNA gene data base project: improved data processing and web-based tools. NucleicAcidsRes 41, D590-6.

Rahimlou, S., Babaeizad, V., Bose, T., Sayari, M., 2016. Determination of lignin-modifying enzymes (LMEs) in *Hyphodermella* species using biochemical and molecular techniques. Mycologia Iranica 3(1), 57-63. doi: 10.22043/mi.2017.47179.1075

Rakotonirainy, M.S., Heude, E., Lavédrine, B., 2007. Isolation and attempts of biomolecular characterization of fungal strains associated to foxing on a 19th century book. Journal of Cultural Heritage 8(2), 126-133. doi: 10.1016/j.culher.2007.01.003

Rastogi G., Sani R.K., 2011. Molecular Techniques to Assess Microbial Community Structure, Function, and Dynamics in the Environment. pp. 45 In: Ahmad I., Ahmad F., Pichtel J. (eds) Microbes and Microbial Technology. Springer, New York, USA. doi: 10.1007/978-1-4419-7931-5_2

Ricelli, A., Fabbri, A.A, Fanelli, C., Menicagli, R., Samaritani, S., Pini, D., Rapaccini, S.M., Salvadori, P., 1999. Fungal growth on samples of paper: Inhibition by new antifungals. Restaurator 20(2), 97-107. Doi:10.1515/rest.1999.20.2.97

Robertson, B., 1984. An alkaline extracellular protease produced by *Cladosporium cucumerinum* and its possible importance in the development of scab disease cucumber seedlings. Physiological Plant Disease 24(1), 83-92

Rodrigues da Silva, R., Ângela, T., Cabral, H., 2013. Comparative evaluation of peptidases produced by *Penicillium corylophilum* and *Penicillum waksmanii* in solid state fermentation using agro-industiral residues. Journal of Agricultural and Technology B, 230-27.

Rosado, T., Mirão, J., Candeia, A., Caldeira, A.T., 2014. Microbial communities analysis assessed by pyrosequencing—a new approach applied to conservation state studies of mural paintings. Anal Bioanal Chem 406, 887–895. doi: 10.1007/s00216-013-7516-7

Rüttimann-Johnson, C., Cullen, D., Lamar, R. T., 1994. Peroxidases of the White Rot Fungus *Phanerochaete sordida*. Applied and Environmental Microbiology 60 (2), 599-605.

Samson, R.A., Hoekstra, E.S., Frisvad, J.C., Filtenborg, O. (eds.), 2000. Introduction to Food and Airborne Fungi (6th ed.), Centraalbureau Voor Schimmelculture, Utrecht, The Netherlands.

Sanmartín, P., De Araujo, A. and Vasanthakumar, A., 2016. Melding the Old with the New: Trends in Methods Used to Identify, Monitor, and Control Microorganisms on Cultural Heritage Materials. Microbiology Ecology pp. 1-17. doi: 10.1007/s00248-016-0770-4

Santos, A., Cerrada, A., García, S., San Andrés, M., Abrusci, C., Marquina, D., 2009. Application of Molecular Techniques to the Elucidation of the Microbial Community Structure of Antique Paintings. Microbiology Ecology 58:692-702. doi: 10.1007/s00248-009-9564-2

Sato, Y., Aoki, M., Kigawa, R., 2014. Microbial deterioration of tsunami-affected paper-based objects: A case study', International Biodeterioration and Biodegradation 88, 142-149. doi:10.1016/j.ibiod.2013.12.007

Schabereiter-Gurtner, C., Piñar, G., Lubitz, W., Rölleke, S., 2001. An advanced molecular strategy to identify bacterial communities on art objects. Journal of Microbiology Methods 45(2), 77-87. doi: 10.1016/S0167-7012(01)00227-5

Schimdt, O., 2007. Indoor wood-decay basidiomycetes: damage, causal fungi, physiology, identification and characterization, prevention and control. Mycological Progress 6, 261.doi: 10.1007/s11557-007-0534-0

Schmieder, R., Edwards, R., 2011. Quality control and preprocessing of metagenomic datasets. Bioinformatics 27, 863-864.

Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L., Levesque, C.A., Chen, W., 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for fungi. In: Proceedings of the National Academy of Sciences of the United States of America. Online at http://www. pnas.org/content/109/16/6241.full.pdfþhtml.

Schubert, M., Lindgreen, S., Orlando, L., 2016. Adapter Removal v2: rapid adapter trimming, identification, and read merging. BMC Res Notes 9, 88.

Seifert K., Morgan-Jones G., Gams W, Kendrick B., 2011.The Genera of Hyphomycetes. (1st ed.) CBS Biodiversity Series 9, CBS-KNAW Fungal Biodiversity Centre, Utrecht, The Netherlands.

Sequeira, S., Cabrita, E.J., Macedo, M.F., 2012. Antifungals on paper conservation: an overview. International Biodeterioration and Biodegradation 74, 67-86. doi: 10.1016/j.ibiod.2012.07.011

Sequeira, S.O., Cabrita, E.J., Macedo, M.F., 2014. Fungal Biodeterioration of Paper: How are Paper and Book Conservators Dealing with it? An International Survey. Restaurator 35 (2), 181-199. doi: 10.1515/res-2014-0005

Shi, D., Lu, G., Mei, H., Sybren de Hoog, G., Samerpitak, K., Deng, S., Shen, Y., Liu, W., 2016. Subcutaneous infection by *Ochroconis mirabilis* in an immunocompetent patient. Medical Mycology Case Reports 11, 44–47. Doi: 10.1016/j.mmcr.2016.04.007

Šimonovičová, A., Gódyová, M., Kunert, J., 2004. *Engyodontium album*, a new species of microscopic fungi for Slovakia and its keratinolytic activity. Biologia, Bratislava 59 (1), 17-18.

Šimonovičová, A., Kraková, L., Pangallo, D., Majorošová, M., Piecková, E., Bodoriková, S., Dörnhoferová, M., 2015. Fungi on mummified human remains and in the indoor air in the Kuffner family crypt in Sládkovičovo (Slovakia), International Biodeterioration and Biodegradation 99, 157-164. Doi:10.1016/j.ibiod.2014.12.011

Staszczac, M., Zdunek, E. , Leonowikz, A., 2000. Studies on the role of proteases in the white-rot fungus *Trametes versicolor*: Effect of PMSF and chloroquine on ligninolytic enzymes activity. Journal of Basic Microbiology 40 (1), 51-63.

Sterflinger , K., Piñar, G., 2013. Microbial deterioration of cultural heritage and works of art - tilting at windmills? Applied Microbiology Technology 97: 9637-9646. doi: 10.1007/s00253-013-5283-1

Sterflinger K., 2010. Fungi: their role in deterioration of cultural heritage. Fungal Biology Reviews 24, 47-55. doi: 10.1016/j.fbr.2010.03.003

Sterflinger, K., Piñar, G., 2013 Microbial deterioration of cultural heritage and works of art – tilting at windmills?. Applied Microbiology Technology 97, 9637-9646. doi: 10.1007/s00253-013-5283-1

Sterflinger, K., Pinzary, F., 2012. The revenge of time: fungal deterioration of cultural heritage with particular reference to books, paper and parchment. Environmental Microbiology 14(3), 559-566. doi: 10.1111/j.1462-2920.2011.02584.x

Sunitha, V.H., Nirmala, Devi, D.M., Srinivas, C., 2013. Extracellular Enzymatic Activity of Endophytic Fungal Strains Isolated from Medicinal Plants. World Journal of Agricultural Sciences 9 (1): 01-09. doi: 10.5829/idosi.wjas.2013.9.1.72148

Szczepanowska, H., Cavaliere, A.R., 2000. Fungal deterioration of 18th and 19th century documents: a case study of the Tilghman Family Collection, Wye House, Easton, Maryland. International Biodeterioration and Biodegradation 46, 245-249.

Szulc, J., Otlewska, A., Ruman, T., Kubiak, K., Karbowska-Berent, J., Kozielec, T., Gutarowska, B., 2018. Analysis of paper foxing by newly available omics techniques, International Biodeterioration and Biodegradation. Article in press. doi: 10.1016/j.ibiod.2018.03.005

Technical note NT-SCE-02 (SCE - Sistema Nacional de Certificação Energética da Qualidade do Ar Interior nos Edifícios) 2009. Metodologia para auditorias periódicas de Qualidade do Ar Interior em edifícios de serviços existentes no âmbito do RSECE, 43. http://www.adene.pt/ptpt/SubPortais/SCE/Destaques/Paginas/Notatecnica2.aspx

Tedersoo, L., Bahram, M., Põlme, S., Kõljalg, U., Yorou, N.S., Wijesundera, R., Villarreal, R. L., Vasco-Palacios, A.M., Thu, P.Q., Suija, A., Smith, M.E., Sharp, C., Saluveer, E., Saitta, A., Rosas, M., Riit, T., Ratkowsky, D., Pritsch, K., Põldmaa, K., Piepenbring, M., Phosri ,C., Peterson, M., Parts, K., PärtelK ,O. E., Nouhra, E., Njouonkou, A.L., Nilsson, R.H., Morgado, L.N., Mayor, J., May ,T.W., Majuakim, L., Lodge, D.J., Lee, S.S., Larsson, K.H., Kohout, P., Hosaka, K., Hiiesalu, I., Henkel, T.W., Harend, H., Guo, L.D., Greslebin, A., Grelet, G., Gem,l.J., Gates, G., Dunstan, W., Dunk ,C., Drenkhan, R., Dearnaley, J., De Kesel, A., Dang, T., Chen, X., Buegger, F., Brearley, F.Q., Bonito, G., Anslan, S., Abell, S., Abarenkov, K., 2014. Global diversity and geography of soil fungi. Science 346, 1256688.

Tiano, P., 2002. Biodegradation of Cultural Heritage: Decay, Mechanisms and Control Methods. Seminar article. New University of Lisbon, Department of Conservation and Restoration.

Tóth, B., Csősz, M., Dijksterhuis, J., Frisvad, J.C., Varga, J., 2007. *Pithomyces chartarum* as a pathogen of wheat. Journal of Plant Pathology 89 (3), 405-408.

Troiano, F., Polo, A., Villa F., Cappitelli, F., 2014. Assessing the microbiological risk to stored sixteenth century parchment manuscripts: a holistic approach based on molecular and environmental studies. Biofouling 30(3), 299–311.

Trovão, J., Mesquita, N., Paiva, D.S., Paiva de Carvalho, H., Avelar, L., Portugal, A., 2013. Can arthropods act as vectors of fungal dispersion in heritage collections? A case study

on the archive of the University of Coimbra, Portugal. International Biodeterioration and Biodegradation 79, 49-55. doi: 10.1016/j.ibiod.2012.10.015

Urzì C. and De Leo, F., 2008. Biodeterioration of Cultural Heritage in Italy: State of Art Online at: http://www.arcchip.cz/w08/w08_de_leo.pdf

Urzì, C., Krumbein, W.E., 1994. Microbiological impacts on the cultural heritage. In: Krumbein, W.E., Brimblecombe, P., Cosgrove, D.E. Staniforth, S.(eds.), Durability and change: the science, responsibility, and cost of sustaining cultural heritage, Wiley, J. and Sons. pp.. 107-135.

van der Wal, A., Klein Gunnewiek, P. J. A, Cornelissen, J. H. C., Crowther, T. W., de Boer, W., 2016. Patterns of natural fungal community assembly during initial decay of coniferous and broadleaf tree logs. Ecosphere 7 (7), e01393. doi: 10.1002/ecs2.1393

Vasconcelos, A., 1991. O Arquivo da Universidade. Reedição de Manuel Augusto Rodrigues. A.U.C, Coimbra, Portugal.

Vukjević, J., Grbić, M.L., 2010. Moulds on Paintings in Serbian fine art museums. African Journal of Microbiology Research 4(13), 1453-1456.

Watanabe T., 2002. Soil and Seed Fungi. Morphologies of Cultured Fungi and Key to Species (2nd ed.), CRC Press, Boca Raton, Florida, USA.

White, T.J., Burns, T., Lee, S., Taylor, J., 1990. Amplification and direct sequencing of ribosomal RNA genes for phylogenetics. In: Innis, M.A. (eds.), PCR Protocols: a Guide to Methods and Applications. Academic Press, New York, USA, pp. 315-322.

White, T.C., Findley, K., Dawson, T. L., Scheynius, A., Boekhout, T., Cuomo, C. A., Xu, J., Saunders, C. W. , 2014. Fungi on the Skin: Dermatophytes and *Malassezia*. Cold Spring Harbor Perspectives in Medicine 4(8), a019802. doi: 10.1101/cshperspect.a019802

Zaferanloo, B., Quang, T. D., Daumoo, S., Ghorbani, M.M., Mahon, P.J., Palombo, E.A., 2014. Optimization of protease production by endophytic fungus *Alternaria alternata* isolated from an Australian native plant. World Journal Microbiology Biotechnology 30, 1755-1762. doi: 10.1007/s11274-014-1598-z

Zyska, B., 1997. Fungi isolated from library materials: A review of the literature. International Biodeterioration and Biodegradation 40(1), 43-51. doi: 10.1016/S0964-8305(97)00061-9

Consulted websites:

https://www.uc.pt/auc/instituicao/historia

http://www.uc.pt/auc/instituicao/missao+ Vasconcelos, 1990+

https://www.cascais.pt/equipamento/casa-das-historias-paula-rego