Identification and control of fungal contamination in ancient heritage documents

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Abstract

Archives, libraries and museums worldwide protect and conserve different historical collections. These buildings frequently present suitable conditions for the growth of fungi and bacteria, which are able to deteriorate documents by exploring small niches made available in items from these collections.

This multidisciplinary work addresses the causes and control methodologies for the prevention of biodeterioration by fungi in ancient documents. Our objectives were to: i) identify organisms responsible for biodeterioration in paper and parchment documents, and relate the different *taxa* with the substrate types; ii) assess the effects of gamma radiation in different biological parameters of fungal isolates; iii) evaluate the role of arthropods as vectors for fungal dispersion in library environments, and the effects of seasonality on the occurrence of different fungal *taxa*.

The first task was a case study on the presence of biodeteriorating fungi in documents from the Archive of the University of Coimbra (AUC). Thirty documents, made of different materials (parchment, laid-paper and wood-pulp paper) were analysed. In total, 14 genera, and 20 species were isolated and identified. The most frequent genera were *Cladosporium, Penicillium* and *Aspergillus*. Less frequent genera, such as *Alternaria, Botrytis, Chaetomium, Chromelosporium, Epicoccum, Phlebiopsys* and *Toxicocladosporium* were also present.

As external comparison, an 18th century papal bull stored in Freamunde (Paços de Ferreira, Portugal) was analyzed. In total, 18 species, belonging to 10 genera, were isolated and identified. The parchment presented a high diversity of species, with prevalence of *Aspergillus versicolor* and *Penicillium brevicompactum.* Other less common species, like *Chrysosporium carmichaelii* and *Fusicladium rhodense*, were also isolated.

Coincident species were found in both studies, namely *Cladosporium cladosporioides, Penicillium chrysogenum* and *A. versicolor*; however there were species present in the Bull that had not previously been obtained. Some of the isolates were found only in one support type, since they are substrate specific, and thus unable to thrive in other supports. We were unable to establish a relation between the different taxonomical groups and the support types.

Cladosporium cladosporioides is a ubiquitous and radio resistant fungal species, found in AUC documents as well as in the papal bull. A strain of *C. cladosporioides* was selected as test organism for the application of different gamma radiation doses, and the effects of this treatment were assessed using two different methodologies. Radiation doses of 8.2 and 15.4 kGy caused a significant decrease in all parameters: viability, growth, biomass and CFU count. Furthermore, 15.4 kGy sterilized all samples. This task provided us with information on the radiation dose required for the disinfection of *C. cladosporioides* (and consequently of other less resistant fungi), and on the effects on biological parameters after irradiation. However, little information regarding the early stages of fungal growth was obtained. In this way, we chose to develop a more straightforward method, using flow cytometry, that allows the assessment of the radiation effects during the early stages of growth of individual cells.

The effects of gamma irradiation (0 to 15 kGy) on spores from *Penicillium chrysogenum, Aspergillus nidulans* and *Aspergillus niger* were assessed. The Forward and Side-Scatter channels were used to assess the size and complexity of particles; Propidium iodide was used to assess the membrane integrity and spore viability; and Dihydroethidium was used to measure metabolic activity. It was possible to analyse these parameters during early growth stages: irradiated spores generally grew less, later, and were less metabolically active.

Our results support the D_{min} of 5 kGy for the disinfection of paper and parchment documents as it will likely inactivate all organisms, or reduce them to very low counts. Because of that, it is an effective control procedure, since doses up 15 kGy are still considerably lower than the D_{max} for both paper and parchment.

After disinfection, re-contamination can occur through different processes, such as cross contamination done by users, settling of airborne propagules or the action of vector organisms (eg. arthropods).

A total of 148 isolates, 25 genera and 59 species were retrieved from arthropods captured during one year, using 80 traps placed in the AUC. The most common genera were *Penicillium*, *Aspergillus* and *Cladosporium.* No relation was found between isolates and the order of arthropods they were retrieved from; nevertheless, an association was found between entomopathogenic fungi (from the genera *Aspergillus, Beauveria, Cladosporium,*

Engyodontium, Lecanicillium, Penicillium and *Paecilomyces*) and the orders of arthropods they were isolated from.

Nearly 36% of the species previously found colonizing documents were also isolated from the arthropods captured in this study. The main examples were *Cladosporium cladosporioides*, *Penicillium chrysogenum*, *Aspergillus versicolor*, *Alternaria alternata*, *Epicoccum nigrum*, *Penicillium canescens* and *Phlebiopsis gigantea*. This suggests that at least on this site, contaminating species can be dispersed by mechanisms that use arthropods as vectors. Additionally, this task also provided information on the seasonality effects on the prevalence of certain genera in the different seasons.

The information provided on this thesis contributes to the understanding of the biodeterioration processes in paper and parchment supports, as well as possible treatment procedures, and the role of insects as vectors of fungal dispersion. I trust it will assist other researchers in fighting the tangible issue of the biodeterioration of cultural heritage items.

Keywords: Biodeterioration, Ancient documents, Fungal identification, PCR, ITS sequencing, Foxing, Paper, Parchment, Molecular characterization, Gamma Irradiation, Disinfection, *Cladosporium cladosporioides*, Sub-lethal effects, Spore viability, Fungal viability, Metabolic activity, Spore growth, Flow cytometry, CFU count, Filamentous fungi, Dihydroethidium, Reactive oxygen species; Entomopathogenic Fungi; Arthropod Vectored dispersion.

Resumo

Os arquivos, museus e bibliotecas são os principais responsáveis pelo armazenamento, protecção e conservação de diversos acervos históricos, um pouco por todo o mundo. Porém, apresentam muitas vezes condições adequadas ao crescimento de fungos e bactérias, que promovem a deterioração de documentos, explorando pequenos nichos favoráveis ao seu desenvolvimento.

Neste trabalho multidisciplinar, pretendemos estudar as causas e metodologias de controlo para a prevenção da biodeterioração em documentos antigos. Os objectivos foram: i) identificar organismos responsáveis pela biodeterioração de documentos em papel e pergaminho, relacionando os diferentes *taxa* com os diferentes substratos; ii) avaliar os efeitos da radiação gama em diferentes parâmetros biológicos de isolados fúngicos; iii) avaliar o papel dos artrópodes enquanto vectores de dispersão de fungos neste ambiente, bem como os efeitos de sazonalidade na ocorrência de diferentes *taxa*.

A primeira tarefa foi um caso de estudo no Arquivo da Universidade de Coimbra (AUC), que consistiu no isolamento e identificação de diferentes fungos presentes em documentos de pergaminho e papel. Foram isolados e identificados 14 géneros e 20 espécies. Os géneros mais frequentes foram: *Cladosporium, Penicillium* e *Aspergillus*; entre outros, menos frequentes: *Alternaria, Botrytis, Chaetomium, Chromelosporium, Epicoccum, Phlebiopsys* e *Toxicocladosporium*.

Uma bula papal do séc. XVIII, proveniente de Freamunde (Paços de Ferreira, Portugal) foi analisada como termo de comparação. No total foram isoladas e identificadas 18 espécies, pertencentes a 10 géneros. O pergaminho apresentou uma alta diversidade de espécies, com predominância de *Aspergillus versicolor* e *Penicillium brevicompactum*. As espécies *Chrysosporium carmichaelii* e *Fusicladium rhodense*, também foram isoladas, embora com menor frequência.

Foram encontradas espécies coincidentes em ambos os estudos: *Cladosporium cladosporioides, Penicillium chrysogenum* e *A. versicolor*, porém, algumas espécies encontradas na bula não tinham sido previamente obtidas. Alguns dos isolados foram encontrados apenas num tipo de suporte, por terem uma maior especificidade para com o substrato. Não foi possível estabelecer uma relação entre os diferentes grupos de fungos, e os diferentes suportes.

Cladosporium cladosporioides é uma espécie ubíqua, e rádio resistente, que foi isolada a partir de documentos do AUC, mas também da bula papal. Uma estirpe de *C. cladosporioides* foi escolhida como organismo de teste para a aplicação de diferentes doses de radiação gama, e avaliação dos efeitos deste tratamento em diferentes parâmetros biológicos.

Doses de radiação de 8.2 e 15.4 kGy causaram um decréscimo significativo em todos os parâmetros: viabilidade, crescimento, biomassa e contagem de UFCs. Além disso, a dose de 15.4 kGy esterilizou todas as amostras. Esta tarefa forneceu informação acerca da dose necessária para a desinfecção de *C. cladosporioides* (e consequentemente de outras espécies menos resistentes), bem como dos efeitos em diferentes parâmetros biológicos após irradiação. Porém, não foi possível obter esta informação durante as fases iniciais de desenvolvimento. Deste modo, decidimos desenvolver um novo método, utilizando citometria de fluxo, por permitir a avaliação dos efeitos da radiação durante as fases iniciais de desenvolvimento em células individuais.

Foram analisados os efeitos da radiação gama (0 a 15 kGy) em esporos de *Penicillium chrysogenum, Aspergillus nidulans* e *Aspergillus niger*: ao nível do tamanho e complexidade (*forward* e *side-scatter channels*), integridade da membrana, viabilidade e actividade metabólica, sendo que estes parâmetros foram analisados com sucesso durante as fases iniciais de germinação dos esporos. Esporos irradiados cresceram menos, mais tarde, e foram menos activos metabolicamente.

Os nossos resultados sustentam uma D_{min} de 5 kGy para a desinfecção de documentos de pergaminho e papel, uma vez que inactivará todos os organismos, ou pelo menos reduzi-los drasticamente em número, confirmando assim a sua eficácia, especialmente porque doses até 15 kGy estão abaixo das D_{max} determinadas para o papel e o pergaminho.

Após um tratamento de desinfecção, a re-contaminação pode ocorrer por diferentes processos: contaminação cruzada promovida por utilizadores; deposição de propágulos presentes no ar; ou por acção de organismos vectores (ex: artrópodes). Um total de 148 isolados, 25 géneros e 59 espécies foram obtidos a partir de insectos capturados ao longo de um ano, em 80 armadilhas colocadas no AUC. Os géneros mais comuns foram *Penicillium*, *Aspergillus* e *Cladosporium*. Não foi encontrada uma relação entre os isolados e a ordem de artrópodes dos quais foram obtidos. No entanto, os fungos entomopatogénicos dos géneros *Aspergillus, Beauveria, Cladosporium, Engyodontium, Lecanicillium, Penicillium*

e *Paecilomyces* surgiram relacionados com as ordens de artrópodes das quais foram isolados.

Cerca de 36% das espécies encontradas previamente nos documentos do AUC foram também isoladas a partir dos artrópodes capturados neste estudo. Os principais exemplos são *Cladosporium cladosporioides*, *Penicillium chrysogenum*, *Aspergillus versicolor*, *Alternaria alternata*, *Epicoccum nigrum*, *Penicillium canescens* e *Phlebiopsis gigantea*. Estes resultados sugerem que, pelo menos neste local, espécies contaminantes são dispersas por artrópodes. Adicionalmente, esta tarefa também forneceu informação acerca dos efeitos da sazonalidade na prevalência de diferentes géneros nas diferentes estações.

A informação produzida nesta tese contribui para o conhecimento dos processos de biodeterioração documentos de papel e pergaminho, bem como da aplicação de radiação gama no tratamento destes documentos, mas também do papel que os insectos podem ter no processo de contaminação. Acreditamos que ajudará outros investigadores no combate ao problema da biodeterioração do nosso património cultural.

Palavras Chave : Biodeterioração, Documentos antigos, Identificação de fungos, PCR, Sequenciação da região ITS, Foxing, Papel, Pergaminho, Caracterização molecular, Radiação gama, Desinfecção, *Cladosporium cladosporioides*, Efeitos sub-letais, Viabilidade de esporos, Viabilidade de fungos, Dihidroethidium, ROS, Fungos entomopatogénicos, Dispersão vectorizada por insectos.

Thesis outline

This thesis is divided into a General Introduction, Chapters 1-5 and a General Discussion and Conclusions, in which the key results, highlights and future research possibilities are presented. The diversity of fungal organisms present in ancient documents from the Archive of the University of Coimbra is studied in Chapter 1. In Chapter 2, a case study is described, in which an 18th century parchment Papal Bull, is analysed concerning the diversity of contaminating fungal organisms and their pathogenic potential. The effects of gamma radiation on the growth and biomass of *Cladosporium cladosporioides* are studied on Chapter 3. The application of Flow Cytometry to the assessment of radiation effects, during the initial growth stages of three different fungal species, is described in Chapter 4. In Chapter 5, the role of insects as fungal dispersion agents is studied.

Following is a detailed outline for Chapters 1-5

Chapter 1 - Fungal diversity in ancient documents. A case-study on the Archive of the University of Coimbra

Thirty documents, made of different materials (parchment, laid-paper and wood-pulp paper) were analysed for the presence of contaminating fungal organisms. The identification of the infecting *taxa* was based on ribossomal DNA *loci* amplification and sequencing, confirmed by morphological identification, using macro and microscopic traits. In general, and considering the number of samples, a high fungal diversity was found in all support materials, and different species were isolated and identified. Some of the species were only found in only one type of support, which may be explained by production of different enzymes by the various organisms. In total, 14 fungal genera, and 20 different species were isolated and identified. The most frequent genera were *Cladosporium, Penicillium* and *Aspergillus*. Less frequent genera, such as *Alternaria, Botrytis, Chaetomium, Chromelosporium, Epicoccum, Phlebiopsys* and *Toxicocladosporium* were also present.

Chapter 2 - Assessing the fungal diversity in an ancient parchment - the pathogenic potential of existing mycoflora as a threat to the health of users

An 18th century Papal Bull made of parchment was analysed for the presence of contaminating moulds, and to check for potentially pathogenic species. Identifying infectious groups is essential in the selection of strategies for the prevention of health issues, both to restorers and other users. Different growth media were used, to maximize the number of obtained isolates. Molecular identification was obtained with the amplification and sequencing of ribossomal DNA *loci*, and confirmed with morphological identification, using macro and microscopic traits. In total, 18 different species, belonging to 10 genera, were isolated and identified. Overall, the parchment presented a high diversity of species, with the most common being *Aspergillus versicolor* and *Penicillium brevicompactum.* Other less common species, like *Chrysosporium carmichaelii* and *Fusicladium rhodense*, were also isolated. Apart from the degradation of the library material, most of these organisms can cause adverse human health effects in conservators, restorers and users. In fact, some of the species found in this parchment are producers of toxins that are dangerous to humans.

Chapter 3 - Assessing gamma radiation effects in a *Cladosporium cladosporioides* strain isolated from an ancient document: CFU-count vs. Growth parameters

A strain of *Cladosporium cladosporioides* that was isolated from the collection of the Archive of the University of Coimbra was submitted to different doses of gamma radiation. This fungus is a common contaminant in library and archive documents, it is moderately radio-resistant, and therefore was selected to estimate the minimum radiation dose required for decontamination. The irradiation effects on its growth and biomass production were assessed using two different methodologies: A) Colony forming unit count; and B) Colony biomass and radial growth. By using different methods to assess the effects of gamma radiation, we were able to test the spore survival and germination potential, but also the post germination effects on viability, growth and biomass of colonies. Radiation doses of 8.2 and 15.4 kGy caused a significant decrease in all tested parameters: spore viability, colony growth and biomass. Furthermore, 15.4 kGy sterilized all samples, in the sense that no growth or biomass increase was observed after irradiation with this dose.

Chapter 4 - Flow cytometry as a tool to assess the effects of gamma radiation on the viability, growth and metabolic activity of fungal spores

Flow cytometry is frequently used for viability and vitality assessment in bacteria and yeast. However, its application to the study of fungal spore development is still unusual. Flow cytometry was used to assess the effects of different gamma irradiation doses (0 to 15 kGy) on the survival, growth and metabolic activity of spores from *Penicillium chrysogenum, Aspergillus nidulans* and *Aspergillus niger.* The Forward and Side-Scatter channels of the cytometer were used to assess the differences in size and complexity of particles; Propidium iodide, a non-vital fluorescent dye, was used to assess the membrane integrity and spore viability; and Dihydroethidium (DHE), a vital fluorescent dye, was used to measure the changes in metabolic activity. Overall, we were able to analyse the viability, growth and metabolic activity of irradiated spores during the early growth stages. Results were consistent between radiation doses on all species: irradiated spores generally grew less, later, and were less metabolically active. The fluorescence intensity of Propidium Iodide in unviable cells was 5 to 7x higher than in viable cells, allowing the assessment of the effects of a sterilization treatment without the need to re-culture the cells. These analyses can be performed immediately after a given treatment, without the need to re-inoculate, incubate and perform colony counts, therefore providing a rapid insight into the condition/resistance of different organisms. Furthermore, unculturable, yet viable spores can also be screened.

Chapter 5 - Can arthropods act as vectors of fungal dispersion in heritage collections? A case study on the archive of the University of Coimbra, Portugal

Following the work described in Chapter 1, where a high diversity of colonizing fungi was isolated from ancient documents - *Cladosporium*, *Aspergillus* and *Penicillium* were the most frequent genera in biodeteriorated documents - we studied the role of arthropods as vectors for fungal dispersion. The presence of various arthropods in the archive suggested that zoochoric dispersion could play a significant role in the dispersion of fungal propagules. In this work, the role of arthropods as fungal dispersers and potential vectors of contamination was assessed, as well as the effects of seasonality in the diversity of fungal species associated with invertebrates, to allow a better understanding of the relationship between identified fungal species, and the captured arthropod *taxa*. The use of

ITS sequencing combined with micro and macroscopic analysis of fungal isolates provided information on the fungal diversity carried by the arthropods. Results showed a high fungal diversity associated with invertebrates. From a total of 148 isolates, 25 genera and 59 species were identified, from which the most common genera were *Penicillium*, *Aspergillus* and *Cladosporium.*

General Introduction

Archives, libraries and museums worldwide protect and conserve many historical document collections, as well as items of important cultural value. Sometimes, however, these repositories present suitable conditions for fungal growth, which is mainly dependent of temperature and humidity. Parchment and paper books and documents stored in such places are subject to decay, promoted by a variety of organisms (da Silva et al., 2006), such as insects, bacteria and fungi. Items in these collections are often complex objects, usually made from a combination of several organic and inorganic materials, where each responds to external agents in a different manner (Harvey, 1992). If we take into account the vast number of libraries spread worldwide and the number of items that they store at a given moment, once within the biosphere, their degradation by different biological mechanisms is likely to occur. It has been the role of conservators to prevent and delay these weathering processes and of restorers to repair the damages, however, new fields of research in biology and other sciences have been developed to assist in better understanding the role of biological degradation, from the smallest of molecular components, up to the macroscopic analysis of organisms, such as fungi and insects, and their influence on the biodeterioration processes.

The biotransformation processes caused by microorganisms are usually more severe in warm and humid climates, where the environmental conditions better fit their requirements. This degradation phenomenon is often referred to as biodeterioration, and is a true and global concern. Biodeterioration can be defined as an irreversible loss of value and/or information of an object of art following the attack by living organisms (Urzì and Krumbein, 1994), or any undesirable change in the properties of a material caused by the vital activities of organisms (Hueck, 1968). From incunabula to maps, old and recent books, as well as other materials, they all have a physical shape and substance; if we consider that every part of a library collection is a substance, we should also consider that everything within a library is susceptible to an external environment. The action of these environmental and biological factors will result in changes to the physical, chemical and mechanical properties of the different supports (Zyska, 1997).

As already stated, temperature and humidity are key factors in the development and growth of most microorganisms, and for that reason they are usually monitored and

controlled in these institutions. However, it is traditionally performed using devices that analyse a determined area of the repository, a process that is not sensitive enough if one considers the microniches that are potentially available for microorganisms to thrive in (Sterflinger, 2010). Poor ventilation and lack of surface temperature homogeneity can promote water condensation and thus, microclimates with higher water availability that allows fungal organisms to proliferate; these microclimates are created inside of compact shelving or the wrapping of objects into plastic bags and tight boxes; the lack of ventilation, daily temperature changes, air and vapour exchange as well as the water retention by hygroscopic materials are the most important reasons for heavy mould infections in heritage collections (Sterflinger, 2010; Sterflinger and Pinzari, 2012), and since a fungal colony can grow up to 4mm per day, an outburst of fungi can potentially contaminate a whole collection within a few days, if the proper conditions are presented (Sterflinger and Pinzari, 2012).

"A library or a single book can be compared to a virgin land that can be reached by some colonizing organisms that behave like pioneer species on a nude soil" (Michaelsen et al., 2010)

Fungal contamination is of great concern for libraries and archives that store paper-based documents and books, as well as parchment and leather items. For adequate storage and maintenance of these materials it is important not only to control fungal growth but also to remove the greatest possible amount of fungal propagules, such as ascospores and conidia (Michaelsen et al., 2012). The spores are resistance forms in most fungi, which allow them to resist and tolerate adverse environmental conditions while in their dormant state. Spores have low water content, and a reversibly dormant metabolism, allowing them to germinate once the adequate environmental conditions are met. This way, spores are the major threat in the long-term, and their control may prevent several different contaminations from occurring. Furthermore, keeping a controlled environment (low temperature, and low humidity) will prevent, or at least slow the growth of microorganisms and further contamination of the support.

Fungi

A great part of our cultural heritage has, for many centuries, been recorded in parchment, paper or leather, which are vulnerable due to the biodeterioration of their organic components, through the direct and indirect action of fungi, given their tolerance to desiccation, high salt concentrations and heavy metal compounds (Corte et al., 2003; Cappitelli and Sorlini, 2005, Michaelsen et al. 2010), but also of bacteria (Scharbereiter-Gurtner *et al.*, 2001; Michaelsen et al. 2010; Kraková et al., 2012), insects and other organisms.

In the course of their metabolism, most organisms produce different organic acids (oxalic, fumaric, succinic, citric, etc.) that reduce the pH of paper and other supports, facilitating the occurrence of new attacks by fungi and bacteria. Cellulolytic and keratinolytic strains are substrate specific, and can damage the chemical structure of paper and parchment, while non-specific strains may stand as opportunists in these materials. These strains are usually able to grow on almost all types of material if sufficient humidity is present, although they are not able to enzymatically degrade the substrate material to use it as a carbon source. Both groups are able to promote the degradation of the materials, but only the substrate specific are actually responsible for the decay of the material itself (Sterflinger and Pinzari, 2012).

Most of the mould species that are found colonizing library documents are Ascomycetes. The term 'mould' usually refers to the asexual state of the fungi, or Anamorph, in contrast with their sexual state, the Teleomorph (Sterflinger and Pinzari 2012). Fungi are the most common biodeteriorating agents, since they show a great tolerance to environmental conditions, and require lower water activity than bacteria, that play a more limited role in the biodeterioration of ancient documents. Bacteria that tolerate drying do exist, but they are mostly non-sporogenic species, whereas bacteria described as paper biodeteriorants are primarily sporogenic, common airborne species (Corte, 2003). Tiano (2002) refers to several works in which bacteria were isolated from papers stored in environments with high relative humidity (RH>85%).

The importance of water activity (A_w) was first addressed by Scott (1957), who related it with the total water of the substrata. In solid substrates, water consists of 'bound water', which is chemically held to the substrate by strong forces, and of 'free water', which is weakly bound. The latter is effortlessly available for microbial growth, but even so, the

degree to which it can be used depends on the water content and type of substrate - the total water content is not the sole determinant in this process (Magan, 2007). Scott (1957) suggested that water activity – biologically available water – was defined by the ratio between the vapour pressure of water in a substrate (P), and that of pure water (P_o) at the same temperature and pressure ($A_w=P/P_o$). Thus, the A_w of pure water is 1.00, whereas a substrate containing no free water has a smaller vapour pressure than does pure water, and A_w is consequently lower (Magan, 2007). Sterflinger and Pinzari (2012) state A_w to be the most important reason, and a key factor, for fungi to be predominant in museum and library environments when compared to bacteria or archaea.

The prevention of mould growth in museums – by climate control, regular cleaning and monitoring – as well as the development of appropriate treatment methods for contaminated objects are a challenge for restorers, curators and architects, given their potential for causing aesthetic damage, but also pathogen and allergen effects as well as mycotoxin production (Sterflinger, 2010). Fungi are some of the most harmful organisms associated to the biodeterioration of both organic and inorganic materials. A wide variety of enzymes is excreted by these biodeteriorating fungi: cellulases, glucanases, laccases, phenolases, keratinases and many more, making fungi the most important agents of deterioration in museums (Sterflinger and Pinzari, 2012). Among cellulolytic strains, several species of Deuteromycetes (*Alternaria* spp., *Aspergillus* spp., *Fusarium* spp., *Penicillium* spp., *Trichoderma* spp., *Ulocladium* spp.) and Ascomycetes (*Chaetomium* spp.) are frequently isolated from books and other documents (Corte et al., 2003; Capitelli and Sorlini, 2005; Mesquita et al., 2009). Some cellulolytic species of the genera *Aspergillus* and *Penicillium* are specially dangerous to paper because most of them are able to grow on substrates with low available moisture (~8%), usually provided in paper with RH levels of ~65% (Tiano, 2002). These microorganisms, which are able to tolerate and grow under water stress conditions, are frequently defined as xerophilic (from Greek, 'dry-loving'). Pitt (1975) defined a xerophile as a fungus that is able to grow in a given phase of its life cycle at $A_w=0.85$.

It is the metabolic versatility of fungi that promotes their efficiency in colonizing different kinds of substrata. Several works have been performed to study the presence of different microorganisms in various materials, such as: glass (Krumbein et al., 1991; Scharbereiter-

Gurtner *et al.*, 2001), stone (Wollenzien et al. 1995; Sterflinger and Krumbein, 1997; Warscheid et al, 2000), paper (Szczepanowska and Cavaliere, 2000; Gonzalez et al. 2002; Kraková, 2012), parchment (Kraková, 2012; Nunes et al., 2013a), paintings and painting canvas (Rizzo et al., 2002; López-Miras et al., 2012), clothes (Pangallo et al., 2013), wall paintings (Gurtner et al., 2000) and even cinematographic film (Abrusci et al., 2005; Vivar et al., 2012), among several others.

Monitoring the exposure to fungi in indoor environments is a complicated task, due to the unavailability of standard methods to evaluate the interactions between indoor microclimates, outdoor environment and microscopic fungi (Piecková, 2012). The ability of *Penicillium* spp. and *Aspergillus* spp., the so-called first colonizers, to grow at a relative humidity of 76~80% (requiring an A_w<0.8), explains their prevailing in normal, otherwise 'healthy' buildings. Secondary colonizers such as *Cladosporium* spp., *Alternaria* spp. and *Chaetomium* spp. (that grow at ~85% RH; A_w between 0.8 and 0.9) and tertiary colonizers (*Fusarium* spp., *Acremonium* spp. and yeasts; that grow at >90% RH) are able to deteriorate most building materials within optimal growth conditions (Piecková and Wilkins, 2004). In moderate to humid climates, fungal communities are often dominated by hyphomycetes such as *Alternaria*, *Cladosporium*, *Epicoccum*, *Aureobasidium* and *Phoma*, whereas in arid and semi-arid environments, fungal communities are often black yeasts and microcolonial fungi (Sterflinger, 2010).

Mycotoxins

Mycotoxins are a structurally diverse group of mostly small, low molecular weight compounds, that are secondary metabolites produced by certain filamentous fungi as a result of their growth; some mycotoxins can cause a toxic response in mammals, poultry and fish, whereas others display antitumor, cytotoxic and antimicrobial properties (Steyn, 1995). Mycotoxins can induce a variety of powerful biological effects, ranging from skin sensitivity to necrosis or extreme immunodeficiency (Sweeney, 1998), and their impact on health depends on factors such as the toxicity of the compound, the input amount, the body weight of the individual and the presence of synergistic effects with other mycotoxins (Steyn, 1995). For these reasons, fungal presence in libraries and storage rooms can pose a

threat to librarians, restorers and users, due to the allergen potential of mycotoxins, and the ability to cause systemic infections in humans (Sterflinger and Pinzari, 2012).

According to Zyska (1997), some of the fungal organisms involved in the deterioration of library materials may also be dangerous to humans, due to the production of mycotoxins. In fact, the author refers that from the 84 genera and 234 species compiled in his review article, regarding library and museum isolates, 44 of them (19%) could be a source of different diseases caused by mycotoxins. The genus *Penicillium* alone includes nearly 100 toxicogenic species, and a very broad range of mycotoxin classes, when compared to other genera. Some examples of common mycotoxins are: Aflatoxin B_1, naturally produced by *Aspergillus flavus*, and regarded by many as the most potent liver carcinogen known for a wide variety of animal species, including humans (Dragan and Pitot, 1994); Ochratoxin A (OTA), produced by *Aspergillus ochraceus* and related species, as well as some *Penicillium* species (e.g. *P. verrucosum*), is a potent nephrotoxin, teratogen and carcinogen (Krogh, 1997); Sterigmatocystin, produced essentially by *Aspergillus versicolor*, is acutely toxic and carcinogenic (Terao, 1983); and cyclopiazonic acid (CPA), produced mainly by *Aspergillus flavus*, but also by some *Penicillium* strains, can cause liver or gastrointestinal necrosis (Cole, 1986; Pitt, 1997). Chronic low-level exposure to a mixture of fungal toxins and other indoor stress causing agents may have synergistic effects, and eventually lead to neuroendocrine-immune changes (Piecková, 2012).

Mycotoxin production is influenced by various parameters such as nutrient availability, water activity (A_w) and temperature. For example, aflatoxins can be produced in the temperature range of 12-40ºC (Koehler et al. 1985), and pH range of 3.5-8.0 with an optimal pH near 6.0 (Buchanan and Ayres, 1976). The minimum A_w needed for aflatoxin production in *A. flavus* and *A. parasiticus* (the main producers of these mycotoxin) is of 0.82 and 0.87, respectively (ICMSF, 1996), which is higher than the A_w value required for their growth (Sweeney, 1998). The minimum water activity required for the production of some of the most relevant mycotoxins starts in values as low as 0.80, which at 25ºC corresponds to an RH of approximately 80%, but can reach A_w values of up to 0.99 (Patulin production by *Aspergillus clavatus*; see Table 1). In environmentally controlled libraries, the microniches that exist within small gaps and spaces made available in books and paper, and the inherent water condensation in those places, facilitates the growth of deteriorating

organisms present in samples, allowing them to produce mycotoxins, even with low RH values.

Table 1 – Mycotoxin related species and minimum water activity required for growth and toxin production

Mycotoxin	***Species***	***Min A_w Growth***	***Min A_w Toxin***	***Reference***
Aflatoxin	*Aspergillus flavus*	0.80	0.82	Kozakiewicz and Smith, 1994
Aflatoxin	*Aspergillus parasiticus*	0.84	0.87	Kozakiewicz and Smith, 1994
Ochratoxin A	*Aspergillus ochraceus*	0.77	0.80	Pitt and Christian,, 1968; Adebajo et al. 1994
Ochratoxin A	*Aspergillus ochraceus*	0.77	0.85	Kozakiewicz and Smith, 1994
Sterigmatocystin	*Aspergillus versicolor*	0.76	0.80	ICMSF, 1996; Patterson and Damoglou, 2008
Ochratoxin A	*Penicillium verrucosum*	0.80	0.86	Pitt and Hocking, 1997; ICMSF, 1996
Patulin	*Penicillium expansum*	0.82	0.95	Mortimer et al., 1985
Patulin	*Aspergillus clavatus*	0.88	0.99	Kozakiewicz and Smith, 1994
Fumusin B1	*Fusarium moniliforme*	0.87	0.92	Marin et al., 1995

There are many different ways in which fungal organisms may harm us besides mycotoxin production. According to Nielsen (2003), there are other agents released by moulds that can be implicated in human health issues; such as proteins that cause different allergies, volatile organic compounds, or structural elements such as β-1,3-glucans that may potentially trigger inflammatory reactions. Since we do not know every species present in these environments, nor their potential to exert a toxic effect, the control of fungal development and spreading should be a priority in libraries, museums and other repositories, in order to stop the damage from biodeterioration processes.

Damage and damage assessment

The biodeterioration of paper and parchment in ancient books and documents is a cause of great concern for libraries, archives and collections all over the world. One of the main goals of scientists, who deal with the problems of “solving” biodeterioration, is to determine what are the causes of these phenomena; in this scenario, they are confronted with the relation between non-living materials (substrate of the deterioration process), and the living organisms that thrive in those materials. Libraries and archives, from the microbiological perspective, represent wide sources of organic material that is available for

colonization by different decomposers (biodeteriogens), and in human related environments, their development and subsistence depends on few and scarcely predictable factors: the nature of the propagules that reach the material's surface; the microenvironment to which they are exposed (temperature, relative humidity and light); the water activity of the substrate; and casual events that someway aid on the establishment and spreading of these biodeteriogens (Pinzari et al., 2011).

If one considers paper or parchment stored in a closed environment, the potential for its colonization and degradation depends more on the species identity and composition, than on species diversity, since essentially cellulolytic or collagenolytic species, respectively, can exploit the bulk of the substrate (Michaelsen et al., 2010). However, this identification procedure is somewhat complex, since many of the organisms actually found in the degraded objects are not culturable, are no longer viable, or may not even be related with the degradation process itself (Pinzari et al., 2011). On the other hand, if one takes an approach such as DGGE analysis, which provides information on the whole species community present in the analyzed substrate, there are also the risks of finding organisms that are only present by chance, or no longer viable nor potentially damaging, so there is no sole key strategy in these sort of assessments. One should consider, however, that even if the isolated organisms are no longer viable or actively degrading the documents, they might have done so in the past, potentially facilitating further contamination from other organisms, or might do so in the future. The culturability of the retrieved isolates is, in fact, an issue, since many potentially harmful organisms are not easily grown in culture media, or only do so in very specific substrates, either because of their specific metabolic needs, or because of inter-species competition; However, in contrast with bacteria, for which it is generally accepted that less than 1% of the present *taxa* can be isolated by culture isolation methods, the recovery rate for fungal organisms is presumed to be more than 70%, making culture dependent approaches still extremely valuable in mycology (Sterflinger, 2010). These analyses are often complemented with *in situ* microscopical observations of the contaminant organisms (while on the actual substrate in study) and with the assessment of their cellulolytic, proteolytic and other enzymatic potentials, in order to evaluate their potential as substrate degraders.

Many different organisms are related with the biodeterioration phenomenon, and it is essential to know the interaction processes between the organisms, substrate and

environment; fungi as well as bacteria, though with different physiologies, can behave in very similar ways regarding the biodeterioration processes, if not even synergistically (Pinzari et al. 2011). Since the fungal cell walls are composed of chitin (nitrogen containing polymer), cellulosic materials get enriched with nitrogen after fungal colonization becoming more susceptible for microorganism contamination because of the higher nitrogen-carbon ratio, leading to a succession of colonizing *taxa* on these substrates (Michaelsen et al., 2010). The action of these organisms by means of their normal metabolic activities leads to the release of organic substances - chelating compounds, organic acids, inorganic acids and pigments - while the mechanical pressure exerted by the growing structures and shrinking/swelling phenomena induces different kinds of damage to the support materials: mechanical (abrasion and mechanical stress) and chemical (solubilisation and new-reaction products). The growth process and development of fungal organisms has a direct consequence on the conservation of cultural assets: by growing on these materials, hyphae penetrate deeply leading to physical detachment, material loss, acid corrosion, enzymatic degradation and mechanical attack, on both contents and substrates (Sterflinger, 2010; Sterflinger and Pinzari, 2012).

All these processes promote the degradation of organic components that consequently affects the aesthetics of the documents or art pieces, either by pigmentation or by various other chemical alterations to the substrate. For example, dark spots and stains are usually attributed to the presence of Dematiaceous fungi - their mycelium contains water, insoluble organic products and melanin pigments; while brown spots might be caused by the oxidation of cellular debris that remains in materials after cyanobacteria and algae colonization (Tiano, 2002). The physiological state of the organism and the maturity of the mycelia and fungal structures can have different effects on the substrate – for instance, senescent hyphae may release chemicals that young fungal mycelia do not produce, therefore causing diverse types of degradation that are somewhat age-related (Pinzari et al., 2011).

Foxing

In general, fungi cause changes in the aesthetics of paper and parchment by producing stains, in a phenomenon usually referred as foxing. These spots appear in different shapes and colors, usually due to the presence and nature of pigments of the mycelia, but also to the release of metabolites by the infecting fungi. The color of these pigments can depend on the conditions of fungal growth but also on certain properties of the support, such as the pH, presence of starch, metals, salts, etc. Besides that, sometimes the foxed substrate shows an increased ability to absorb water when compared to the surrounding area, probably because of the relaxation of the cellulose fibers as a consequence of the fungal activity (Corte, 2003). Stains might pass through successive pages, and on the long-term, they will affect the legibility of the document; since the number of stained areas increases with time, this will frequently lead to utter degradation (Rakotonirainy et al., 2007).

Arai (2000), has done a vast research on the study of foxing. In his concept the process of foxing occurs as follows: xerophilic fungi produce conidia and/or ascospores that are carried and dispersed through the air, eventually attaching to all sorts of materials. If the adequate conditions for fungal growth are present, they will germinate and grow their hyphae, forming colonies around dust particles. In many cases, the fungal organism will exploit those dust particles and the reserves from the spores, what helps to explain the small size of some foxing stains, and why the growth stops. In case the fungal organism is able to produce cellulases, it is likely to feed on the underlying cellulose fibers, unless there are inhibitors associated with the matrix (Corte, 2003). These fungi, metabolize mainly malic acid, cello-oligosaccharides and gamma-aminobutyric acid in the colonies, that will react together through the Maillard reaction over the materials at A_w 0.80 and temperature of 20-35ºC, resulting in melanoidin production and the consequent formation of foxing stains. Gallo (1992), however, reported a temperature of 10-20ºC and relative humidity of 40-60%.

Arai (2000) also concluded in his studies that foxing is not exclusive to paper, since cellulose in paper fibers is not always an essential nutrient for fungal growth Foxing can be formed on other non-cellulosic materials (e.g. silk), as long as the adequate environment conditions are met. This is usually done by absolute tonophilic (xerophilic) fungi, with the most frequent being common, airborne, cosmopolitan conidial fungi, mainly belonging to the genera *Aspergillus* and *Penicillium*, and Fungi imperfecti (Deuteromycetes). Press (2001), in

another work, found a relation between the areas where foxing is formed and their iron content - lower iron concentrations promote the occurrence of foxing, as opposed to what was previously accepted.

It is worth mentioning that, regarding foxing, there are two complementary theories for its occurrence (Florian and Manning, 2000; Corte et al. 2003):

- *The biotic theory* – In which the stains are the result of the activity of microorganisms. These stains are usually circular in shape and, even when uncolored, they display a natural fluorescence that is yellow under UV light. This is likely due to the presence of aromatic aminoacids in the proteins of fungal structures, lipoproteins, β-glucans, or to the release of melanoidins (Meynell, 1978; Arai, 2000; Florian and Manning 2000; Press 2001).
- *The abiotic theory* – In which the stains are the result of chemical phenomena such as oxidizing and/or heavy metal deposits. The stains are usually irregular, according to the shape of the contaminating substance (metals fragments or crystals of chemicals incorporated in paper during its manufacturing), and display a blue fluorescence under UV light (Cain and Miller, 1982; Florian and Manning 2000).

Sampling and analysis

A basic principle in restoration is to reduce damage to the least possible amount when processing the object being restored, since it usually handles antique and culturally invaluable items. Therefore, when collecting samples for analysis (except when using fragments that are already detached), one should be wary on the methods used. Regarding library materials, and according to Pinzari et al. (2011) the following methods are recommended:

- *Swab sampling* – Sterile cotton swabs are wiped across spots showing visible signs of damage; they are transferred to the lab in sterile tubes, and inoculated in culture media for the present microorganisms to grow, allowing their identification; Alternatively, the surface of the documents can be lightly scraped with a scalpel, to remove the cellular structures that are present, and place them in sterile tubes for posterior analysis.

- *Adhesive tape sampling* – A transparent adhesive tape is gently pressed against the damaged spots and transferred to a sterile bag, in order to collect cells, spores, mycelia and fruiting structures. This tape can be placed on a glass slide for direct microscope observation (using a stain such as Lactophenol Cotton Blue), or used for DNA extraction.

- *Nitrocellulose membrane* – Small sterile membranes (2-6cm in diameter) are gently pressed over the spots, and then immediately transferred to petri plates with culture medium, or used in other tests.

The direct observation of the contaminated surface, or the use of the abovementioned techniques may provide a valuable insight on the range of contaminating organisms that are present in the sample; apart from being important to know the diversity of organisms, their identification is important in developing adequate control methods.

To complement these strategies, techniques based on DNA analysis of communities affecting heritage items have been recently developed (Piñar et al., 2001; Schabereiter-Gurtner et al., 2001; González and Saiz-Jiménez, 2005; Michaelsen et al., 2006; Michaelsen et al., 2010). These techniques provide researchers with additional and complementary tools in the identification of individual and/or communities of infecting organisms, sometimes without the need for cultivation. These techniques are frequently based on ribosomal sequences or ITS regions, nested in the rDNA repeat, and are often used as phylogenetic markers in molecular level investigations; they contain both variable and highly conserved regions that allow the discrimination between organisms at different phylogenetic levels (White et al., 1990; Pinzari et al., 2011). The retrieved genetic sequences of obtained isolates can be crosschecked with databases such as NCBI's Genbank, providing a molecular-based species identification of otherwise unknown organisms. Different kinds of genetic analyses can be performed in a given sample; in culture-dependent approaches, DNA is extracted from isolates in axenic cultures, originally retrieved from contaminated items, to be amplified using PCR and sequenced to obtain species-specific sequences, and hopefully an insight on their identification (Michaelsen et al., 2006). This presupposes that the organisms or their spores are viable, and that we are able to provide the required medium and conditions for their growth; colony growing also allows a morphological confirmation, whenever needed. Most fungal organisms are likely

able to grow *in vitro*, but because of inter-species competition some species might be lost in the process.

Culture-independent approaches, such as the extraction of all DNA present in a sample, allows obtaining genetic material from several different organisms simultaneously, which can afterwards be amplified and separated using processes such as DGGE. After sequencing the different bands, this method provides insight on the different species that are present in the sample. The main disadvantages are that it does not allow a morphological confirmation, and also that there is no indication of the viability of the retrieved organisms, nor their degradative potential. It does, however, provide good information on the diversity of organisms that have been present in the sample, even if they were only formerly active. In a way, both these methods are complementary in information, and when interpreting results, one should assess if the obtained species actually have a degradative potential (i.e. knowledge on their enzymatic ability), if they can be (or might have been) harmful to the substrate, or if they are just present in samples by chance, either by indiscriminate contamination or dust deposition.

An analysis of the air microbiota or other vectors of fungal spreading (i.e. insects), will also provide information on whether these species are regular and ubiquitous airborne species that ended up deposited in documents, or if there is no connection between them to this regard. In the end, the information obtained with the different techniques (molecular analysis, micro and macro morphology, biochemical analyses, metabolic activity, etc.) should be integrated to better understand the role of the identified contaminant species in the actual degradation potential they might exert on the different heritage items and materials.

Support materials

Paper

Paper is primarily composed of cellulose – the major source of energy for microorganisms – but may also include other substances, essentially of organic nature, related to the origin of the materials used in its manufacturing process, from which lignin, hemicelluloses, waxes, tannins and proteins are examples. During the middle ages, paper was of higher quality, in what regards to its constituents – it was made essentially from cotton rags, with a high

cellulose amount, and few impurities. The use of rags for the production of paper occurred for over 1000 years (8th to 19th century), being gradually replaced with wood pulp as raw material, from the end of the 17th century (Mendes, 2008). The latter, derived from wood pulp, contains a great amount of polymers and non-fibrous materials other than cellulose, as well as impurities (Tiano, 2002). In fact, modern papers are more vulnerable to microorganism attack than older papers. Most of the filamentous fungi that are associated with paper damage can, as previously stated, dissolve cellulose fibres by using cellulolytic enzymes, but they can also colour/discolour paper, and degrade glues and inks; and most of these fungi comes from the dust and dust inhabitants (Sterflinger and Pinzari, 2012). Mold can start to develop in a very short period of time, and once it has bloomed on an item, it will reappear whenever favourable environmental conditions (warm temperature and high humidity) are present; a mold is very difficult to eradicate from a paper or book, unless it is killed (Sinco, 2000).

Chapter 1 of this thesis, "**Fungal diversity in ancient documents. A case-study on the Archive of the University of Coimbra"** describes the very first step in this whole study, and it was the first analysis of the mycoflora present in documents from a Portuguese heritage repository – in this case, the Archive of the University of Coimbra. Little was known about the population of biodeteriorating species present in documents and books within the Archive. In order to develop adequate control methods it is imperative, at a first step, to perform the analysis of the fungal diversity present in documents. For this purpose, thirty documents, made of different materials (parchment, laid-paper and wood-pulp paper) were analysed for the presence of contaminating fungal organisms. The identification of the infecting fungal *taxa* was performed based on ribossomal DNA *loci* amplification and sequencing, as well as morphological identification, using macro and microscopical traits. In general, and considering the number of samples, a high fungal diversity was found in all support materials, and different species were isolated and identified. The most frequent genera were *Cladosporium, Penicillium* and *Aspergillus*. Less frequent genera, such as *Alternaria, Botrytis, Chaetomium, Chromelosporium, Epicoccum, Phlebiopsys* and *Toxicocladosporium* were also present.

Parchment

Parchment as a writing support was originally produced from sheep, cow or goatskin, which underwent a process of liming, by drying at normal temperatures while under tension, in a tension frame. Because it is limed and not tanned (like leather), it is more susceptible and reactive to changes in relative humidity. Parchment was quite popular, because it had the advantages of being stronger, more durable and flexible, and overall more resistant than other supports, and allowed the scraping during its manufacturing process, as well as the correction of errors while writing; it could be cut to fit different sizes, and it could be written in both faces. The main limitation was the high cost, and limited supply, as it involved the raising and upkeep of animals, their slaughter and a very labour-intensive and tarry process.

Structurally and chemically, it is mainly composed of collagen, keratin, elastin, and residual amounts of albumin and globulin. Collagen is one of the most resistant proteins in what concerns to microbiological attack; it is degradable only by specific enzymes – collagenases – from filamentous bacteria and proteolytic fungi. Proteinases start the breakdown process, which release soluble peptides that are degraded into their amino acid components by hyphal peptidases (Mandrioli et al., 2003). However, the mechanic disruption of skin during the manufacturing process depolymerizes some of the collagen chains allowing nonspecific proteolytic enzymes (produced by several bacteria and fungi) to degrade this already denatured collagen.

The vulnerability of parchment to the effects of biodeterioration depends on several factors: the method of production that, as stated, might start the mechanical disruption of collagen; the raw material itself, since other components (proteins, lipids, carbohydrates and minerals) can also be involved in the decay process; external environmental factors, such rapid temperature or relative humidity increases, pH changes and UV radiation; and finally, the action of organisms. Under aerobic conditions some bacteria (e.g. *Bacillus* spp., *Pseudomonas* spp., *Sarcina* spp.); and fungi (*Cladosporium* spp., *Fusarium* spp., *Ophiostoma* spp., *Scopulariopsis* spp., *Aspergillus* spp., *Penicillium* spp.) can attack collagen in parchment, leading to the loss of its original properties, making it hard and brittle, and sometimes physically deformed.

In Chapter 2 of this thesis **"Assessing the fungal diversity in an ancient parchment - the pathogenic potential of existing mycoflora as a threat to the health of users",** an 18th century Papal Bull made of parchment was analysed for the presence of contaminating moulds, and to confirm the presence of potentially pathogenic species. Different growth media were used to maximize the number of obtained isolates. Molecular identification was obtained with the amplification and sequencing of ribossomal DNA *loci*, and confirmed with morphological identification, using macro and microscopical traits. In total, 18 different species, belonging to 10 genera, were isolated and identified. Apart from the degradation of library materials, most of these organisms can also cause adverse human health effects in conservators, restorers and users. In fact, some of the species found in the tested parchment are producers of toxins that are dangerous to humans.

Biodeterioration Control

In order to control the biodeterioration processes, adequate and non-invasive methods should be used. Regarding the nature of the employed technique, we can classify the treatment methods as: mechanical, physical, biological or chemical. All described as 'mechanical' share the common process of physically displacing the biodeteriogens (by hand, or with the use of a scalpel, etc.). These were mostly used in the past, and usually do not produce lasting results, especially because the removal of the organisms does not always prevent the effects of their prior presence. It can, in some cases, assist in spreading the contaminating propagules (e.g. fungal conidia), or even cause mechanical damage to the substrate. However, it had the advantage of not adding new substances that could result in unexpected degradation effects on the substrate itself.

Throughout the years, different techniques to control microorganism contamination have been developed and put to use. One of the most important is the use of the ethylene oxide, and its application to cultural heritage assets dates back to the beginning of the 20th century. When pure, ethylene oxide has a boiling point of 10.73ºC (at atmospheric pressure) so it can be used at room temperature; it does not require activation energy; and it possesses a high reactivity and diffusive power, allowing the inactivation of most infecting organisms. Briefly, ethylene oxide is injected into containers, infiltrating the objects placed inside, and it is later removed by changing the atmosphere back to standard

air, purging the noxious gas out of the container. Ethylene oxide adds alkyl groups to DNA, RNA and proteins, interfering with the normal cellular metabolism and reproduction (Rutala and Weber, 1999), and it can also have carcinogenic effects in humans (Bolt, 1996) - these facts led many countries to ban its application. One of the main problems of ethylene oxide application is that, no matter how many times the air is purged in the process, the ethylene oxide that remains will eventually free itself from the objects, endangering staff and users (Sinco, 2000).

Another technique of interest in the control of biodeteriogens is freeze-drying, which consists of frozen water being removed from objects by sublimation, bypassing the liquid state, therefore allowing its removal without the side-effects of water's evaporative forces, that can cause dimensional changes. This dehydration procedure also has the useful effects of killing hydrated conidia and spores, and preventing the growth of fungi and bacteria. The main disadvantages are that thawed water might not be completely removed from the objects, becoming available to organisms; and the formation of ice that might increase the porosity and thickness of organic materials, making them more hygroscopic, and therefore more susceptible to future contaminations (Michaelsen et al, 2012).

The third alternative, and of bigger interest for this thesis, is the application of gamma radiation to contaminated materials. There are two main types of radiation to which organisms are usually exposed: non-ionizing radiation - solar radiation and ultraviolet light; and ionizing radiation (e.g. gamma radiation) - from natural (e.g. decay of radioisotopes in underlying rock strata) and manmade sources (Magan, 2007; Hooley and Clipson, 1995).

Non-ionizing radiation refers to any type of electromagnetic radiation that does not carry enough energy per quantum to ionize atoms or molecules – that is, to completely remove an electron from an atom or molecule. Examples of this are radio waves, visible light and microwaves. This type of radiation can damage the DNA of exposed organisms to some extent, however, some of these organisms are able to repair radiation-induced damage relatively quickly, maintaining essential metabolic functions, either by the pigmentation of mycelium and spores, or by having developed rapid mechanisms for DNA-Repair (Battista, 2000, Magan, 2007). Ultraviolet radiation can distort the shape of DNA molecules by forcing adjacent chemical bases on the same strand to bond with each other instead of

pairing with the corresponding base on the opposing strand (Hooley and Clipson, 1995). It has germicidal properties when between 200-300nm, and a maximum effect when between 230-275nm, and is generally more effective at low relative humidity levels (RH<50%). The sensitivity of organisms to this type of radiation varies according to their current development stage (being greater during the logarithmic growth phase), and nature of the substrate. Ultraviolet radiation has poor penetration power and can produce undesired effects on some materials, such as cellulose, proteins, pigments and dyes (Tiano, 2002).

Ionizing radiation, on the other hand, has very high energy; it is capable of removing electrons from molecules, breaking chemical bonds, or even breaking the atom nucleus. Some examples of electromagnetic radiation are X-rays and gamma radiation. Industrial sources of ionizing radiation often use Cobalt-60, a radio-isotope that emits gamma photons with a mean energy of 1.23 MeV (million electron volts); and electron beam machines, that emit accelerated electrons up to 10 MeV, used directly or transformed in X-Rays (Adamo, 2004).

Gamma radiation

Frequently used as a sterilization procedure, gamma rays are highly penetrative electromagnetic waves that are able to pass through materials without leaving any residue. In the decontamination treatment of heritage, low radiation energy is used, which does not induce radioactivity; repeated treatments, however, are not recommended since the effects of radiation are cumulative (Tiano, 2002; Adamo, 2004), however, this does not mean that materials will become radioactive. The use of gamma radiation in conservation science dates back to the 1960´s, through the work of Belyakova (1960), who analysed the radio resistance of different moulds related to heritage collections. This method is a good alternative when compared with other disinfection procedures, since it allows the safe handling of items right after the irradiation treatment; furthermore, the uniformity of the applied dose during irradiation allows a controlled treatment procedure, and the treatment of large amounts of objects simultaneously, at a low cost (Negut et al., 2012). Therefore, gamma radiation has widely been used as a successful sterilization method in many different areas, from medical items, to food, herbs, spices and medicinal plants, paper,

wood and others (Adamo et al, 1998; Pointing et al, 1998; Adamo et al., 2001; Adamo et al. 2004; Aquino, 2011).

The 'absorbed dose' is measured in Gray (Gy), and is defined as the amount of energy that is absorbed per mass unit of irradiated matter. The dose rate is the rate of change of absorbed dose with time, measured in Gray per second (Gy/s). Irradiating with the same dose, but different dose rates may lead to different results. The uniformity in radiation dose is very important as it allows the reproducibility between treatments, and besides that, it ensures us that the doses administrated during treatments correspond to those required to achieve a set of pre-desired chemical, physical or biological effects in irradiate materials (Mendes et al., 2007). When setting the irradiation procedure for a given treatment, there are usually two reference doses that are taken into consideration: a 'minimum dose' (D_{min}), that corresponds to the dose that allows the intended effect to be obtained; and a 'maximum dose' (D_{max}), above which undesirable changes may occur to the treated material (Adamo, 2004). Different authors suggest the dose of 10kGy as the threshold below which mechanical properties of most heritage materials (wood, paper, parchment, paintings, etc.) are not significantly affected, but regarding paper alone, doses over 20 kGy have been used without significant changes in the physical integrity of the support (Adamo et al., 1998; Adamo et al., 2001, Nunes et al., 2012).

Adamo (2004) refers that in cultural heritage restoration procedures, gamma radiation is the most suitable method when aiming for the most uniform bulk irradiation of any single object. It is relatively easy to perform, even with large dimension and irregularly shaped objects, and it is not expensive when compared to other traditional treatments. Although this treatment method presents all the abovementioned advantages, its application in the contamination of cultural heritage objects is still an exotic topic, as conservers are often reluctant in using it, because of the common negative connotation of 'radiation', but also because its effects on materials is still being assessed by many researchers (Negut et al., 2012).

"Knowledge of a phenomenon is the best remedy against unfortunate misconceptions of a topic" (Adamo et al., 2004)

The time it takes for a given substrate to be re-contaminated by biodeteriogens depends on different aspects, such as the considered preventive measures, or the susceptibility of the material itself to contamination. It can take several years, if objects are kept in adequate conditions suggested by the principles of conservation (16-20ºC and 40-60% RH (Corte et al. 2003)) and safe confined spaces, preventing cross-contamination; but if these conditions are not met, the treated items are also subjected to re-colonization. After a decontamination treatment, objects are clean from most living forms, so there is no competition to delay the establishment of new organisms on the materials.

Effects on organisms

Gamma radiation, as a high energy electromagnetic radiation, causes direct and mostly irreversible damage to cell DNA through ionization effects, and production of reactive oxygen species (ROS) that denature DNA by producing double-strand breaks (the most severe form of DNA damage in irradiated cells); it also induces mutations, destroys proteins (by exerting oxidative damage, it inactivates enzymes, including those necessary for DNA repair) and eventually kills the cells. If classic radiation toxicity models identify DNA damage as the most relevant lesion in cells, the level of oxidative damage on proteins caused during irradiation is now a significant research topic as well; if particular DNA repair enzymes are impaired, the cell may lose some of its capability to repair the damage from irradiation (Hooley and Clipson, 1995; Daly, 2012); DNA can be repaired, but for that to happen, the repair proteins must be functional. Double strand breaks are considered the most lethal effects, since a pair of double strand breaks can lead to the deletion of a segment of a cell´s genome, and thus, to the loss of potentially essential genetic information (Battista, 2000). Furthermore, since radiation damages cellular molecules indiscriminately, and since genes exist in far lower abundance than their products, DNA assumes the role of the most important target for irradiation (Daly, 2012). Also, the indirect effect of the radiolysis of cellular and extra-cellular water molecules leads to the formation of active oxygen species, free radicals, peroxides and ions, that react with biological molecules, such as single and double strand DNA, leading to their denaturation.

Its application in conservation procedures is capable of a fast devitalisation of all infecting microorganisms (McNamara et al., 2003; Maggaudda, 2004). Michaelsen et al. (2012)

applied an irradiation dose of 5kGy to different paper samples, finding it able to cause DNA fragmentation after DGGE analysis; after one year, however, the previously irradiated strains no longer displayed DNA fragmentation, suggesting that the surviving fungal population had recovered from the genetic damage, since only intact DNA was detected. Nevertheless, the same authors concluded that gamma rays could be used to treat large amounts of paper simultaneously, without a subsequent chemical hazard (as opposed to the application of ethylene oxide), considering it adequate as decontamination treatment that allows biodeteriorating organisms to be reduced to a controllable or blank level. Adamo (2003) states that in general, dose ranges from 3 to 10 kGy are sufficient to clean materials from microscopic fungi, while doses of only 0.2 to 0.5 kGy are necessary to eradicate insect populations. The same author, in a different work (Adamo, 2001) concluded that doses of up to 10 kGy do not cause evident changes in irradiated paper of different types, and that higher doses are needed in order for these to occur.

"The book conservation world has been wary of treating infested books and documents with radiation, but sometimes nothing works better" (Sinco, 2000)

The susceptibility of microorganisms and their spores to gamma radiation is well established, and the lethal radiation dose varies between organisms. In general, the vegetative forms of bacteria are more sensitive to radiation than fungi, partly because of the natural radio-protective agents present in the mycelia. Numerous metabolites (such as alcohols, acids, pigments enzymes), intercellular components (pigments, amino-acids, proteins, fatty acids), and lipid fractions contained on the cell walls of some fungi, may provide radio-resistance (Aziz, 1997). Melanin is a good example of that; it is a black pigment (polymer) that protects many organisms from UV radiation as well as ionizing radiation; dematiaceous fungi are a group of fungal organisms that are known to have above-average resistance due to their strong pigmentation. Saleh, (1988) described a higher resistance of dematiaceous fungi (*Alternaria alternata*, *Cladosporium cladosporioides*, and others) to gamma radiation.

In organisms in general, the fate of irradiated cells rests not on the number of double strand breaks caused during irradiation, but rather on their capacity to accurately mend them; in the end, the decay of cellular robustness – that leads to cell death – is a direct

result of the progressive accumulation of oxidative damage to the proteome, diminishing their catalytic activities and interactions; cellular recovery is essentially limited by the oxidative sensitivity as well as the rate of turnover for a given set of enzymes needed for DNA maintenance, repair and cellular growth on a given environment (Hooley and Clipson, 1995; Daly, 2012). In order to survive irradiation exposure an organism should avoid the consequences of DNA double strand breaks, either by passively preventing their occurrence, or by repairing them in a manner that prevents the loss of information (Battista, 2000). Fungi usually do not rely on a single repair system; different DNA repair enzymes, with overlapping functions, increase the probability of repair in every individual lesion (Hooley and Clipson, 1995). Resistant bacteria have evolved efficient antioxidant chemical defences that protect proteins and the functions they catalyse (Daly, 2012). Also, certain chemical compounds (e.g. lactic acid, acetic acid and alcohol) may act as scavengers, and therefore are able to protect the irradiated organisms, as they react with the available free radicals, which result from water radiolysis, and thus, reduce the damage from that indirect effect. The induction by gamma radiation of the production of certain enzymes or chemical substances can also lead to the recovery of radiation damage – the variation in radiation resistance among fungi is extremely variable, from supersensitive to highly resistant (Nahed, 1999). Curiously, gamma radiation can, in some cases, promote the growth and germination of fungal spores (Salama, 1977; Geweely and Nawar, 2006).

Regarding radiation and DNA double-strand breaks, the most tolerant organism known to man is in fact a non-spore forming bacteria – *Deinococcus radiodurans* – which is known to withstand high levels of gamma radiation, by having developed extraordinarily efficient antioxidant chemical defences, which specifically protect proteins and the functions they catalyse, as well as presenting a great capacity for scavenging ROS (Daly, 2012). Its DNA repair ability is quite surprising as well: a dose of 5 kGy fragments its 3.2 million base pair genome introducing nearly 200 DNA double-strand breaks, however, this organism is able to repair this damage within a 6-hour period, maintaining the linear continuity of its genome. Also, it has as many as ten genome copies per cell, and since the distribution of the strand breaks is expected to be random, the probability that any specific site will be damaged in all copies simultaneously is very small (Battista, 2000).

Another indirect effect of gamma radiation is the decrease in water activity within the substrate, and therefore it assists in preventing further infection by fungi and bacteria, by limiting the water available to organisms and toxin production. Oppositely, water present in the mycelia can also work as a natural radio protector, shielding the cellular components from radiation (Geweely and Nawar, 2006). On another perspective, gamma radiation is able to destroy harmful molecules such as mycotoxins: Aflatoxin B1 and Aflatoxin B2, for instance, are destroyed by radiation and the effect of free radicals formed from water radiolysis. An increase in A_w in the substrate will empower the effect of gamma radiation in the destruction of aflatoxins (Aquino et al. 2005). Tests have also been performed using Alternaria alternata, with similar results (Braghini et al. 2009). Fumunisins (mycotoxins produced by some Fusarium strains), however, are very stable molecules and have a greater resistance to gamma radiation, requiring a higher dose to be neutralized (Aquino, 2011).

A curious fact is that, in some cases, the size of the inoculum correlates inversely with the amount of mycotoxin production (Karunaratne and Bullerman, 1990). According to Aquino (2011) a medium inoculated with a larger number of aflatoxigenic fungal spores will produce less aflatoxin than if inoculated with a smaller number of spores: apparently, toxin production is suppressed above a determined spore concentration. Thus, if the applied radiation dose is not enough to destroy all mycotoxins, the consequent reduction of inoculum may lead to an increase in the production of mycotoxins.

Effects on materials

Several studies in this field have demonstrated that the damage to materials and to their mechanical and physical properties is not significant at the doses that are usually applied in the decontamination treatment of heritage items (Adamo et al. 1998; Adamo et al. 2001; Adamo et al. 2004; Rizzo et al. 2002; Nunes et al. 2013a). During irradiation, free radicals can be released in the cellulose matrix and quickly react with oxygen, breaking the cellulose molecules, and degrading the paper (Sinco, 2000).

Adamo (1998) found that doses of 10 kGy did not have negative effects on the physical and mechanical properties of paper, even after a 12-day artificial ageing process. A reduction in the polymerization state of cellulose fibres can occur, especially while using lower dose

rates, as longer irradiation periods will allow more time for the formed reactive species to interact with the cellulose polymers by chemical modification. This depolymerisation, however, didn´t significantly change the mechanical properties of paper in these works, and although proportional to the absorbed radiation dose, it was negligible at the gamma radiation doses needed to prevent entomological and microbiological growth (Adamo et al., 1998; Adamo et al., 2004; Gonzalez et al., 2002). Gonzalez (2002) also states that no changes in colour were observed in any of the tested samples after irradiation and/or artificial ageing treatment; furthermore, optical microscopy and SEM detected no difference in fibre structure between control and irradiated samples.

"A slightly yellowed or embrittled document can be tolerated when the alternative is no document at all." (Sinco, 2000)

Regarding inks and painting pigments, Rocchetti (2002) and Adamo (2004) have described the resistance of printing inks on irradiated paper in doses ranging from 3-10 kGy, but also in an extreme testing at 100 kGy. Rizzo (2002) tested doses up to 25 kGy on different colour pigments used in paintings and also demonstrated by colorimetric analysis that no significant changes happened. In the same work, no significant modifications were observed in the physical stability of the polymers and pigments after the irradiation procedure: it is worth mentioning that the author obtained good disinfection results using an irradiation dose of just 6 kGy. Negut et al. (2012), in his analysis of radiation effects on 22 pigments, stated that gamma rays can induce defects, which he referred to as 'colour centres', that are likely to modify the original painting colours by altering the embedded pigments; the author found that colour changes induced by radiation in nearly all pigments disappeared after 30-40 days. All these tests support safety of the application of gamma radiation as a sterilization procedure of the referred materials, using doses that are adequate for most microorganisms and insects, while maintaining the integrity of the substrates, especially considering the cost-benefit analysis, as the benefits are usually greater than the costs (Magaudda, 2004). It is clear, however, that when very high doses are used, gamma radiation can, and will deteriorate paper materials by severely depolymerizing cellulose, and colour changes may eventually occur (Sinco, 2000).

Gamma radiation effect assessment

After the application of any biocide agent, it is important to assess the effects of that treatment on the target organisms. Several studies address this matter, using different techniques and experimental contexts: focusing gamma radiation and fungi in particular, Geweelly and Nawar (2006) tested the effects of gamma radiation on total protein, soluble sugars and lipids, where higher doses (near 3 kGy) inhibited most metabolic processes; da Silva et al. (2006) analyzed the effects on fungal growth by measuring the diameter of growing colonies, and concluded that there is a dose-related effect on growth, and also that doses of 16 kGy were efficient in total sterilization (including *Cladosporium* spp. and *Aspergillus niger*), whereas 5 kGy and 10 kGy were sufficient to inactivate *Penicillium* spp. and *Fusarium* spp. respectively; more recently, Michaelsen et al. (2012) monitored the effects of different conservation treatments on paper-infecting fungi - in regard to gamma radiation (one of the addressed treatments), the DNA fragmentation, the long-term DNA recovery and metabolic activity (using RNA as an indicator) were used to assess the radiation effects.

It is important to highlight that all the abovementioned tests were able to obtain the inactivation of biodeteriogens using doses below 16 kGy, a dose which is known to be safe to paper and parchment documents (Adamo et al., 1998; Nunes et al., 2012).

Chapter 3 of this thesis "**Assessing gamma radiation effects in a *Cladosporium cladosporioides* strain isolated from an ancient document: CFU-count vs. Growth parameters",** describes the assessment of gamma radiation effects on the growth, biomass and survivability of irradiated spores (resistance forms) of the dematiaceous fungi *Cladosporium cladosporioides*, using different methods: 'Colony forming unit count' and 'Colony biomass and radial growth'.

We were able to monitor the spore survival and germination potential, but also the post germination effects on viability, growth and biomass of colonies, up to 21 days after the irradiation procedure. Radiation doses of 8.2 and 15.4 kGy caused a significant decrease in all tested parameters: spore viability, colony growth and biomass. Furthermore, 15.4 kGy sterilized all samples, in the sense that no growth or biomass increase was observed after irradiation with this dose.

The task described in Chapter 3 provided information on the survival and growth of colonies that originated from irradiated spores; however, there were some limitations inherent to the procedure: irradiated spores were not individually tested; the first visible information was only observed a couple of days after irradiation; the tests had a long duration period (21 days) and were labour intensive. Therefore, we planned, developed and tested a different approach to this matter, which would provide a different assessment method and a complementary set of information; this task is described in Chapter 4.

Following the results obtained in Chapter 3, Chapter 4 of this thesis "**Flow cytometry as a tool to assess the effects of gamma radiation on the viability, growth and metabolic activity of fungal spores"** describes the development of a new technique that allows performing viability, as well as metabolic activity tests, directly after the application of a biocide agent, during the very first stages of spore germination.

Flow cytometry was the selected tool for this purpose, since it has the capability of analysing the size, the complexity and different fluorescence intensities of thousands of particles (spores) each minute, allowing differentiating particles with a determined set of characteristics. By using adequate stains, different properties of the cells can be tested, namely their viability, metabolic activity, DNA ploidy, among others. In this work, spores from three different species were irradiated with doses ranging from 0-15 kGy. Overall, we were able to successfully analyse the viability, growth and metabolic activity of irradiated spores during the early growth stages. Obtained results were consistent between radiation doses on all species: irradiated spores generally grew less, later, and were less metabolically active. We were able to assess the effects of a sterilization treatment without the need to re-culture the cells. This procedure can be performed immediately after a biocide treatment is applied, without the need to incubate and perform colony counts, providing a rapid insight into the condition/resistance of the different organisms. Furthermore, unculturable, yet viable spores can also be screened.

Contamination spreading and vectors

Most of the fungal species that were retrieved from the documents in this work, as well as the ones described in the literature in regard to deterioration of cultural heritage, belong to the Ascomycetes group. 'Mould' is a term applied for the asexually reproducing forms – *Anamorphs* – in contrast to the fungi that produce spores by sexual reproduction – *Teleomorphs* (Sterflinger and Pinzari, 2011). It is crucial not only to identify the present organisms, but also to prevent cross-contamination between objects, users and documents.

Most Ascomycetes are able to produce very high quantities of spores; dry spores are generally dispersed through the air (anemophilous dispersion), while sticky spores usually rely on water or other vectors for dispersal, such as insects and other arthropods that are able to transport spores to new substrata for colonization (Abbott, 2002). The production of fungal propagules capable of adhering to other materials also contributes to their dispersal to different substrates (Holz et al., 2004). In fact, most of the fungal species that attack library materials come from dust and dust inhabitants (Sterflinger, 2012).

Air as vector

In museums and libraries, as well as in any building, microorganisms enter from outdoors, by means of the ventilation systems, in addition to being transported inside by visitors and personnel; surfaces and objects are also important sources of air contamination because of the dust settling and allowing the development of colonies that will generate more air-dispersible propagules (Vivar et al., 2012). López-Miras et al. (2012) analyzed the airborne fungal diversity while studying an oil painting, and found a correspondence between fungal species isolated from the air and present on the painting, probably due to the gravitational settling of spores. In his review article, Zyska (1997) referred that from the 34 genera of fungi isolated from the air of archives, only 3 of them had, until that date, not been reported in library materials.

Borrego et al. (2010) tested the cellulolytic and proteolytic ability of fungi isolated from the air in two Cuban repositories. The prevalent fungal genera were *Cladosporium, Aspergillus* and *Penicillium*, are all commonly found in indoor environments of houses, churches, libraries and museums (Piecková and Wilkins, 2004; Aira, 2007; Nunes et al. 2013b); the

authors also stated that the majority of the fungal strains isolated from the abovementioned environments, frequently exhibit cellulolytic, proteolytic and amylolytic activities, and are therefore supporting and accelerating the degradation of different substrates (Borrego et al., 2010; Borrego et al., 2012). The air is therefore considered the most important vector for fungal spore dispersion in both indoor and outdoor environments, but we should also highlight the role of the direct handling and potential cross-contamination by users, as well as the almost invisible, but quite successful work of different insects and other arthropods, roaming inside as well as outside the archive, carrying different sorts of fungal propagules that are potentially capable of initiating new degradation processes.

Insects as vectors

Insects are the most serious source of damage for wooden and paper objects kept in museums and indoor environments; they use wood as shelter and egg deposit, but also as nutrient source. Some are able to digest cellulose (or products of its digestion by other organisms), as well as other available materials. They are also frequently involved in the deterioration of paper; similarly to their action in wooden objects, they are able to feed from the paper's cellulose. Others may cause damage by utilizing fillings, glues, leather or other substances used indifferent parts of the documents. Other proteinaceous materials, such as leather and parchment, are also susceptible to attack by insects that feed on collagens and keratins, causing direct damage to the support, causing erosion, holes or loss of material (Tiano, 2002). However, for this work, we were more interested in the role of insects, not as biodeteriorating agents themselves, but rather as potential vectors for fungal propagule dispersion.

Fungi and insects have various and very interesting relations; insects can feed on fungal spores, but can also feed some types of entomopathogenic fungi. Spores can be spread by adhering to insects that move through contaminated substrata, or by the ingestion and excretion of fungal propagules (Dromph, 2003). In nature, many fungi are subject to predation by arthropods, due to their ability to accumulate high amounts of nitrogen and phosphorous (Ruess and Lussenhop, 2005). Some produce spores that in natural environments are mainly dispersed by mites and insects (Deacon, 1997). The high

resistance of fungal spores is usually associated with the cell wall properties, which allow the passage through the digestive tract of arthropods while maintaining spore viability, and allowing an efficient spreading by invertebrates (Coluccio et al., 2008). Furthermore the presence of entomopathogenic species is linked to arthropod vectored dispersion since the conidia of these species can adhere to the cuticle of a host, and with proper conditions of humidity and temperature, develop hyphae that penetrate the cuticle and invade the circulatory system, leading to the host's death, body mummification, and posterior fungal spreading (Jurado et al., 2008). In another perspective, Sterflinger (2012) highlights that insect infestations in libraries are also be associated with fungi infected materials; the metabolic water, cell debris and insect droppings produced by insects represent a perfect medium for fungal germination and growth.

As previously stated, the study of the airborne fungi population in the air of the Archive of the University of Coimbra revealed a low diversity in the air of the archive (Nunes et al. 2013b), which led us to consider that other complementary mechanisms could be assisting in the dispersion of fungal propagules between documents. However, very little was known on insect mediated fungal dispersion in indoor environments.

In Chapter 5 of this thesis, **"Can arthropods act as vectors of fungal dispersion in heritage collections? A case study on the archive of the University of Coimbra, Portugal"**, the role of insects in the dispersion of and cross-contamination of contaminating fungal propagules was assessed. In fact, the presence of various arthropods in the archive suggested a zoochoric dispersion could play a significant role in this dispersion.

Therefore, insects were captured in different areas of the archive, and monitored for the presence of fungal propagules; the sequencing of the total ITS region of the obtained fungal species, combined with the analysis of macroscopic and microscopic taxonomic traits, provided data on the fungal diversity carried by the arthropods. Results show a high fungal diversity associated with invertebrates: from a total of over 148 isolates, 25 fungal genera and 59 species were identified. To our knowledge, these are the first results on the identification of fungi associated with arthropods living in libraries/archives, at least in Portugal.

About 36% of the species found colonizing documents from the work described in Chapter

1 have also been isolated from the captured arthropods. These species were *Cladosporium cladosporioides*, *Penicillium chrysogenum*, *Aspergillus versicolor*, *Alternaria alternata*, *Epicoccum nigrum*, *Penicillium canescens*, and *Phlebiopsis gigantea*. This co-occurrence indicates that, at least on this site, these species are likely to be dispersed by animal-vectored dispersion; some of the fungal species were consistently present both in arthropods and in the documents, therefore playing an important role in the process of document biodeterioration.

Objectives

This thesis describes a multi-disciplinary and exploratory research, which addresses the causes as well as the control methodologies for the prevention of biodeterioration phenomena in ancient documents. This work focused the main group of organisms responsible for paper and parchment deterioration – fungi. Gamma radiation was used as a disinfection procedure, and its effects on resistant strains were assessed using different methodologies. Also, the role of insects as agents of fungal propagule dispersion was analyzed within the Archive of the University of Coimbra.

It is important to know which organisms are causing the damage, in order to apply an adequate disinfection procedure and assess its effects. Also, it is important to know how they are spread, in order to prevent further contamination.

The aims of this study were to:

i) Identify the infecting organisms that are responsible for the biodeterioration processes on ancient paper and parchment documents and to relate the different *taxa* with the substrate types;

ii) Assess the effects of gamma radiation in different biological parameters of fungal isolates - colony growth, weight, metabolic activity, spore growth, viability - by using conventional techniques, but also by developing a new approach using flow cytometry;

iii) Evaluate the role of arthropods as vectors for fungal dispersion in library environments, and the effects of seasonality on the occurrence of different fungal *taxa*.

Chapter 1

Fungal diversity in ancient documents:

A case-study on the Archive of the University of Coimbra

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Chapter 1 – Fungal diversity in ancient documents: A case-study on the Archive of the University of Coimbra.

Abstract - This multidisciplinary research combines knowledge in molecular biology with fungal morphology, aiming at the identification of infecting fungi from historical documents on the Archive of the University of Coimbra. The identification of infecting fungi on several bibliographic documents and support materials was based both on ribosomal DNA *loci* amplification and sequencing, as well as on morphological identification, using macro- and microscopical traits. A high fungal diversity was found in all types of support: parchment, laid paper and wood pulp paper. Fourteen fungal genera were isolated, identified, and kept in culture. The most frequent were *Cladosporium*, *Penicillium* and *Aspergillus*, and other less frequent genera, such as *Alternaria*, *Botrytis*, *Chaetomium*, *Chromelosporium*, *Epicoccum*, *Phlebiopsys* and *Toxicocladosporium* were also present. Within these genera, 20 different species were identified, from which 15 were found only in a single support type. *Cladosporium cladosporioides* and *Penicillium chrysogenum* were the only species present in all support types.

Keywords - Fungal identification, PCR, ITS sequencing, Foxing, Ancient documents.

1.1 Introduction

The Archive of the University of Coimbra holds a vast documentation asset produced and received by the University, essential for the analysis of the history of the University of Coimbra since its foundation by King D. Dinis in 1290, but mainly since its definitive establishment in the city of Coimbra in 1537. The oldest document in the Archive is the parchment of ‘‘Colegiada de Guimarães’’, dated from 983, prior to the foundation of Portugal. The past preservation and conservation conditions of some of the Archive collection were not adequate and the documents are now susceptible to biodegradation.

Library documents are generally composites of different materials (natural, semi-synthetic or synthetic compounds), each with different possible responses to environmental changes. This is a setback in understanding the biodegradation processes (Harvey, 1992). The biodeterioration of organic materials is very important as a recycling process, however, in some cases, this process also destroys historical records, resulting in the loss of valuable information (Cappitelli and Sorlini, 2005). Microorganisms are important biodegrading agents. The presence of spores or vegetative cells on the surface of documents may indicate a possible degradation in the future. Fungi are considered as serious degrading agents of bibliographic documents, particularly cellulolytic fungi (Fabbri *et al.*, 1997). One of the major concerns regarding fungal colonization is the change in the document aesthetics, either by discoloration by weak acids produced by fungi, or by the accumulation of pigments that may stain its support in a phenomenon referred to as foxing (Arai, 2000). Some foxing stains occur as reddish and/or brownish marks on the paper support, and are thought to have both biotic and abiotic origins, from the metabolic activity of microorganisms, to metal or ink oxidation (Meynell and Newsam, 1978; Arai, 2000). However, the majority of foxing stains are supposedly due to the effect of microorganisms, or their metabolites (Corte *et al.*, 2003). Foxing may occur on other non-cellulosic materials, as long as an optimum environment for ‘‘foxing-causing fungi’’ is present, because cellulose is not always an essential nutrient for fungal growth (Arai, 2000).

Some fungi involved in the deterioration of library material may be dangerous to library professionals and users, due to the production of mycotoxins. They enter the body via inhalation of toxicogenic spores and direct dermal contact, and can cause several diseases (Bennet and Kilch, 2003) from which, airway infections, mycosis, immune system issues,

and asthma are examples (Nielsen, 2003). Zyska (1997) reported 84 genera and 234 species of filamentous fungi isolated from library material (different supports), 19% of which could be a source of different health issues.

Our main goal was to assess the diversity of infecting fungi, in documents from the Archive of the University of Coimbra. Thirty documents, made of three different types of support (parchment, laid paper and wood pulp paper) were analyzed. Parchment, made of treated animal leather, was sampled from a 13th century document and from the parchment cover of an 18th century book. The second support type, laid paper, is a hand-made paper made of cotton, hemp and linen fibers, and was sampled from 12 different sources, from the 16th to 18th century. Wood pulp paper, industrially produced from wood pulp processing, was sampled from 6 documents from the 19th and 20th centuries.

For routine use, the molecular approach gives more reliable results than traditional morphological analysis (Scharbereiter-Gurtner *et al.*, 2001b). Therefore the identification of infecting fungi was assessed by fungal genomic DNA analysis, using PCR (Polymerase Chain Reaction) and DNA sequencing, complemented with microscopy and other morphological analysis techniques.

1.2 Materials and methods

1.2.1 Sample isolation and culture

Several contaminated documents, made of different materials were initially selected from the Archive of the University of Coimbra, according to biodeterioration signs, such as paper coloring/discoloring, presence of microfungal structures or any other observable texture changes. Only areas without any text, or close to the bookbindings were sampled using a scalpel to scratch the paper surface or to remove a small portion, whenever biodegradation symptoms were observed. Around 150 small samples (max. 0.5 cm^2) were retrieved from 30 different documents, and stored in sterile Petri dishes until further processing. The sample's texture, coloring and surface pigmentation were quite diverse: white, pink, purple, brown, black and green (among others) were found, and this diversity suggested different origins for the observed deterioration. All sample manipulations were made aseptically with previously sterilized material, in order to prevent cross contaminations. Paper fragments were then incubated between 25ºC and 28ºC, on several culture media,

such as malt extract agar (MEA) and potato dextrose agar (PDA), with streptomycin (0.5 g/L) to prevent bacterial growth.

1.2.2 Molecular and morphological identification

After culturing the samples in the different mediums, colony DNA extraction was performed, using a commercial kit (Nucleon Phytopure Plant DNA Kit, Amersham Pharmacia Biotech). A PCR was performed to amplify the total ITS region of all DNA samples, using primers ITS 4 and ITS 5 (White *et al.*, 1990). The reaction components for the PCR were 5 ml Taq (DNA polymerase) buffer 10x concentrated (Pharmacia Biotech, USA), 1 ml of 10 mM dNTPs (deoxynucleotides) (Pharmacia Biotech, USA), 2 ml of each primer (10 mM) (Pharmacia Biotech, Sweden), 1.5 ml of 50 mM MgCl2 (Sigma, St. Louis, USA), 2 U (enzymatic units) of Taq Polymerase (Pharmacia Biotech, Sweden) and 1 ml of template DNA, combined in a final volume of 50 ml for each amplification.

The PCR reactions were done in a Applied Biosystems 9700 Thermocycler (Norwalk, USA), according to the following conditions: initial denaturation at 95ºC for 2 min, followed by 35 cycles of denaturation at 95ºC for 1 min, annealing at 51–53ºC for 1 min, extension at 72ºC for 1 min, with a final extension at 72ºC for 10 min. Negative controls without DNA template were prepared in all amplifications. The PCR products were separated by 1.2% agarose gel electrophoresis (Pharmacia Biotech, Uppsala, Sweden), stained with ethidium bromide (Sigma, St. Louis, USA) and photographed under UV-light on a fluorescent table (Vilber Lourmat, Marne La Valée, France) to confirm the amplified fragment size.

The PCR products were purified and directly sequenced using an ABI 3730 Genetic Analyzer, using the Big Dye v.3 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), using the primer ITS4. Sequences were analyzed using Geneious software (www.geneious.com), and were ran against NCBI's BLAST (Basic Local Alignment Search Tool) database in order to assess the similarity with published sequences, belonging to identified fungal species. The sequences obtained in this study were deposited in GenBank under accession numbers FJ791124–FJ791161 (see Tables 1–3).

To confirm these identifications, upon colony growth and fruiting bodies emergence (if visible), the different cultures were identified to the genus level, according to their macro-morphology and micro-morphology (Barnett and Hunter, 1998; Watanabe, 2002) and, whenever possible, to the species level. This was performed using optical microscope

observation with Lactophenol Cotton Blue (Sigma) as staining solution. Different culturing methods were used, from the direct observation of small colony samples in microscope slides to the use of Riddell's chamber (Riddell, 1950) and other variants of this method (Kawato and Shinobu, 1960; Nugent *et al.*, 2006).

1.3 Results

The first step for fungal identification was the sequencing of the total ITS region, followed by genetic alignments with the NCBI's GenBank database to determine the fungal species. Determined sequence similarities ranged from 95.5% to 100.0% (Tables 1–3). This identification was subsequently confirmed by morphological examination of the isolates.

Table 1 – Fungi isolated from ancient parchment documents: original source document, estimated date, GenBank accession numbers as well as genetic similarity with existing NCBI sequences are presented. The ID number corresponds to each fungal isolate. The * refers to isolates that have only been identified using the molecular approach.

ID	Original Document	Century	Isolated Species	Accession number	Similarity (%)
32	*Register book of Jorge Botelho, private notary of the Cárquere Monastery*	18th (1755 to 1761)	*Phlebiopsis gigantea*	FJ791151	96.7
33	*Register book of Jorge Botelho, private notary of the Cárquere Monastery*	18th (1755 to 1761)	*Penicillium chrysogenum*	FJ791152	99.8
34	*Register book of Jorge Botelho, private notary of the Cárquere Monastery*	18th (1755 to 1761)	*Cladosporium cladosporioides*	FJ791153	100.0
35	*Register book of Jorge Botelho, private notary of the Cárquere Monastery*	18th (1755 to 1761)	*Cladosporium cladosporioides*	FJ791154	99.8
36	*Registry book of councils from the Santa Cruz Monastery (Coimbra)*	13th to 14th	*Thanatephorus cucumeris* *	FJ791155	99.8
37	*Registry book of councils from the Santa Cruz Monastery (Coimbra)*	13th to 14th	*Epicoccum nigrum*	FJ791156	99.8

Table 2 - Fungi isolated from ancient laid paper documents: original source document, estimated date, GenBank accession numbers as well as genetic similarity with existing NCBI sequences are presented. The ID number corresponds to each fungal isolate (omitted ID numbers correspond to isolates that were lost previous to DNA extraction). The * refers to isolates that have only been identified using the molecular approach.

ID	Original Document	Century	Isolated Species	Accession number	Similarity (%)
2	*Ordination process of João Correia, from Espinhal*	17th (1690)	*Alternaria alternata*	FJ791124	99.5
3	*Postil of teachers from the Faculty of Law*	17th	*Cladosporium cladosporioides*	FJ791125	100.0
4	*Postil of teachers from the Faculty of Law*	17th	*Cladosporium cladosporioides*	FJ791126	99.8
5	*Ordination process of João Correia, from Santo Varão*	18th (1787)	*Penicillium chrysogenum*	FJ791127	100.0
6	*Postil of teachers from the Faculty of Law*	17th (1620)	*Cladosporium cladosporioides*	FJ791128	100.0
8	*Postil of teachers from the Faculty of Law*	17th (1620)	*Skeletocutis* sp. *	FJ791129	99.0
9	*Ordination process of João Correia, from Espinhal*	17th (1690)	*Penicillium* sp. *	FJ791130	97.4
10	*Judicial sentence (copy)*	16th (1574)	*Penicillium helicum*	FJ791131	99.2
11	*Postil of teachers from the Faculty of Law*	17th	*Coprinus* sp. *	FJ791132	99.3
12	*Postil of teachers from the Faculty of Law*	16th to 17th	*Cladosporium cladosporioides*	FJ791133	100.0
13	*Ordination process of João Correia, from Espinhal*	17th (1690)	*Phlebia subserialis*	FJ791134	95.5
14	*Postil of teachers from the Faculty of Law*	17th (1620)	*Toxicocladosporium irritans* *	FJ791135	99.2
15	*Judicial sentence (copy)*	16th (1574)	*Cladosporium cladosporioides*	FJ791136	100.0
16	*Registry of accounts from the University of Coimbra*	17th (1650)	*Penicillium chrysogenum*	FJ791137	100.0
17	*Royal Hospital of Coimbra - registry of rents*	18th (1757)	*Cladosporium cladosporioides*	FJ791138	100.0
25	*Postil of teachers from the Faculty of Law*	17th	*Aspergillus nidulans*	FJ791144	99.3
26	*Judicial process from the Diocesan court of Coimbra*	18th (1725)	*Chaetomium globosum*	FJ791145	97.0
27	*Judicial process from the Diocesan court of Coimbra*	18th (1725)	*Chaetomium globosum*	FJ791146	99.7
31	*Ordination process of João Correia, from Condeixa-a-Nova*	18th (1779)	*Penicillium chrysogenum*	FJ791150	100.0
39	*Ordination process of João Correia, from Espinhal*	17th (1690)	*Botrytis cinerea*	FJ791158	99.8
42	*Postil of teachers from the Faculty of Law*	17th	*Chromelosporium carneum*	FJ791160	99.6

Table 3 – Fungi isolated from ancient wood pulp paper documents: original source document, estimated date, GenBank accession numbers as well as genetic similarity with existing NCBI sequences are presented. The ID number corresponds to each fungal isolate (omitted ID numbers correspond to isolates that were lost previous to DNA extraction).

ID	Original Document	Century	Isolated Species	Accession number	Similarity (%)
19	*Petitions to the Rector of the University of Coimbra*	20th (1937 to 1939)	*Penicillium* sp.	FJ791139	100.0
20	*Petitions to the Rector of the University of Coimbra*	20th (1937 to 1939)	*Cladosporium cladosporioides*	FJ791140	100.0
21	*Petitions to the Rector of the University of Coimbra*	20th (1937 to 1939)	*Penicillium canescens*	FJ791141	100.0
22	*Petitions to the Rector of the University of Coimbra*	20th (1937 to 1939)	*Penicillium chrysogenum*	FJ791142	100.0
23	*Petitions to the Rector of the University of Coimbra*	20th (1937 to 1939)	*Aspergiullus fumigatus*	FJ791143	100.0
28	*Petitions to the Rector of the University of Coimbra*	20th (1937 to 1939)	*Chromelosporium carneum*	FJ791147	99.6
29	*Petitions to the Rector of the University of Coimbra*	20th (1937 to 1939)	*Chromelosporium carneum*	FJ791148	99.6
30	*Letter from Francisco Gomes de Almeida Branquinho, Secretary of the Civil Governor of Coimbra*	19th (1860)	*Aspergillus versicolor*	FJ791149	99.8
38	*Petitions to the Rector of the University of Coimbra*	20th (1937 to 1939)	*Penicillium chrysogenum*	FJ791157	100.0
40	*Correspondence of the Coimbra University Rectorat*	20th (1936)	*Chaetomium globosum*	FJ791159	100.0
44	*Letter from Francisco Gomes de Almeida Branquinho, Secretary of the Civil Governor of Coimbra*	19th (1860)	*Aspergillus versicolor*	FJ791161	98.6

According to both methodologies, 20 species and 14 genera were isolated and identified from the various sample sources. The results of the identification are presented in Tables 1–3, for the three different support types. Overall, all support types presented a high diversity of species. The most common species were *Cladosporium cladosporioides* and *Penicillium chrysogenum*, however other less common species, like *Chromelosporium carneum* and *Toxicocladosporium irritans* were also isolated from these materials (Table 4).

In the laid paper samples, *C. cladosporioides* was the most recurrent species. The most frequent genera were *Cladosporium*, and *Penicillium*, in almost half the samples. However, less common species such as *T. irritans* and *C. carneum* were found. In wood pulp paper, *C. carneum*, *Aspergillus versicolor* and *P. chrysogenum* were the most frequent species, and the less common *Chaetomium globosum* was also isolated. Regarding parchment, *C. cladosporioides* was the most common, and *Epicoccum nigrum*, *Thanatephorus cucumeris* and *Phlebiopsis gigantea* were found exclusively in this type of support (Table 4).

Table 4 – Frequency table for the isolated fungal species in all support types. The * refers to isolates that have only been identified using the molecular approach.

Species	**Parchment**	**Laid paper**	**Wood pulp paper**	**Total**
Cladosporium cladosporioides	6	1	2	9
Penicillium chrysogenum	3	2	1	6
Chaetomium globosum	2	1	-	3
Chromelosporium carneum	1	2	-	3
Aspergillus versicolor	-	2	-	2
Alternaria alternata	1	-	-	1
Aspergillus fumigatus	-	1	-	1
Aspergillus nidulans	1	-	-	1
Botrytis cinerea	1	-	-	1
Coprinus sp. *	1	-	-	1
Epicoccum nigrum	-	-	1	1
Penicillium canescens	-	1	-	1
Penicillium helicum	1	-	-	1
Penicillium sp. *	1	-	-	1
Penicillium sp. *	-	1	-	1
Phlebia subserialis	1	-	-	1
Phlebiopsis gigantea	-	-	1	1
Skeletocutis sp.*	1	-	-	1
Thanatephorus cocumeris	-	-	1	1
Toxicocladosporium irritans *	1	-	-	1

1.4 Discussion

The ITS region has been frequently used, and with good results, for the identification and inventory of fungal organisms that contaminate documents and art objects (e.g. Michaelsen *et al.*, 2006 or Rakotonirainy *et al.*, 2007). The sequences we obtained from the total ITS region, together with the morphological analysis, allowed the identification of most isolates. These are the first results on the identification of Archive fungi reported in Portugal.

Considering the number of samples, a great variety of species have been isolated and identified. Some of the species were only found in one type of support, which may be explained by production of different enzymes by the various organisms. As example, the cellulase enzyme complex is responsible for the degradation of cellulose from paper fibers (Deacon, 1997). In parchment and leather, keratin is the most abundant structural protein together with collagen and proteases like keratinases and collagenases are responsible for its degradation (Popescu *et al.*, 2008).

Regarding parchment, the majority of the species found were already reported in this support (Zyska, 1997), with *C. cladosporioides* being the most abundant. *Epicoccum nigrum and T. cucumeris* appeared in parchment samples only. Strzelczyk *et al.* (1989) state *Chaetomium globosum* to be a very active organism in the decay of leather from bookbindings, and Sharma and Sharma (1979) described the presence of *Alternaria alternata* in finished leather, but we didn't find these species in this support.

Chaetomium globosum and *C. carneum* were found in both wood pulp paper and laid paper, while some other species were only found in one support type (see Table 4), like *Alternaria alternata* and *Toxicocladosporium* irritans, only found in laid paper, and *Penicillium canescens* and *A. versicolor*, only found in wood pulp paper. Less frequent genera such as *Botrytis*, *Chaetomium*, *Chromelosporium*, *Epicoccum* and *Phlebiopsys* have also been described in different supports and in various countries (Zyska, 1997; Szczepanowska and Cavaliere, 2000; Corte *et al.*, 2003).

Overall, the high frequency of *C. cladosporioides*, *Aspergillus spp*. and *Penicillium spp*. is in agreement with other works (Zyska, 1997; Hyvärinen *et al.*, 2002; Corte *et al.*, 2003; Nielsen, 2003; Da Silva *et al.*, 2006). These are almost ubiquitous *taxa*, and can produce numerous mitospores and conidia that are easily dispersed by air (Abrusci *et al.*, 2005). However, for a parallel research, we sampled the air from the Archive, to test if air

dispersion of airborne fungal spores or propagules occurs. The first analysis showed little diversity, with *Aspergillus fumigatus* clearly being the most frequent (data not shown), and in the current work, it was found in only one wood pulp sample. At least in this case, the fungal diversity found in the documents is probably due to other reasons, such as the storing conditions these documents were subjected to in the past.

Apart from the degradation of the library material, most of these organisms (e.g. *Penicillium*, *Aspergillus* and *Alternaria*) can also cause adverse human health effects in both Archive workers and users. A good example of this, found in one of the laid paper samples is *T. irritans*, that produces ample amounts of volatile metabolites, which cause a skin rash within minutes of opening an inoculated dish for microscopic examination (Crous *et al.*, 2007). It is important for library workers and users to be aware of this problem so that adequate care is taken when handling ancient documents.

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Chapter 2

Assessing the fungal diversity in an ancient parchment:

the pathogenic potential of existing mycoflora as a threat to the health of users

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Chapter 2 - Assessing the fungal diversity in an ancient parchment: the pathogenic potential of existing mycoflora as a threat to the health of users.

Abstract - The contamination of documents with fungi is a widely spread problem. Most of these fungi and their metabolites can promote the degradation of rare and valuable documents. The health of users, conservators and restorers is at stake, since many of these fungi produce different allergens and toxins. A previous review work reported that 84 genera and 234 fungal species had been isolated from library materials, 19% of which were potentially harmful to humans. In Portugal, the first results on fungal diversity in archive documents were recently reported.

The aims of this investigation were to assess the fungal diversity and the presence of pathogenic species in a specific parchment - The Papal Bull to the Confraria de Santo António, which dates back to 1629, and belongs to the pontificate of Urbano VIII. Samples were inoculated in two different culture media, Malt Extract Agar e Potato Dextrose Agar (MEA and PDA), and fungal colonies were isolated into axenic cultures. After DNA extraction, the molecular identification of the fungal organisms was performed through PCR amplification, sequencing and analysis of the Total ITS region of the rDNA, followed by morphological confirmation using optical microscopy. Data on fungal diversity were analyzed using the Shannon-Wiener Index, followed by a *t* test. No statistical differences in diversity were found with the use of the two culture media (MEA and PDA). The most common genus was *Penicillium*, followed by *Cladosporium* and *Aspergillus*. Less common genera like *Chrysosporium*, *Pseudocercosporella* and *Trichosporon* were also found. From all isolated species, 12 are potentially pathogenic.

This Papal Bull was the subject of a conservation intervention, which consisted of the hydration of the document, planning, stabilization and mechanical cleaning. Its restoration was quite difficult since it started from a very degraded and fragile condition. In the end, the parchment was sterilized with ionizing radiation in order to prevent future issues with the health of users.

Keywords – Papal bull, Parchment, Fungi, Molecular characterization.

2.1 Introduction

The Papal Bull to the Confraria de Santo António is a parchment from the 18th Century, with ferrogalic ink inscriptions. It was written by Nicolas C. Colinos, and sealed by the Pope Urbano VIII, whose Latin name is Urbanos Octavus. This Pope exercised his pontificate between August 6th of 1623 and the 29th of July of 1644. In the 7th of July of 1626, the Pope Urbano VIII, granted indulgences to privileges for members of the church, of any gender and profession, especially to the confreres in the confraternity of Santo António. This document was stored in the sacristy of the parish church of Freamunde, Paços de Ferreira (Portugal), however, the temperature and humidity of the room were favorable to fungal growth, and contributed to the inadequate preservation conditions of the Bull, making it susceptible to degradation by these organisms for many years.

2.1.1 Inherent problems to the fungal contamination of documents

One of the main concerns about fungal colonization is the change that occurs in the aesthetics of the document. This can occur by discoloration, induced by weak acids produced by fungi, by the accumulation of pigments that can stain the documents in a phenomenon known as foxing (Arai, 2000), and also other biotic and abiotic factors. Foxing can cause the deterioration of these documents, and is potentially dangerous to human health, through fungal contamination, mycotoxins and allergenic substances (Gonzalez *et al.,* 2002): fungi can enter the body through the inhalation of mycogenic spores or by direct dermal contact, causing several diseases (Bennett and Kilch, 2003), from which the infection of the respiratory tract, different types of mycosis, immune system problems and asthma are examples (Nielsen, 2003). Zyska (Zyska, 1997) reported the existence of 84 genera and 234 species of filamentous fungi isolated from library material (different media), 19% of which could be a source of adverse effects on human health. Conservators and restorers are special targets for fungal contamination when exposed to documents and pieces of art that are being the subject of intervention, and therefore, are potentially contaminated.

2.1.2 Fungal contamination forms and corresponding diagnosis

The biodegradation of organic material is an essential process in the natural environment, for recycling complex organic matter, however, this process can destroy historical records that are essentially composed of organic matter, natural and synthetic polymers, leading to the loss of valuable cultural goods in archives and libraries (Cappitelli and Sorlini, 2005). Some of these documents are made of paper and parchment, like the Papal Bull to the Confraria de Santo António, which makes them a favorable support for microbial growth in general, but particularly to microscopic fungi (Cappitelli and Sorlini, 2005).

Fungi can degrade a wide variety of polymers, including cellulose, as an effect of their efficient degrading enzymes and enzymatic complexes (Bennett and Faison, 1997), because of this, microscopic fungi, namely cellulolytic fungi, are considered serious degrading agents of bibliographic documents (Fabbri *et al.,* 1997). By using cellulose as substrate, and growing in favorable environmental conditions, they are able to destroy paper in short periods of time (Adamo *et al.,* 2003). Thus, the presence of spores or vegetative cells on the surface of the documents indicates the potential for fungal development and biodegradation in the future (Mesquita *et al.* 2009).

Foxing stains occur as reddish and/or brownish spots, usually present in documents that are stored in warm and moist environments (Arai, 2000) and they are a serious problem in paper preservation. The name comes from the similarity with the spots of foxes (Rakotonirainy *et al.,* 2007). They can have a mineral or biological origin (Meynell and Newsam, 1978); mineral contamination results from metallic impurities (i.e. iron salts) incorporated in paper during its production, and the subsequent chemical changes of these impurities; on the other hand, biological foxing is caused by the presence of fungi that react slowly with the support. They sometimes contain structures that resemble filamentous fungi (Rakotonirainy *et al.,* 2007), however, most spots are presumably due to the effect of microorganisms or their metabolites (Corte *et al.,* 2003). Foxing spots are common in paper artifacts from the 16th to the 19th century; however, it is known that even modern papers can present this phenomenon (Arai, 2000; Corte *et al.,* 2003). Spot size increases with time, causing irreversible damage by deteriorating documents, and difficulting its readability (Rakotonirainy *et al.,* 2007). Nevertheless, they can also occur in non-cellulosic materials, since cellulose is not always an essential nutrient for fungal growth (Arai, 2000).

The humidity and temperature of storage and exhibition sites, that host documents and pieces of art, are very influential factors in the development and spreading of fungi. However, some insects also intervene with this process. Wood-boring insects that feed on cellulose and other materials that compose these documents (some organisms of the Coleoptera order, or others from the Psocoptera, Thysanura and Blattaria orders), contribute to their degradation. Besides, they can also act as vectors in the spreading of fungal propagules.

The primary aim of this work was to assess the fungal diversity in the Papal Bull; to identify the infecting fungi; to compare the fungal diversity between the two different culture media - MEA and PDA - but also to check for potentially pathogenic species. The correct identification of infectious groups is essential to the selection of strategies for the protection of restorers. As a future perspective, this work will allow to evaluate the need for the sterilization of old documents (e.g. with ionizing radiation), to prevent health issues to users. Using molecular tools for identification and confirmation provides better results than the traditional morphological analysis alone (Scharbereiter-Gurtner *et al.,* 2001b) Fungal isolates were kept in culture to be used in future studies.

2.2 Materials and methods

2.2.1 Sample isolation and culture

The Papal Bull was sampled in twelve different areas (Fig. 1), according to the presence of signs of degradation or biological activity, such as the presence of fungal structures. Samples were obtained by gently scraping the parchment surface with a scalpel, without damaging the support, which was in extremely weak condition already. The texture, thickness, colour and shape of sampled areas were diverse. This suggests different origins for the observed deterioration. The handling of all samples was performed using aseptic material, previously sterilized to prevent cross-contaminations.

Collected samples were incubated between 25ºC and 28ºC, on two culture media, Malt Extract Agar (MEA) and Potato Dextrose Agar (PDA), with added streptomycin (0.5 g/L) to prevent bacterial growth.

Fig. 1. Sampled areas from the Papal Bull to the Confraria de Santo António: general a) and detailed b) views.

2.2.2 Molecular and morphological identification

After culturing the samples in the different media, and isolating them into axenic cultures, fungal DNA was extracted from the colonies using an Applied Biosystems 6100 NucPrep DNA extraction station, according to the manufacturer's standards.

A PCR was performed to amplify the total ITS region of all DNA samples, using primers ITS 1F and ITS 4. The PCR mixes were composed of 25 µl of Jump Start Taq DNA Polymerase wit $MgCl_2$ (Sigma D9307), 1 µl of each primer (10mM), 20µl of ultra-pure water and 2µl of template DNA, for a total reaction volume of 25µl.

The PCR reactions were performed in an Applied Biosystems 9700 Thermocycler (Norwalk, USA), according to the following conditions: initial denaturation at 95ºC for 2 min, followed by 35 cycles of denaturation at 95ºC for 1 min, annealing at 51–53ºC for 1 min and extension at 72ºC for 1 min, and a final extension at 72ºC for 10 min.

Negative controls (without template DNA) were prepared in all amplifications. The PCR products were separated through agarose gel (1.2%) electrophoresis (Pharmacia Biotech, Uppsala, Sweden), stained with Gel Red (Biotium) and photographed under UV-light on a fluorescent table (Bio Rad Gel Doc XR+).

The PCR products were purified and sequenced using an ABI 3730 Genetic Analyzer, using the Big Dye v.3 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), with the primer ITS4. Sequences were analyzed with the Geneious software

(www.geneious.com), and were run in NCBI's BLAST (Basic Local Alignment Search Tool) database, in order to assess the similarity with published sequences of identified fungal species.

To confirm these identifications, upon colony growth and fruiting bodies emergence (when visible), the different cultures were identified to the genus level, according to their macro-morphology and micro-morphology (Barnett and Hunter, 1998; Watanabe, 2003), and whenever possible, to the species level. This was performed using optical microscope observation with Lactophenol Cotton Blue (Sigma) as staining solution. Different culturing methods were used, from the direct observation of small colony samples in microscope slides to the use of Riddell's chamber (Ridell, 1950) and other variants of this method (Kawato and Shinobu, 1960; Nugent *et al.*, 2006).

2.2.3 Statistical analysis

The fungal diversity from each sampled area was quantified through the Shannon-Wiener Index (H') (Shannon & Wiener, 1966). STATISTICA software version 7 was used for the statistical analysis. Statistical tests for significant differences between the Shannon-Wiener Indexes (of both MEA and PDA cultures) were performed using an independent *t* test by groups, and assuming a normal data distribution (α=0.05).

2.3 Results

A total of 18 different species belonging to 10 genera were isolated and identified (see Table 1). One of the isolates was only identified to the genus level. Results concerning the identification obtained for each type of culture medium (MEA and PDA) are described in Tables 2 and 3. Overall, the sampled parchment presented a high diversity of species: the most common were *Aspergillus versicolor* and *Penicillium brevicompactum*. Nevertheless, other less common species, like *Chrysosporium carmichaelii* and *Fusicladium rhodense*, were also isolated.

Table 1 - Frequency table for the isolated fungal species in each sampled area.

Fungal species	Isolates		Sampled areas											
	MEA	PDA	1	2	3	4	5	6	7	8	9	10	11	12
Aspergillus conicus		1									1			
Aspergillus versicolor	6	2		2				2		1			2	1
Chrysosporium carmichaelii		1											1	
Cladosporium cladosporioides		7	2	1						3	1			
Cladosporium langeronii	1												1	
Cladosporium sp.		1								1				
Cladosporium sphaerospermum		3	1				1					1		
Cladosporium xylophilum		1					1							
Fusicladium rhodense	1				1									
Lenzites betulinus		1							1					
Microascus brevicaulis	1					1								
Penicillium brevicompactum	7	3	4		1		3			1			1	
Penicillium chrysogenum	3	2		2			2						1	
Penicillium corylophilum	5	6		2		2						1		6
Penicillium dipodomyicola		1									1			
Penicillium expansum	1										1			
Penicillium griseofulvum	1	1		1				1						
Penicillium sp.	1				1									
Pseudocercosporella fraxini		1										1		
Tricholoma flavovirens		1									1			
Trichosporon sp.		1				1								
Total of isolates	27	33												
Total number of *taxa*	10	16												
Number of unique *taxa*	5	11												
Shannon Index	1.97	2.47												

The comparative analysis of the fungal diversity between the two types of culture media was performed using an independent *t* test, by groups, and the Shannon-Wiener Index (Tables 2 and 3). The *p* value was 0.3717 ($\alpha = 0.05$), which indicates no significant differences between MEA and PDA culture media (Fig. 2), however, as displayed in the same image, a greater diversity of species was found when using PDA (H'=0.685655) rather than MEA (H'=0.490015). Four species were present in MEA exclusively (Table 2),

Fusicladium rhodense, *Microascus brevicaulis*, *Penicillium expansum* and *Cladosporium langeronii*, and ten species were present only in PDA, from which *Aspergillus conicus*, *Pseudocercosporella fraxini* and *Lenzites betulinus* are some examples.

Table 2 - Fungi isolated from the Papal Bull (MEA culture medium), between 25ºC and 28ºC. H' represents the Shannon-Wiener Index for each sampled area.

Isolated Species	**Sampled area**	**H'**
Penicillium brevicompactum	1	0
Aspergillus versicolor *Penicillium chrysogenum*	2	0,6365142
Fusicladium rhodense *Penicillium brevicompactum* *Penicillium sp.*	3	1,0986123
Microascus brevicaulis *Penicillium corylophilum*	4	0,6365142
Penicillium brevicompactum *Penicillium chrysogenum*	5	0,6365142
Aspergillus versicolor *Penicillium griseofulvum*	6	0,6931472
Penicillium brevicompactum	8	0
Penicillium expansum	9	0
Aspergillus versicolor *Cladosporium langeronii*	11	0,6365142
Aspergillus versicolor *Penicillium corylophilum*	12	0,5623351

Table 3 - Fungi isolated from the Papal Bull (PDA culture medium), between 25ºC and 28ºC. H' represents the Shannon-Wiener Index for each sampled area.

Isolated species	**Sampled area**	**H'**
Cladosporium cladosporioides *Cladosporium sphaerospermum*	1	0,636514
Cladosporium cladosporioides *Penicillium chrysogenum* *Penicillium corylophilum* *Penicillium grisofulvum*	2	1,332179
Trichosporon sp.	4	0
Cladosporium sphaerospermum *Cladosporium xylophilum* *Penicillium brevicompactum*	5	1,386294
Aspergillus versicolor	6	0
Lenzites betulinus	7	0
Aspergillus versicolor *Cladosporium sp.* *Cladosporium cladosporioides*	8	0,950271
Aspergillus conicus *Cladosporium cladosporioides* *Penicillium dipodomyicola* *Tricholoma flavovirens*	9	1,386294
Cladosporium sphaerospermum *Penicillium corylophilum* *Pseudocercosporella fraxini*	10	1,098612
Chrysosporium carmichaelii *Penicillium brevicompactum* *Penicillium chrysogenum*	11	1,098612
Penicillium corylophilum	12	0

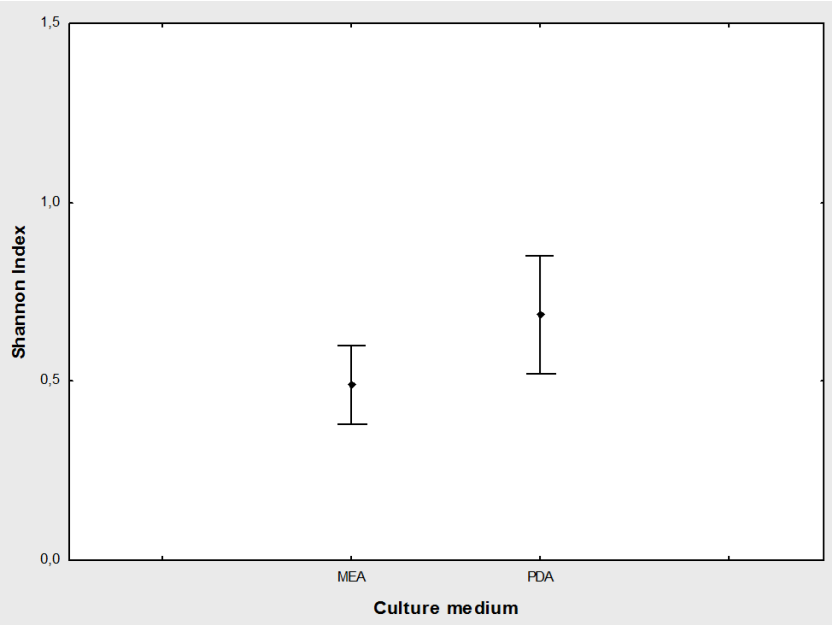

Fig. 2. Comparison between the Shannon-Wiener Indexes determined for MEA and PDA culture media. The graph presents average values ± standard error. The result of independent t-test, by groups, was not significant (P>0.05) for a t value of 0.914854 and a df of 19.

2.4 Discussion

The ITS region has frequently been used, and with good results, in the identification and inventory of fungal organisms that contaminate documents and art objects (e.g. Rakotonirainy *et al.,* 2007; Michaelsen *et al.,* 2006). The sequences we obtained from the total ITS region, together with the morphological analysis, allowed the identification of most isolates.

Considering that we just sampled one parchment document, a great variety of species was isolated and identified. Some of the species, however, were found only in one culture medium, but no significant differences were found. A greater diversity was found when using PDA, and 10 species were present only in this medium, which may be explained by the fact that PDA is a very rich medium. Since some species were found while using only one of the two culture media, we advise that more than one culture medium should be used, to allow more species to grow and be isolated. The growth rate and development of these organisms is related to the available nutrients. It is easier to isolate slower-growing species using culture media with fewer nutrients, since they are not outgrown by other species.

Some of the species that were found in this parchment had already been reported for this support type (Zyska, 1997; Mesquita *et al.,* 2009), with *P. chrysogenum* being one of the most abundant in these studies. Strzelczyk *et al.* (1989) stated *C. globosum* to be a very active organism in the decay of leather from bookbindings, and, in 1979, Sharma and Sharma (1979) described the presence of *Alternaria alternata* in finished leather, but we didn't find any of these species in this document.

Overall, the high frequency of *Aspergillus* spp., *Cladosporium* spp. and *Penicillium* spp. is in agreement with other works (Zyska, 1997; Hyvärinen *et al.,* 2002; Nielsen, 2003; Corte *et al.*, 2003; Da Silva *et al.,* 2006). These are almost ubiquitous *taxa*, that can produce numerous mitospores and conidia, which are easily dispersed through the air (Abrusci *et al.*, 2005).

As previously referred, apart from the degradation of the library material, most of these organisms can also cause adverse human health effects in conservators, restorers and users. In this work alone, 12 of the obtained species have been described as potential human pathogens. *Aspergillus*, *Penicillium* and *Cladosporium* (and their toxins) are frequently involved in human disease, and are related with abnormalities in T and B cells, rhinorrhea, sinus tenderness, wheezing, physical signs and symptoms of neurological dysfunction (Didriksen, 2003). A good example is the high resistance of *A. conicus* to the antimycotic 5-Fluorocytosine, proving to be the most virulent in a case of study on human isolates of *Aspergillus* (Nobre, 1977). *Aspergillus versicolor* is also related with some forms of aspergillosis (Tashiro *et al.,* 2011), and has been reported as associated with asthmatic symptoms (Cai *et al.*, 2011). Its well-studied mycotoxin RTI 3843 is reported deadly to the human monocytic THP-1 cell line, contributing to inflammatory responses (Pei and Gunsch, 2011). Another example is Sterigmatocystin, its toxic effects are much the same as those of aflatoxin B1, being considered as a potent carcinogen, mutagen and teratogen, and a possible cause for gastric cancer in Mongolian gerbils (Kusunoki *et al.,* 2011). The species *C. carmichaelii* is a keratinophilic fungus that produces keratinases, which act on the degradation of hair, nails, among others (Kushawaha and Gupta, 2008). *C. langeronii* was described as a pathogen on human skin as well (Zalar *et al.*, 2007). Both *C. cladosporioides* and *P. chrysogenum* produce transaldolases, which are a novel and IgE cross-reactive allergen family (Chou *et al.,* 2011). Keratomycosis is a frequent cause of ocular morbidity and blindness, and can be caused by *C. cladosporioides* infection (Chew *et al.,* 2009).

Furthermore, this species can provoke cutaneous phaeohyphomycosis (Duquia *et al.,* 2010). Another example is *C. sphaerospermum* that is involved in endodontic infections (Gomes *et al.,* 2010).

Vega et al. (2006), have reported that Ochatoxin A was produced by *P. brevicompactum*. This mycotoxin is weakly mutagenic, possibly by induction of oxidative DNA damage, and is potentially carcinogenic to humans (Palma *et al.,* 2007). Pen b 26 is one of the allergens produced by *P. brevicompactum*, a source of respiratory problems, including asthma (Serdal Sevinc *et al.,* 2009). Likewise, *P. chrysogenum* has been reported as a robust allergen, in particular for asthma development (Ward *et al.,* 2010). The alkaline serine protease of this species (Pen ch 13) induces histamine release from the basophils of asthmatic patients; it degrades tight junction proteins, and stimulates the release of pro-inflammatory mediators from human bronchial epithelial cells (Shen *et al.,* 2007); *P. corylophilum* presented a possible connection with chronic hypersensitivity pneumonia (Ohnishi *et al.,* 2002); *Penicillium dipodomycola*, *P. expansum* and *P. griseofulvum* produce the mycotoxin patulin (Dombrink-Kurtzman, 2007) which has been reported as genotoxic, and therefore potentially carcinogenic (Hopmans, 1997). As a last example, regarding the genus *Trichosporon*, there is trichosporonosis, which is a lethal opportunistic infection, occasionally found in immunocompromised patients, particularly those who have hematological malignancies (Nagai *et al.,* 1999).

It is not yet clear if environmental strains can directly infect humans, however, it is necessary to take extra care if the users, restorers and conservators are immunocompromised (Tokimatsu and Kadota, 2006). It is commonly said that most fungal organisms, are 'potentially' pathogenic, in a broad sense. However, some of the species found in this work are directly related to the production of dangerous toxins, which is why we think is important, that users and conservators are aware of this problem, so that adequate measures are taken when handling ancient documents or art pieces.

After the sampling process took place, the Papal Bull was the subject of a conservation process, following all the safety guidelines to prevent further contamination. Its full restoration was very difficult, since it was in a very degraded and fragile condition in the beginning. According to Clavaín (2009), parchment is a material that is somewhat similar to paper regarding its nature and demeanor. The dimensional instability to the exchange of

moisture makes the surface smooth, and rarely found without ripples. For this reason, the Papal Bull was cleaned using a soft rubber to remove all traces of mycelium and spores of contaminating fungi, and other surface dirt. A strong exposure to moulds caused the dehydration of parchment, which became very rigid and brittle. Thus, it was necessary to proceed to document hydration to be able do the relaxation and smoothing of the Bull. These treatments are designed to restore the parchment's original flexibility, based on the introduction of new products, in an attempt to preserve its integrity. The simple humidification and drying under maintenance constitutes the most appropriate treatment, the document was dried under tension to avoid wrinkling (Clavaín, 2009). Due to the fragility, disruptions were not repaired with grafts from another parchment or restoration of inscriptions, but only the consolidation and reinforcement of the Bull took place, through the application of metil-cellulose. This glue penetrates the fibers, increasing cohesion and producing the physical union of the breakdown field. On the backside, Japanese paper was applied to provide a better mechanical reinforcement.

After this process, a sterilization procedure with gamma radiation was performed, in order to prevent future health issues with its users. The universal biocide effect of ionizing irradiation is based on its ability to cause chemical damage to the DNA molecules, and in this way, to the genetic material of all living organisms that are present as potential biological contaminants (Katušin-Ražem *et al.,* 2009) making it an universal biocide, and a very effective bio-inactivation method.

2.5 Acknowledgments

We would like to thank the Centre for Functional Ecology of the University of Coimbra for the support and the Priest Dr. Luis Manuel Pacheco Leão de Brito for letting us borrow the Papal Bull to the Confraria de Santo António, which belongs to his parish in Freamunde, Portugal.

Chapter 3

Assessing gamma radiation effects in a Cladosporium cladosporioides strain isolated from an ancient document: CFU-count vs. Growth parameters.

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Mesquita, N.; Nunes, I.; Santos, G.L.; Cabo-Verde, S.; Alves, L.; Botelho, M.L.; Portugal, A.; Freitas, H. Assessing gamma radiation effects in a *Cladosporium cladosporioides* strain isolated from an ancient document: CFU-count vs. Growth parameters.

Chapter 3 - Assessing gamma radiation effects in a *Cladosporium cladosporioides* strain isolated from an ancient document: CFU-count vs. Growth parameters.

Abstract - Recent research in paper biodeterioration aims at the development and optimization of methodologies that help preventing the degradation of old historical documents. In this work, we studied the effects of gamma radiation on the survival and growth of a common archive contaminant fungus – *Cladosporium cladosporioides*. Our results can be used to evaluate the applicability and effects of gamma radiation as an alternative technology in the decontamination treatment of archive documents.

A *C. cladosporioides* strain was isolated from an ancient document from the collection of the Archive of the University of Coimbra. This organism was identified using molecular and morphological methods. Due to its documented resistance to gamma radiation, this organism was selected to estimate the minimum radiation dose required to decontaminate documents. Spores were placed in paper disks and were submitted to different radiation doses. The effects of this bio-inactivation procedure were assessed using two different methodologies: 1) Colony forming units (CFU) count, and 2) Colony biomass and radial growth.

Radiation doses of 8.2 and 15.4 kGy caused a significant decrease in all tested parameters: spore viability, colony growth and biomass. Furthermore, 15.4 kGy sterilized all samples, in the sense that no growth or biomass increase was observed after irradiation with this dose.

The two methods provided different and complementary information regarding the inactivation: CFU analysis allowed the quantification of total spore viability (pre-germination), according to the obtained survival curves; on the other hand, the growth analysis provided qualitative data concerning medium term effects in sub-lethal doses (e.g. biomass, germination/growth delay and colony diameter).

Keywords: gamma irradiation, ancient documents, disinfection, *Cladosporium cladosporioides*, microfungi, sub-lethal effects.

3.1 Introduction

The use of different materials, such as stones, metals and bark, to draw signs, marks, images and other forms of communication is part of human history. During the last centuries, however, papyrus, parchment and paper have been the most commonly used graphic supports. They are composed of various organic materials, and the different responses they present to environmental changes difficult their study (Capitelli and Sorlini 2005). The restoration and maintenance of written cultural heritage is a complex task due to the yet incomplete knowledge about the deteriorating agents (Michaelsen et al. 2000).

Fungi are very relevant degrading agents of bibliographic documents, in particular cellulolytic fungi (Fabbri et al. 1997), because they can destroy valuable collections in a short period of time, if the conditions for their development are met. A little moisture and a suitable substrate (in this case cellulose) is usually enough for these organisms to grow and spread, feeding on the support, and ultimately spreading to contaminate other documents. However, some fungi involved in the deterioration of library material are also dangerous to librarians due to the production of disease-causing mycotoxins (Zyska 1997). Concerned and alerted to this problem, but without logistic means and financial support, the institutions that manage archive collections welcome all collaborations from different fields of expertise to better handle this problem.

The research on the application of ionizing radiation to decontaminate paper documents dates back to 1960 (Belyakova 1960). Further research on this field was almost stopped with the emergence of other sterilization treatments, such as the use of fumigant gases (e.g. ethylene oxide), fast and efficiently used against microorganisms. Similarly to what had happened with DDT, the application of these sterilization methods spread rapidly, being offered as quick and easy treatments, until the discovery of their serious harmful effects on human health, which eventually led to their prohibition in many countries (Adamo et al. 2001; Gonzalez et al. 2002; Magaudda 2004). The lack of safety mechanisms, the risk for human health and the undesirable physical effects on the treated materials has caused a growing interest in the search for non-chemical alternatives (Laguardia 2005). These facts, together with currently available safer methods, placed the application of gamma irradiation as a central research topic for the control of infecting microorganisms (Magaudda 2004).

The physical properties of electromagnetic waves, such as gamma radiation emitted by a Cobalt-60 source, allow the penetration of materials without leaving any residue. If properly applied, gamma radiation is lethal to infecting microorganisms, and it does not damaging the support materials (Adamo et al. 2005). Previous studies show that gamma radiation treatment is a reliable cost-benefit alternative, and a valuable option when compared to dangerous and expensive chemical substances. Radiation doses up to 14 kGy are safe to paper supports, in what concerns their physical resistance and colour changes (Gonzalez et al. 2002).

The fungus *Cladosporium cladosporioides* (dematiaceous) was the selected test species. It is a very resistant fungus, in part due to the dark pigments that are produced and accumulated in the mycelium, protecting the organism from environmental extremes (Da Silva et al. 2006). This fungus is also frequently found in different sampling sites and materials (Abrusci et al. 2005; Blank and Corrigan 1995; Botelho et al. 1988; Da Silva et al. 2006; Michaelsen et al. 2006; Szczepanowska and Cavaliere 2000). In fact, Zyska (1997) reports it as one of the most frequent moulds, isolated from different support materials: paper, parchment, textiles, wax seals, art of archives, and others.

The used strain of *Cladosporium cladosporioides* was isolated from an ancient paper document, belonging to the Archive of the University of Coimbra, in a previous work (Mesquita et al. 2009). The spores were submitted to different gamma radiation doses to evaluate the effects on biomass, radial growth and colony forming units (CFU) count, as assessment tools for this bioinactivation procedure. In this work, these different fungal viability assessment methods were compared (since they are often used, but rarely together), and the applicability of gamma radiation as a fungal decontamination process in paper was also tested, taking into consideration the safety of the support materials.

3.2 Materials and methods

3.2.1 Sampling, fungal isolation and identification

The dematiaceous fungus *Cladosporium cladosporioides* was selected for this work because of its known radio resistance. It was obtained by incubating paper samples in standard Malt-Agar Extract (MAE) medium (Difco Laboratories, Sparks, MD, USA), in the course of a

previous work (Mesquita et al. 2009). It was morphologically identified using Lactophenol Cotton Blue (Sigma) staining and considering the macro- and micro-morphology of the culture (Barnett 1998; Watanabe 2002). This identification was confirmed using molecular methods (Scharbereiter-Gurtner et al. 2001). Briefly, DNA extraction was performed using a commercial kit (Nucleon Phytopure Plant DNA Kit, Amersham Pharmacia Biotech, UK). The total ITS region was amplified using primers ITS4 and ITS5 as described by White et al. (1990). The reaction components for the PCR were 5 µl Taq buffer 10X (Pharmacia Biotech, USA), 1 µl of 10 mM dNTPs (Pharmacia Biotech, USA), 2 µl of each 10 mM primer, 1.5 µl of 50 mM $MgCl_2$ (Sigma, St. Louis, USA), 2U (enzymatic units) of Taq Polymerase (Pharmacia Biotech, USA) and 1 µl of template DNA for a total volume of 50 µl for each amplification. The PCR reactions were performed in an Applied Biosystems 9700 Thermocycler (Norwalk, USA).

The PCR conditions were: initial denaturation at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 51°C for 1 min and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. Negative controls without DNA template were prepared in all amplification sets. PCR products were separated by 1.2% agarose gel electrophoresis (Pharmacia Biotech, Uppsala, Sweden), stained with ethidium bromide (Sigma, St. Louis, USA) and documented by photography under UV Transillumination (Vilber Lourmat, Marne La Valée, France).

The PCR products were purified and directly sequenced using an ABI Prism 310 DNA sequencer (Norwalk, USA), using the same primer (ITS4) that was used for PCR amplification.

3.2.2 Sample preparation for the irradiation process

A spore suspension was prepared by scraping 6 plates of sporulated cultures, into a porcelain mortar, using a glass slide. Three millilitres of saline solution (0.9% NaCl) with 0.5% of Tween 80 were added to the mortar, and the suspension was then homogenized. The resulting spore concentration was estimated using a Neubauer chamber and corrected to 10^{12} spores/ml. This spore suspension was kept at 4ºC until use.

Paper disks (~Ø 6mm) were impregnated with 10 µl of the spore suspension (approximately 10^7 spores), and left to dry overnight in a biohazard vertical laminar airflow cabinet (Baker

Company Inc., Maine, USA). These test pieces were individually placed in Amilon® plastic containers, and were hermetically sealed. This methodology was adapted from Christensen and Holm (1964).

Test pieces were irradiated at 1, 5, 10 and 15 kGy, (3 replicates/dose). Non-irradiated samples (0 kGy, 3 replicates) were manipulated in the same conditions as the irradiated samples. Routine dosimeters were used to monitor the absorbed doses (batch 63 and batch JB; Harwell, UK). Real absorbed doses were of 1.3, 5.2, 8.2 and 15.4 kGy.

All irradiations were performed at a semi industrial Co-60 source located at the Nuclear and Technological Institute (ITN) campus (source activity of 186 kCi). The dose rate at the irradiation site was of 21 kGy/h.

3.2.3 Colony Forming Unit count method

After irradiation, each sample was placed in a test tube, containing glass beads and 1 ml of saline solution (0.9% NaCl) with 0.5% of Tween 80, and was homogenized by vortex for 5 minutes. For samples with doses of 0, 1.3, 5.2 and 8.2 kGy, an additional 9 ml of saline solution with 0.5% of Tween 80 was added to the test tubes. For samples irradiated with 15 kGy, 4 ml of saline solution with 0.5% of Tween 80 was added. These dilutions were performed according to the preliminary testing and validation of the CFU methodology for this species.

Decimal dilution series were performed for all 3 replicates of each absorbed dose, for countable representative colony-forming units (CFU) numbers ($30 < n < 300$). Aliquots were spread into Tryptic Soy Agar (TSA) Petri plates, and incubated at 30±2ºC for 21 days. The CFUs were counted after 1, 2, 5, 6, 14 and 21 days of incubation.

3.2.4 Colony growth and biomass evaluation method

Separate sets of samples were used to evaluate both biomass and radial growth, since biomass measurement was a destructive process. These methods were carried out in parallel, and followed the same irradiation procedure as described for the CFU method.

For radial growth measurement, Petri plates with MAE medium were prepared. Two perpendicular lines (marked X and Y) were drawn on the bottom of each Petri plate, with a centred intersection. Furthermore, in order to facilitate the extraction of the fungal

colonies for biomass measurement, cellophane disks were pre-boiled twice for 15 minutes before autoclaving (to remove plasticizing agents), and placed over the MAE medium surface, so that the fungal colonies would grow over it after incubation.

Irradiated and non-irradiated test pieces (~10^7 spores each) were placed in the centre of the cellophane disk, over a small drop of sterile water, to help the adhesion of the paper disk to the cellophane membrane, so that it would remain in the centre of the plate during initial manipulation. Each Petri plate was marked according to the irradiation dose and test duration, and was incubated at 25ºC.

Radial growth measurements (axis X and Y) were registered daily until the 21st day in order to evaluate the colony area and growth rate.

For biomass evaluation, each of the cellophane membranes was detached from the culture medium, with the colony adhered, and dried at 50ºC until stable weight was reached (approximately 24 hours). The cellophane and colony were weighted and the mean cellophane disc weight was subtracted. Biomass measurement was assessed at days 3, 7, 14 and 21.

3.2.5 Data analysis

The number of CFU was log-transformed and the log survival count was plotted against the absorbed dose (kGy) to analyse the test strain inactivation kinetics (Daintith et al. 2000; McLaughlin et al. 1988; Morrisey and Phillips 1993).

Statistical differences in the growth and biomass results - days 3, 7, 14 and 21 - were assessed using one-way ANOVAs, followed by *post-hoc* Bonferroni tests at 5% significance level. All statistical tests were performed using SPSS statistical package 20.0 (SPSS 2011).

3.3 Results

3.3.1 Fungal Identification

Macro- and micro-morphological observations of the cultures led to the identification of the isolated fungi as *Cladosporium cladosporioides.* These observations were confirmed by sequencing the purified PCR products. The NCBI BLAST (Basic Local Alignment Search Tool)

of these sequences resulted in 100% similarity with other *Cladosporium cladosporioides* sequences. Our sequence was deposited in Genbank under the accession number FJ791125.

3.3.2 CFU Count method

Data obtained by the CFU count method are presented in Figure 1. A 3-log reduction of viable counts was observed with 5.2 kGy, corresponding to an inactivation of 99.9%. The 8.2 kGy dose resulted in a 5-log decrease, and 15.4 kGy caused a 6-log decrease in viable counts (99.9999%).

The obtained survival response suggests a sigmoidal inactivation curve, with a small "shoulder" for doses lower than 1.3 kGy, an exponential inactivation kinetics until 8.2 kGy and a final "tail" for doses higher than 8.2 kGy.

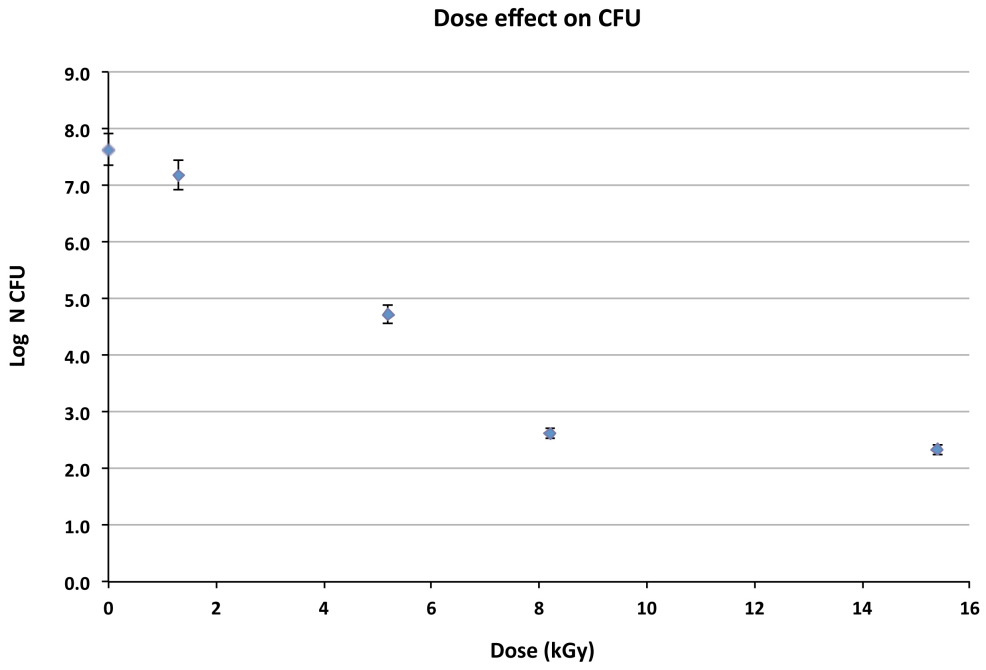

Fig. 1 – Survival plot of *Cladosporium cladosporioides* spores to gamma radiation in paper test pieces. Average log CFU count (Log N) per test piece (n=18) in function of the absorbed irradiation dose (kGy).

3.3.3 Radial Growth evaluation

The radial growth results are displayed in Figure 2. This was obtained by plotting the average of X and Y measurements (diameter) for 21 days. Significantly different groups were assessed for days 3, 7, 14 and 21 only, in order to simplify the displayed information, but also because the same periods were used in the destructive biomass assessment.

The control colonies (0 kGy) had an average diameter of 8 mm on the second day, reaching the full size of the plate (~80 mm) at approximately the 18th day. Nearly similar results were obtained with the dose of 1.3 kGy, although the colonies were slightly smaller in the later stages of the experiment. This difference becomes more relevant after the 9th day of incubation, but it never attained significance when compared with the control.

The dose of 5.2 kGy caused a delay in germination, with the colonies starting to grow at the 3rd day. Results were significantly different from the control at this stage, however, colonies were still able to grow at almost the same rate, and after day 7 these differences were no longer significant. More expressive effects were observed with the 8.2 kGy dose in the first 18 days. In fact, significant differences from lower radiation doses were obtained in days 3, 7 and 14. Colonies started to grow 3 to 4 days later, and their diameter was always smaller than in colonies irradiated with lower doses.

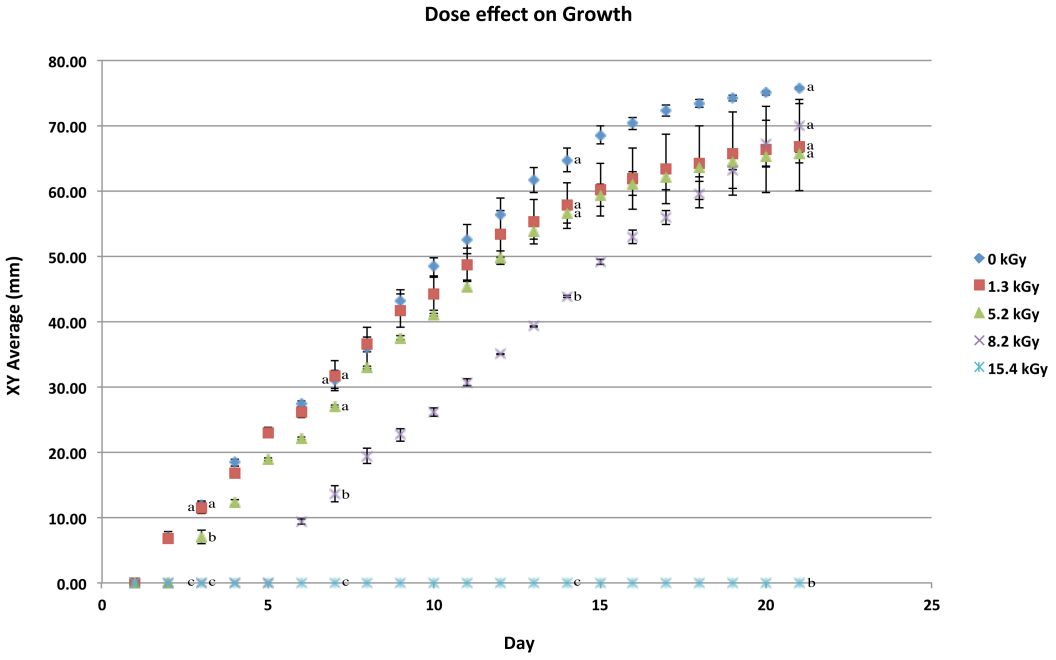

Fig. 2 – XY measurements for each irradiation dose during the 21-day test. Values are means ± SE of 3 replicates per treatment. For days 3, 7, 14 and 21, different letters correspond to significant differences after Bonferroni post-hoc test ($P < 0.05$).

However, in the later stages of this experiment, some of these colonies outgrew lower radiation doses. At the 21st day, the 8.2 kGy dose was only significantly different from the 15 kGy dose. This was probably due to the fact that most of the spores were killed before germination, which led to less competition effects between the surviving spores.

Colony growth was completely inhibited during the 21-day test period after irradiation at 15.4 kGy, with significant statistical differences from all other doses.

3.3.4 Biomass evaluation

Since the biomass assessment was a destructive process, different sets of samples were used for biomass measurement at days 3, 7, 14 and 21. The dose effect on biomass is represented in Figure 3.

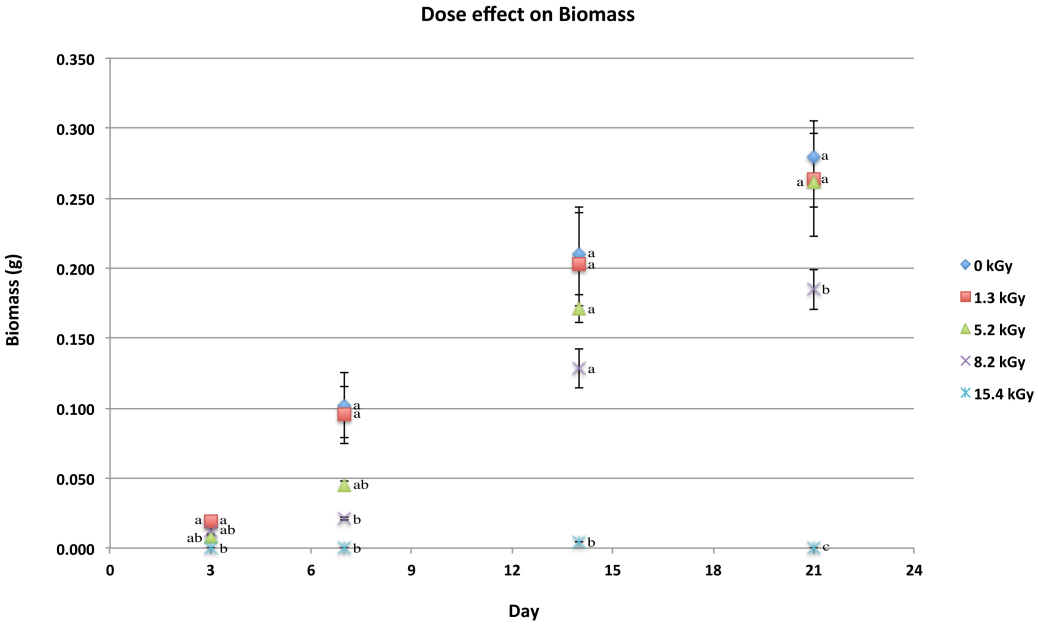

Fig. 3 – Biomass measurements for each irradiation dose for the four different test durations. Values are means ± SE of 3 replicates per treatment. Different letters correspond to significant differences after Bonferroni post-hoc test ($P < 0.05$).

As displayed, the 1.3 kGy dose presented smaller biomass values than the control, throughout the duration of the test, showing that low radiation doses are not efficient in preventing the fungal development. These differences were not statistically significant.

The 5.2 kGy dose did cause a delay in germination (as was observed in the growth assessment) and therefore, biomass values at day 3 were almost irrelevant. Of course this late start in germination led lower biomass values on the 7th day, which were smaller than with the two lower doses (almost reaching significance; P=0.155). Nevertheless, biomass values did increase during the remaining period, and after 14 and 21 days, they were very close to the biomass values obtained with the 1.3 kGy dose. This was probably due to lower competition effects, since 5.2 kGy did inhibit the growth of 99.999% spores (Fig. 1), which allows the few resisting spores to germinate and grow in better conditions.

With the use of 8.2 kGy, biomass was more affected. This was significant at the 7th day, with biomass values for this dose being significantly lower than for the lower doses ($P<0.05$). Similarly to what was previously observed in the growth assessment, the 15.4 kGy dose completely inhibited the colony growth. Biomass was significantly different from control in all tested moments ($P<0.05$).

3.4 Conclusion

By using different methods to assess the effects of gamma radiation, we were able to test the spore survival and germination potential, but also the post germination effects on viability, growth and biomass of colonies. Results between the different tests are concordant, and in general, doses of 8.2 kGy and 15.4 kGy caused a significant decrease in all tested parameters ($P<0.05$). Furthermore, the results for these two higher doses were also significantly different from one another ($P<0.05$), since 15.4 kGy was near to complete sterilization. Our results for this species, in terms of radio resistance, are in agreement with those previously reported for *Cladosporium cladosporioides* (Botelho et al., 1988).

The 15.4 kGy dose caused severe effects in spore viability and fungal survival. The 8.2 kGy dose decreased spore viability by approximately 5 decimal logs, as shown by the CFU test. This radiation dose, according to the growth evaluation tests, has caused a significant decrease in the colony growth (diameter), and nearly 30% decrease in its biomass. Gamma irradiation doses ranging from 3 to 10 kGy have proven to be sufficient to clean paper

material from microscopic fungi and contraindications of this treatment are negligible (Adamo and Magaudda 2003).

Results point out that it is possible to disinfect documents colonized by fungi, decreasing their biological activity using radiation doses lower than 15 kGy. This dose has been tested and described to be safe to paper supports in terms of physical resistance and colour changes (Gonzalez et al. 2002; Magaudda 2004; Rizzo et al. 2002; Rochetti et al. 2002). Da Silva et al. (2006), working with *Cladosporium* sp. and other dematiaceous fungi, and by using radial growth parameters only, reported that there was no need for irradiation doses of 20 kGy or higher, to completely inactivate these organisms, stating that the 16 kGy dose was already effective.

The 3-log decrease (*i.e.* 99.9%) on viable counts obtained after exposure to a dose of 5.2 kGy, suggests that this dose is probably enough to decontaminate most environmentally contaminated samples. The initial spore load in the tested samples (10 million spores) was very high when compared to environmental contamination, so even a high inactivation rate will still allow some of the spores to resist. Also, a shielding effect can occur with high spore concentrations because of the protective effect of melanins and other substances usually present in radio resistant species. For suited minimal dose estimation on paper, the natural fungal bioburden values should be assessed, together with a thorough characterization of the fungal diversity. Magaudda (2004) presented equivalent results, and stated that books and documents that are to be kept and used under unsterile conditions, should be *recovered*, rather than completely *disinfected* or *sterilized,* by reducing the fungal bioburden values to naturally occurring ones.

In order to evaluate the effect of irradiation after disinfection of contaminated supports (such as paper), the CFU count method can provide quantitative information about the total spore viability; it is easy to apply and presents reliable results considering total contaminant population. The analysis of different growth parameters, however, provides more information on the medium-term effects, by gathering information about biomass, germination delay, colony diameter and other factors to be considered for a qualitative evaluation.

Further work should be developed for better understanding the effects of radiation in fungal organisms at the physiological level. It is known that, in general, electromagnetic radiation causes genetic damage, which passes to following generations, if DNA is not

properly repaired (Hooley and Clipson 1995; Mironenko et al. 2000). Furthermore, melanized fungi, such as *Cladosporium cladosporioides,* are able to cope with higher radiation doses, because of the shielding effects of melanins (Dadachova 2008). Research at the genetic and proteomic levels would definitely provide more information on the physiological and molecular effects of gamma radiation in contaminating fungi. Furthermore, better understanding these resistance mechanisms (such as radiation shielding and DNA repair) might help to develop more efficient decontamination treatments.

In order for a thorough assessment to be performed, it is recommended that the chosen inactivation assay is related with the study purpose. CFU counts provide quantitative information, while colony growth assessment, together with biomass evaluation, provides qualitative information on the medium term effects of the applied inactivation procedure.

Chapter 4

Flow cytometry as a tool to assess the effects of gamma radiation on the viability, growth and metabolic activity of fungal spores.

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Chapter 4 – Flow cytometry as a tool to assess the effects of gamma radiation on the viability, growth and metabolic activity of fungal spores.

Abstract - Flow cytometry is often used for viability and vitality assessment in bacteria and yeasts. However, its application to the study of fungal spore development is uncommon, probably due to the difficulties in successfully staining these cells.

In the current study, we used flow cytometry for the first time to assess the effects of a disinfection treatment on the survival, growth and metabolic activity of fungal spores (Penicillium chrysogenum, Aspergillus nidulans and Aspergillus niger) submitted to gamma radiation (0-15 kGy). The Forward and Side-Scatter parameters of the cytometer were used to assess the differences in size and complexity of particles. Furthermore, two fluorescent dyes were used: Propidium Iodide to assess the membrane integrity and spore viability, in a culture-independent procedure; and Dihydroethidium to measure the changes in metabolic activity of irradiated spores in their first 10 h of growth in a liquid culture medium.

Our results support that flow cytometry is a valuable tool in assessing different biological parameters and biocide effects, as it allowed accurate determination of the viability, growth and metabolic activity of gamma-irradiated spores. The fluorescence of Propidium Iodide was 5-7x more intense in unviable spores. The Dihydroethidium fluorescence increase was associated with faster growth. Control and low radiation doses allowed the germination and growth of spores, while higher doses led to growth inhibition and lower fluorescence.

Keywords: Flow cytometry, Spore viability, Fungal viability, Metabolic activity, Spore growth, Filamentous fungi, Gamma radiation, Dihydroethidium, Reactive oxygen species, ROS.

4.1 Introduction

Flow cytometry (FCM) allows the simultaneous analysis of several characteristics of particles from a heterogeneous population (size, complexity, fluorescence). The light scattered by each particle is filtered, and routed to appropriate detectors that measure the magnitude of pulses, which represent the amount of scattered light. Flow cytometry has many different applications in several fields of research: blood cell counts, viability and vitality of both bacteria and yeasts and microbial discrimination (Davey and Kell 1996; Veal et al. 2000; Davey 2002). Each particle is analysed individually, and several hundred can be analysed each second, providing information on single particle properties instead of population averages. It is therefore a powerful analysis tool for qualitative and quantitative purposes.

The size and complexity of particles is assessed using light-scattering measurements; forward-scattered light (FSC) provides information on the particle size, whereas side-scattered light (SSC) is associated with particle complexity. However, using fluorescent stains and probes, many other cell properties, and cell contents can be analyzed. According to the flow cytometer settings and stain specifications, various fluorochromes can be used simultaneously to assess different particle parameters (Shapiro, 2003). The use of multiple parameters to detect and select different particles is one of the technical advantages of flow cytometry, as one can differentiate fungal propagules from biotic debris as well as other cells (Prigione et al. 2004). These analyses can be performed right after a given treatment is applied, without the need to re-inoculate, incubate and perform colony counts, in a process that would usually require several days.

Propidium Iodide is a fluorescent dye that binds to DNA, and is generally unable to penetrate the membrane of live cells, however it stains dead cells, working as a dye-exclusion viability probe (Williams et al. 1998; Steinkamp et al. 1999) and it is suitable for the study of fungal conidia (Brul et al. 1997). When excited by a 488-523 nm laser, it fluoresces orange-red, and can be detected using a 562-588 nm band pass filter. It is commonly used in the evaluation of cell viability or to assess DNA content in cell cycle analysis by flow cytometry. Experiments with bacterial cells show that PI staining is usually independent of cell growth phase, and that cells can be stored in glycerol for long periods while retaining the stain (Williams et al. 1998).

Dihydroethidium is a chemically reduced ethidium derivative that has no positive charge. When chemically reduced, this dye exhibits blue fluorescence within the cytoplasm. In viable cells, it can be oxidized to ethidium by reactive oxygen species (ROS), which intercalates with DNA, fluorescing red (Breeuwer and Abee 2000). In general, spores and conidia have thick and resistant cell walls to protect protoplasts from physical, chemical, and biological damage, but sometimes this inhibits effective staining (Prigione et al. 2004). In this study, controls were made using an epifluorescence microscope (Nikon Optiphot) to confirm that the fluorochromes were actually entering the cells when expected.

Gamma rays are electromagnetic waves with high penetrating power that are able to pass through materials without leaving any harmful residue, which is a big advantage when compared with other disinfection treatments (Adamo et al. 1998, 2001; Da Silva et al. 2006). This sterilization treatment directly damages cell DNA through ionization, induces mutations, and ultimately kills the cell. Through the radiolysis of cellular water, it also promotes the formation of reactive oxygen species (ROS), free radicals and peroxides that cause single and double strand DNA breakages (McNamara et al. 2003). Fungi have successfully been inactivated from different materials, such as paper, wood and soil, using radiation doses ranging from 6 to 15 kGy (Hanus 1985; Pointing et al. 1998; McNamara et al. 2003; Da Silva et al. 2006).

Reactive oxygen species are also formed in the course of metabolic activity, and can therefore be used as metabolic indicators, since aerobic energy transduction depends on a complex electron transport chain and a proton pumping system in the mitochondrial membrane, which is susceptible to electron leakage (Bradner and Nevalainen 2003). This leads to the formation of the superoxide anion, which is a ROS that can promote the formation of other ROS molecules, and at higher concentrations, they can damage cellular components and lead to cellular dysfunctions (Osiewacz 2002). They may serve as regulators of fungal population development, for example, by inhibiting growth in excessively dense spore suspensions. In fact, self-suppression of germination in dense spore suspensions can be explained by the deficiency of vital resources, and self-intoxication with vital activity products: germinating spores produce ROS, which can suppress spore development.

Some fungi cope with increasing ROS concentrations using different strategies: by decreasing the surface area; by developing other mechanisms to limit the penetration of

external substances. Melanin and other anti-oxidants, eventually help to balance the formation of ROS inside the cell. Reactive oxygen species regulate the most vitally important processes in fungi: phase development change, intercellular communications, and protection from interspecies competition (Gessler et al. 2007).

The aims of this work were to analyse the viability of irradiated fungal spores with flow cytometry without the need to culture them; and, coincidently, to assess the metabolic activity and growth of fungal spores in their first 10 hours of growth in a liquid culture medium. Different doses of gamma radiation (0-15 kGy) were applied to fungal spores of three different species - *Penicillium chrysogenum, Aspergillus niger* and *Aspergillus nidulans* - whose strains were isolated from library documents in a previous work (Mesquita et al. 2009). They are common contaminant fungi of documents, and have been reported in different substrates: paper, parchment, leather, textiles and film (Zyska 1997; Sterflinger 2010; Sterflinger and Pinzari 2011). All three species are potentially harmful to humans (Bennett and Klich 2003; Samson et al. 2010).

Most microorganisms living in, or on, objects of art usually depend on special nutrients, so only a minority of species can be cultivated under laboratory conditions (Schabereiter-Gurtner et al. 2001b). Cultivation-independent methods enable the assessment of slower growing or even non culturable microorganisms and, in some cases, render the analysis faster and less expensive.

4.2 Materials and Methods

4.2.1 Tested fungal species

Three fungal species were selected for this study - *Penicillium chrysogenum, Aspergillus niger* and *Aspergillus nidulans.* They were isolated previously by Mesquita et al. (2009). *Penicillium chrysogenum* is a ubiquitous cellulolytic fungus that is a halotolerant, mesophile and psychrotolerant species. Both *A. niger* and *A. nidulans* are cosmopolitan cellulolytic species that are also xerophilic and mesophilic, although they are able to grow at warm temperatures.

4.2.2 Spore suspensions

The three isolates were re-cultured in PDA medium plates, and incubated at 28ºC until sporulating cultures were obtained (6-8 days). Fresh conidia were harvested by washing with a sterile saline solution (0.9 % w/v) containing Silwet L-77 surfactant (0.01 % v/v) (GE Silicones, USA), and this fluid was filtered using 10 µm Partec CellTrics filters (Partec, Germany) to remove the residual mycelia. To avoid the presence of particles which were smaller in size, washing of the fungal plates was performed gently to exclude as much debris as possible from the spore suspensions.

The presence of spores was confirmed using an optical microscope. The original concentration of the spore suspensions was estimated using the Neubauer chamber method, and adjusted to 10^7 spores/ml. Samples were stored in the cold (4ºC) and without light until the irradiation procedure.

4.2.3 Irradiation procedure

Spore suspensions were prepared in triplicate for each species tested, using 2 ml micro tubes, and these were irradiated in a Cobalt 60 experimental source (Precisa 22) located at the Nuclear and Technological Institute campus (Sacavém, Portugal). Five gamma radiation doses were applied: 3, 6, 9, 12 and 15 kGy, using a dose rate ranging between 2.45 kGy/h and 2.87 kGy/h. Absorbed doses were monitored using calibrated routine dosimeters (maximum variation range of ± 2.5%; Perspex, Harwell). After the irradiation procedure, the tubes containing the irradiated spore suspensions were stored in the cold (4ºC) and without light until the flow-cytometry analyses.

4.2.4 Flow cytometric analysis

Growth, viability and metabolic activity of fungal spores were assessed in a Partec CyFlow Space (Partec, Germany) flow cytometer, equipped with a green solid-state laser (532 nm / 30 mW). The flow rate was adjusted to approximately 300 particles.s^{-1}. To remove any particles smaller than spores from the analysis, the discriminator was defined for the particle size (FSC) just under the lowest spore signals. The obtained cytograms were analysed using FlowJo 8 software suite (www.flowjo.com), in particular, debris and cell fragments were removed by gating.

4.2.4.1 Growth assessment

Samples were prepared using cytometer tubes (3.5 ml, 55x12 mm; Sarstedt, Nümbrecht, Germany), with 1 ml of Potato Dextrose Broth (PDB; Difco Laboratories, Sparks, MD, USA) liquid culture medium, and 1 ml of the corresponding spore suspension, for final concentration of $5x10^6$ spores/ml). Samples were incubated at 28ºC, for the duration of this experiment (10h). At four different time points - 0, 4, 7 and 10 hours of incubation - 100 µl of sample were added to a flow cytometry tube with 1.9 ml of distilled water for a final spore concentration of $2{,}5x10^5$ spores/ml. These were mixed by inversion and then analysed in the flow cytometer. To assess the effects of different gamma radiation doses on the growth of the fungal spores, this experiment was replicated for each species using the sets of irradiated doses (0, 3, 6, 9 and 12 kGy). The 15 kGy dose results are not displayed in this analysis, since growth was completely inhibited.

4.2.4.2 Spore viability: Propidium Iodide staining

To assess spore viability without the use of culture media, filtered sterile water was used instead.

Cytometer tubes (3.5 ml) were prepared for each species and irradiation dose, with 1 ml of sterilized water, and 1 ml of the corresponding irradiated spore suspension for a final concentration of 5 x 10^6 spores/ml. From this suspension, 100 µl were added to a flow cytometry tube with 50 µl of Propidium Iodide stock solution (1 mg/ml; Sigma, St Louis, USA) and 1.85 ml of distilled water (final concentration of 2,5 x 10^5 spores/ml and 25 µg/ml of PI). Tubes were mixed by inversion and analysed in the cytometer after 10 minutes of staining.

4.2.4.3 Metabolic activity: Dihydroethidium staining

For each isolate, cytometer tubes (3.5 ml) were prepared with 1 ml of Potato Dextrose Broth (PDB; DIFCO, USA) liquid culture medium, and 1 ml of the corresponding spore suspension (final concentration of $5x10^6$ spores/ml). Samples were incubated at 28ºC, for the duration of this experiment (10h).

At four different time points – 0, 4, 7 and 10 hours of incubation – 100 µl were added to 1.85 ml of distilled water in a flow cytometry tube, for a final spore concentration of 2.5 x 10^5 spores/ml, and stained with 50 µl of the DHE stock solution (1 mg/ml, prepared in

dimethylsulfoxide; Sigma, USA). Tubes were mixed by inversion and analysed in the flow cytometer after 10 minutes of staining time. As DHE fluoresces bright red (605 nm) when oxidized by ROS, so it was detected in the red channel of the flow cytometer

To assess the effects of different gamma radiation doses on the metabolic activity of the fungal spores, this experiment was replicated for each species using the sets of irradiated doses (0, 3, 6, 9, 12 kGy). The 15 kGy dose was not used in this analysis, since growth was completely inhibited.

4.3 Results and Discussion

In this study, forward (FSC) and side (SSC) scatter were used as growth indicators in the early stages of development of three different species of filamentous fungi in PDB culture medium. Also, using two fluorescent stains, other physiological aspects were analysed: propidium iodide was employed to assess the membrane integrity and spore viability (without using culture media) while dihydroethidium was used to measure the changes in the metabolic activity of irradiated spores, during their first 10 hours of growth in PDB liquid culture medium. The values for all flow cytometer readings are presented in arbitrary units (A.U.).

Most of the signals from contaminant debris in spore suspensions were excluded during data analysis with the use of gates and regions. In this work, these gates were set to include all particles that were at least as big (FSC) and as complex (SSC) as the smaller spores of the population, thus removing from analysis much of the smaller debris. However, particles with size similar to the spore population could not be filtered using this strategy, which could produce false positives/negatives during analysis. Collected data were methodically gated to remove smaller debris from analysis, therefore the effects caused by the presence of such particles in the obtained results were negligible.

4.3.1 Growth assessment in non-irradiated spores

Throughout the duration of the assay, there was an increase in size and in complexity of spores, as revealed by the geometric mean (GM) values of the FSC and SSC (Table 1, see 0 kGy; Fig 1). After the first 4 h of incubation, a clear increase in spore size (FSC) was observed, generally with smaller changes in complexity (SSC). This is probably due to spore

swelling, which happens before effective germination. This phenomenon was confirmed by observations of the spores under the microscope.

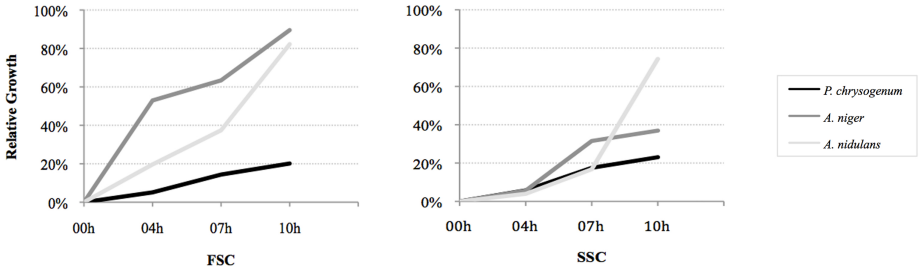

Figure 1 – Relative growth in size (FSC) and complexity (SSC) in the first 10 hours of growth (when compared to initial spore size).

After 10 h of incubation, average FSC values nearly doubled for *Aspergillus niger* and *Aspergillus nidulans*, and increased by approximately 20% for *Penicillium chrysogenum*. Side scatter values increased in a more pronounced manner after 4 hours of incubation, for *A. niger* and *P. chrysogenum*, and after 7 h for *A. nidulans*, as a consequence of the initial hyphae development (also confirmed by microscope observation), meaning that this method can also be used as an indicator for that stage of development. After 10 h, SSC had increased 36.5% for *P. chrysogenum*, 37% for *A. niger* and 75% for *A. nidulans* (Table 1).

Table 1 – Geometric mean values of the FSC and SSC of spores in different time points and irradiation doses. Values are presented in arbitrary units (AU). Approximately 30,000 particles were analysed in each run.

		FSC				SSC			
Species	**Dose**	**00h**	**04h**	**07h**	**10h**	**00h**	**04h**	**07h**	**10h**
P. chrysogenum	**00 kGy**	**6.26**	**6.58**	**7.16**	**7.52**	**10.40**	**12.40**	**14.00**	**14.20**
	03 kGy	5.52	6.21	6.48	6.66	11.00	12.50	13.90	14.00
	06 kGy	5.06	5.39	5.39	5.43	10.70	10.90	11.40	11.20
	12 kGy	4.89	4.78	4.87	4.46	10.50	9.65	10.70	8.13
A. niger	**00 kGy**	**7.65**	**11.70**	**12.50**	**14.50**	**44.40**	**46.90**	**58.40**	**60.80**
	03 kGy	7.79	9.69	11.20	12.20	37.40	39.50	48.80	57.30
	06 kGy	7.70	7.77	8.99	9.05	49.60	43.60	56.70	53.30
	12 kGy	8.11	7.91	8.12	8.32	47.50	43.20	48.00	47.30
A. nidulans	**00 kGy**	**16.30**	**19.50**	**22.40**	**29.70**	**60.80**	**63.20**	**71.00**	**106.00**
	03 kGy	15.50	17.20	17.30	17.60	51.50	51.50	53.50	58.40
	06 kGy	7.98	7.98	8.01	7.41	42.90	45.30	43.60	39.80
	12 kGy	6.82	6.51	6.75	6.64	37.40	34.70	35.90	34.90

4.3.2 Effects of gamma irradiation on spore growth

When spores were subjected to gamma irradiation, FSC and SSC values were affected in a dose dependant manner (Table 1). The 9 kGy dose results are not displayed in Table 1 or Figure 2, since they were very similar to the 12 kGy results. The initial spore size after irradiation differed considerably among each species: whereas for *A. niger*, radiation doses up to 12 kGy had little effects on the FSC or SSC values (in fact, the size of irradiated spores was actually larger at the end of the experiment than in the control). For *P. chrysogenum* and *A. nidulans*, a negative correlation between FSC values and gamma irradiation doses was found (Table 1). In *P. chrysogenum*, 3, 6 and 12 kGy doses reduced the average size of spores in 10, 20 and 22% respectively; comparatively, smaller changes were found in the SSC values. In *A. nidulans*, irradiation with 3 kGy decreased spore size by 5%; in contrast, doses of 6 and 12 kGy caused a decrease of 50 % and 58 % respectively. These data strongly suggest that most spores were severely harmed with these radiation doses, and it is possible that the cell wall structure was compromised; nevertheless such a big decrease in size was unexpected.

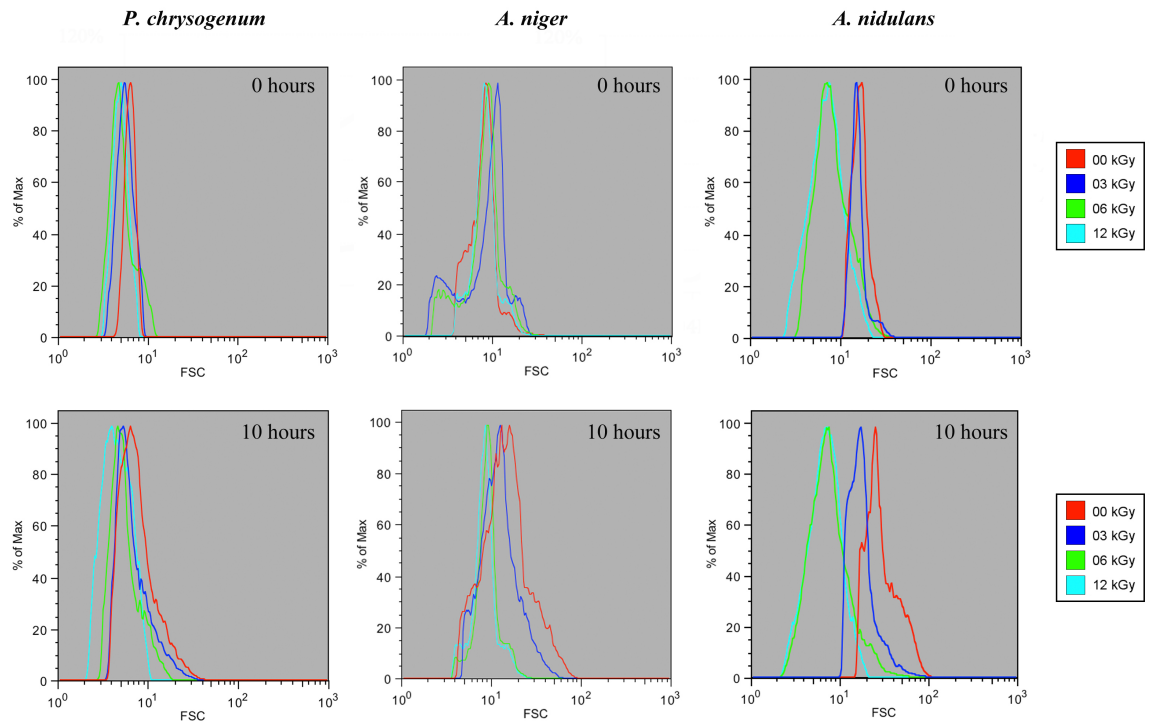

Figure 2 – FSC of irradiated spores after 0 and 10 hours of incubation in PDB. Approximately 30,000 particles were analysed in each run.

In lower irradiation doses (3 kGy), irrespective of the species, some spores kept growing, although at slower rates than in the control. Indeed, at almost every time point, FSC and SSC values of irradiated spores (all species) were lower than in the control. After 10 h, FSC values were 12% lower than the control for *P. chrysogenum*, 16% for *A. niger*, and 40% for *A. nidulans*. With exception, of *A. nidulans*, where a significant decrease was observed after 10h, for the other species, SSC values were similar to those of the control (Table 1).

When 6 kGy were applied, only some of the *P. chrysogenum* and *A. niger* spores grew, causing a slight increase in FSC values in the first 4-7 hours. And if *A. niger* was able to continue growing, *P. chrysogenum* was not, suggesting that even initially viable spores can be rendered unviable after a few hours (Table 1).

A dose of 12 kGy inhibited fungal growth completely in all species, as revealed by the FSC values, which generally decreased over time. At this high dose, SSC was also affected in all species, especially after 10h. Some spores that might have resisted this dose were certainly detrimentally affected by gamma radiation, and were unlikely to survive or to develop normally (Table 1). This is supported by the data provided in Figure 2, which displays the FSC values of irradiated spores from all species, after 0 and 10 hours of incubation in PDB. The effects on growth were generally proportional to the applied radiation dose, with higher doses causing slower growth. In untreated spores, the maximum FSC values (size of bigger spores present in the sample), showed an increase of 3-fold for *A. nidulans*, and 4-fold for *P. chrysogenum and A. niger* (Fig 2). Information gathered from other incubation periods and radiation doses support that some of the irradiated spores that started to grow, had a development arrest after a few hours (data not shown).

We successfully monitored the size and complexity of spores during the first hours of germination, and assessed the radiation effects in these parameters, indicating that this method can provide interesting results on fungal spore growth in liquid culture medium, and that this is in agreement with previous research (e.g. Bradner and Nevalainen 2003).

4.3.3 Spore viability assessment

In this assay our aim was to test a medium-independent analysis of the viability of fungal spores after gamma irradiation treatments; thus water was used instead of culture medium. Propidium Iodide has already been used in flow cytometry to assess the viability

of yeasts and bacteria (Green et al. 1994; Xiao et al. 2010), but in fungal spores it is not a common procedure. Our analyses revealed that in all tested species, independently of the irradiation dose, PI fluorescence intensity was 5 to 7 times higher in irradiated than in non-irradiated spores (Table 2; Fig. 3). Similar differences in fluorescence intensity have been described for yeasts and bacteria (Green et al. 1994; Xiao et al. 2010).

As expected, in general, there was a positive correlation between irradiation doses and the percentage of unviable cells (Table 2).

Table 2 – Geometric mean of the PI fluorescence intensity for the Viable and Unviable spore populations at 0 hours. Values are presented in arbitrary units (AU). Approximately 30,000 particles were analysed each run.

	P. chrysogenum	***A. niger***	***A. nidulans***
Viable	11.0	20.0	11.5
Unviable	62.3	154.0	53.6

The 3 kGy irradiation dose had a more relevant effect in *P. chrysogenum* (70.2% of unviable spores), than in *A. nidulans* (42.8%) and *A. niger* (6.54%). This higher resistance of *A. niger* to 3 kGy dose may be probably a consequence of its high melanin content, being capable to absorb electromagnetic radiation (Dadachova and Casadevall 2008). However, this was not as effective in protecting the interior of the cell when higher doses were used. Nearly 1-log reduction (90%) of viable counts was obtained after exposure to 6 kGy. *P. chrysogenum* was the most sensitive species in this test, with a higher percentage of unviable spores in almost all irradiation doses (98.5% at 15 kGy, almost 2-log).

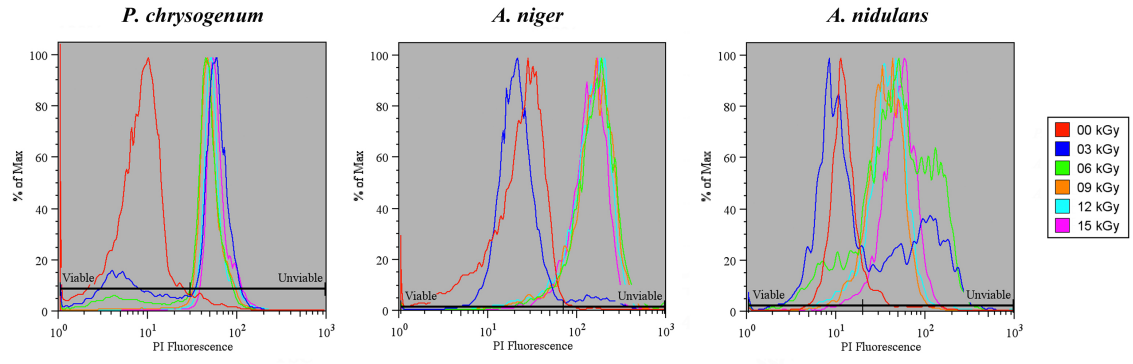

Figure 3 – PI Fluorescence in gamma irradiated spores after irradiation with different doses (at 0 hours). Approximately 30,000 particles were analysed in each run.

In general, the D_{10} dose (that corresponds to 90% of inactivation) fell between 6 and 9 kGy for all three species. In spore survival analysis, direct comparison of D_{10} values between different published works is somewhat difficult because of different testing conditions: the inoculum strain, concentration, irradiation support (water, culture media or others) or even dose/rate values (Saleh et al. 1988; Blank and Corrigan 1995).

4.3.4 Metabolic activity of non-irradiated spores

Non-irradiated spores of all species presented a very low DHE fluorescence at 0 hours, an indicator of very little or even non-existent metabolic activity. This was expected since spores need some time to initiate germination after incubation. Thus, no significant amount of ROS were produced in spores at the beginning of the test, and the detected DHE fluorescence signal was consequently very low (Table 3; Fig 4).

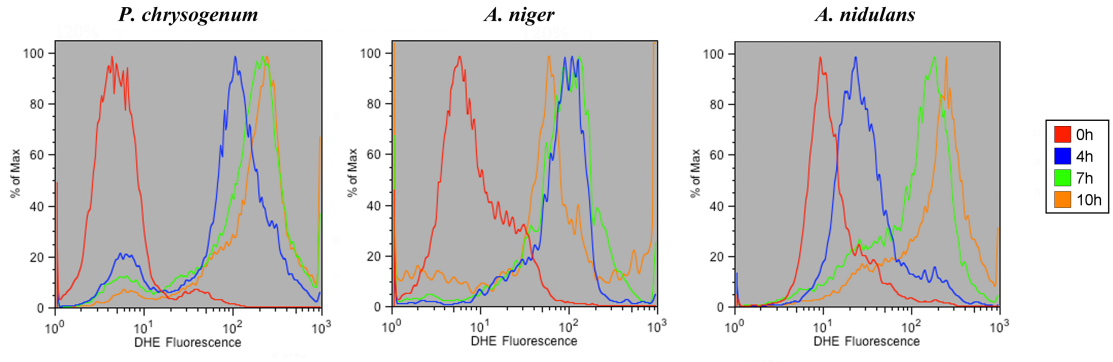

Figure 4 – DHE Fluorescence of non-irradiated spores after 0 and 10 hours. Approximately 30,000 particles were analysed in each run.

After 4 h, there was an obvious increase in fluorescence, with a ~10-fold increase for *P. chrysogenum* and *A. niger* (Table 3, 0 kGy). In comparison, *A. nidulans* started to grow more slowly, as shown in Table 1, so instead, nearly 10 hours were required for this species to display a similar increase. It was interesting to see that fluorescence consistently increased before actual cell growth occurred. As displayed in Table 1, the growth of most *P. chrysogenum* increased only after 7; still, an increase in DHE fluorescence was already clear after 4 h (almost a 12-fold increase), and continued until the end of the experiment.

It is likely that an increase in metabolic activity happens prior to spore germination, while the biological functions of the dormant spores are re-initiating. Accordingly, *A. niger* spores were actively growing after 4 h (50% increase in FSC; Fig 1), and thus were already very

active at that stage, as confirmed by the DHE fluorescence that had already increased 9-fold (Table 3, 0 kGy).

Table 3 – Percentage of unviable spores after irradiation with different doses, according to the PI fluorescence intensity at 0 hours. Approximately 30,000 particles were analysed in each run.

	Radiation Dose					
	0 kGy	**3 kGy**	**6 kGy**	**9 kGy**	**12 kGy**	**15 kGy**
P. chrysogenum	3.93	70.2	82.3	95.6	96.9	98.5
A. niger	0.83	6.54	89.3	90.6	90.1	91.2
A. nidulans	12.5	42.8	83.3	89.2	89.4	95.1

In the subsequent hours, fluorescence continued to increase, but at lower rates, suggesting that *A. niger* spore activity was already optimized after a 4 to 7 h incubation period. Figure 5 shows that after 0, 4 and 7 hours, spores that were bigger presented a higher DHE fluorescence, meaning that they were metabolically more active.

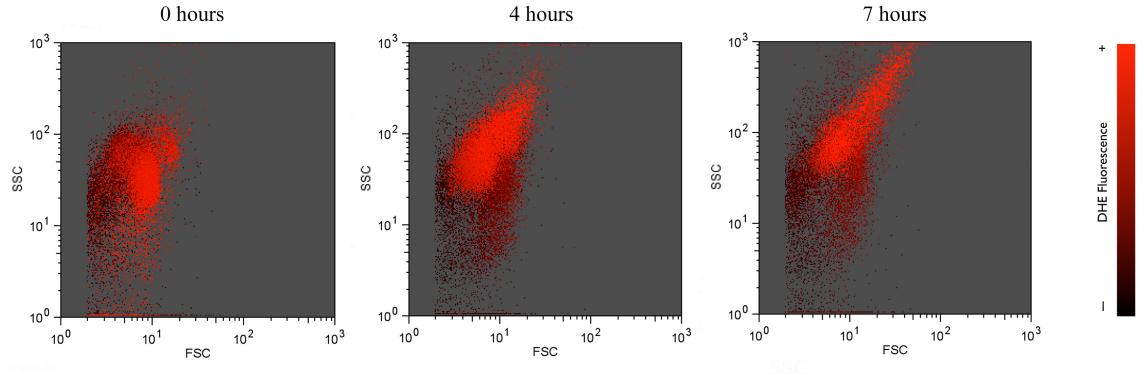

Figure 5 – FSC, SSC and DHE Fluorescence of non-irradiated spores of Aspergillus niger after 0, 4 and 7 hours of incubation in PDB. The colour gradient signals a low (black) to high (red) DHE fluorescence intensity. Approximately 30,000 particles were analysed in each run.

During the first 4 h, *A. nidulans*, the increase in DHE fluorescence was not as significant as in the other species, but after 7h and 10h the values were already comparable (Table 3). Furthermore, *A. nidulans* spores presented the highest complexity values, which had almost doubled after 10 hours (Table 1). Even though it started to grow slower, *A. nidulans*

presented a big increase in FSC and SSC values after 10 hours (Table 1, 0 kGy), which is also supported by the DHE fluorescence results (Table 3, 0 kGy).

4.3.5 Effects of gamma irradiation on metabolic activity

As previously described, gamma radiation promotes the formation of ROS within the cell (McNamara et al. 2003). For that reason, even at 0 h, all irradiated samples presented higher DHE fluorescence than the control, with exception of *A. nidulans* (with 6 kGy and higher doses; Table 3). It is worth mentioning that in this species, exposure to 6 kGy and higher doses decreased the initial spore size (Table 1).

Some spores did survive lower radiation doses and were able to grow, with the ROS fluorescence increasing more intensely afterwards. As an example, the initial DHE fluorescence in *P. chrysogenum* after irradiation with 3 kGy was about 6.8 times more intense than in the control (Table 3), however, the robust spores actually started to grow between 4 and 10 h, and in the end, their DHE fluorescence was nearly 10-fold higher than that of dead, unviable or otherwise stagnant spores (Fig 6). Various environmental factors are responsible for the generation of ROS, ionizing radiation (α, β, γ, and X-rays) and UV radiation are two examples. Recent works show that ROS also have a role in the regulation of proliferation, differentiation, extracellular signal transduction, ion transfer, and immune response (Droge 2002).

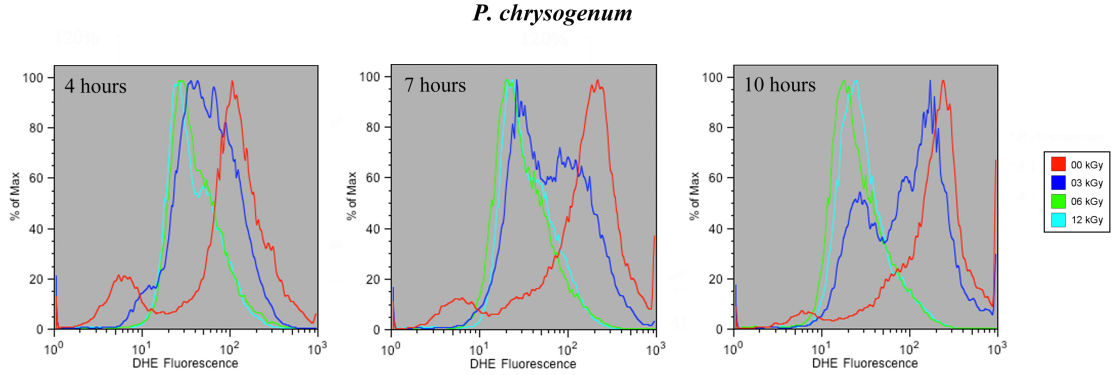

Figure 6 – DHE Fluorescence of Penicillium chrysogenum spores (irradiated with 0-12 kGy) after 4, 7 and 10 hours of incubation in PDB. Approximately 30,000 particles were analysed in each run.

For 6 kGy, the DHE fluorescence of *P. chrysogenum* and *A. nidulans*, is initially high (caused by ROS production during irradiation) but it decreases with time, meaning that there is almost no metabolic activity (Table 3), which is confirmed by its reduced growth (Table 1).

These results indicate that spores that were viable after irradiation with 6 kGy, were able to start growing, but stopped growing after 7 hours of incubation. In *A. nidulans*, since an increase in fluorescence at 0 h was not observed, fluorescence was always low for 6 kGy and higher doses.

Finally, with 12 kGy, the initial DHE fluorescence of *P. chrysogenum* and *A. niger* was considerably more intense than with lower doses, probably a consequence of the higher concentration of ROS formed by the ionizing radiation. Since very little viability levels or growth were detected in spores at that doses, the trend in DHE fluorescence decreased with time, as there was no biological activity to maintain ROS production (Table 3).

4.4 Conclusions

It is common to use the number of colony forming units as a way to assess the bioburden level, and therefore the contamination potential of one room or one document. After a decontamination treatment is applied, some resistant viable spores can nonetheless be severely affected by the treatment they underwent (i.e., genetically, chemically, or other), occasionally to the point that they might not be able to develop normally after germination.

By combining the analysis of FSC, SSC and fluorescence, we verified that, despite some of the spores were viable after irradiation they were incapable of growth after a few hours, meaning that they would be unlikely to damage a document either. This work suggests that lower radiation doses could possibly be applied effectively in the treatment of ancient objects, since effects of doses as low as 6 kGy can prevent continued fungal growth of species we examined, even after they had started developing in liquid culture medium.

Overall, we were able to analyse the viability, growth and metabolic activity of irradiated spores using flow cytometry during the early stages of fungal spore growth. Results were consistent between radiation doses on all tested species: irradiated spores generally grew less, later, and were less metabolically active. Furthermore, FSC and SSC results were concordant with the DHE metabolic activity results: the DHE fluorescence intensity was higher in growing spores, but presented little variation in dead cells, sometimes even decreasing over time. The fluorescence intensity of PI in unviable cells was 5 to 7x higher

than in viable cells, allowing the assessment of the effects of a sterilization treatment without the need to re-culture the cells.

It is worth emphasizing that these analyses can be performed immediately after a given treatment, without the need to re-inoculate, incubate and perform colony counts. This method provides a rapid insight into the condition/resistance of different organisms. Furthermore, unculturable, yet viable spores can also be screened. This study with fungal cells treated with gamma irradiation, opens new doors to similar studies using other bio-inactivation procedures.

Chapter 5

Can arthropods act as vectors of fungal dispersion in heritage collections?

A case study on the Archive of the University of Coimbra.

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Chapter 5 - Can arthropods act as vectors of fungal dispersion in heritage collections? A case study on the Archive of the University of Coimbra.

Abstract - A previous work that used documents from the Archive of the University of Coimbra, described a high fungal diversity colonizing different types of documents. Cladosporium, Aspergillus and Penicillium were the most common genera found in documents where biodegradation was more evident.

The presence of arthropods circulating through the Archive suggested that zoochoric dispersion could play a significant role in the dispersion of fungal species between documents in similar environments. The aims of this study were to evaluate the role of arthropods as fungal dispersers and potential vectors of contamination between the documents from the Archive of the University of Coimbra; to study the effects of seasonality in the diversity of fungal species associated with invertebrates; and to understand the relationship between the identified fungal species and the different arthropod *taxa*. The sequencing of the total ITS region combined with the analysis of macroscopic and microscopic taxonomic traits, provided data on the fungal diversity carried by arthropods. Results show a high fungal diversity associated with invertebrates: from a total of over 148 isolates, 25 fungal genera and 59 species were identified and kept in culture. The most common genera were Penicillium, Aspergillus and Cladosporium.

Keywords: Ancient documents; ITS sequencing; Entomopathogenic Fungi; Arthropod Vectored dispersion.

5.1 Introduction

5.1.1 Biodeterioration by fungal organisms of documents held in the Archive of the University of Coimbra

The Archive of the University of Coimbra (AUC) holds a vast documentation asset, either produced or received by the University since its foundation by King D. Dinis in 1290, reflecting the history of the University in a unique way (Vasconcelos 1991). Dated from 1948, the AUC is a modern building, constructed with a great diversity of materials. It holds documents made of different supports, with several centuries of history, enhancing the possibility of different ecological niches to occur with adequate conditions for the development of microorganisms such as fungi and bacteria (Hyvärinen *et al.* 2002).

The enzymatic capabilities of fungi allow them to easily colonize new substrates. They have been described colonizing paints, oils, paper, leather, textiles, adhesives as well as other materials used in the production of art objects (Jurado *et al.* 2008). Although some of their characteristics have been used to the benefit of humans cultures, others can also lead to the loss of important patrimony and historical assets through the phenomena of biodeterioration and biodegradation (Sterflinger 2010). When present in paper, cellulolytic fungi degrade the cellulose and release metabolites that can be responsible for staining the support. These staining spots, not exclusively a result of fungal activity, are known as Foxing (Meynell and Newsam 1978; Arai 2000).

In a previous work, Mesquita *et al.* (2009) described a diverse fungal community colonizing different paper and parchment documents, which were stored in the Archive of the University of Coimbra. A high frequency of species from the genera *Cladosporium, Aspergillus* and *Penicillium* were found. These are ubiquitous *taxa*, which produce numerous conidia and mitospores that are easily dispersed by air, suggesting that anemophilous dispersion is an important mechanism for fungal dispersion within the Archive (Mesquita *et al.* 2009). However, studies with airborne fungi revealed a low diversity in the air of the Archive (unpublished data), which lead us to consider other complementary mechanisms in the dispersion of fungal propagules between documents.

5.1.2 Arthropod vectored dispersion

Arthropod vectored dispersion of spores is a common spreading mechanism of many fungal species (Roets *et al.* 2011). Spores are, generally, the main fungal propagules, and the different dissemination strategies that are used by the different fungal organisms are one of the key factors that contribute to their success and diversity. Dry spores are generally dispersed through the air, while sticky spores usually rely on water or other vectors for dispersal. For example, some Ascomycota can produce spores that float and use water as a mean of dispersion, but they can also use insects and other arthropods to move their spores to new substrata for colonization (Abbot 2002). The production of fungal propagules that are capable of adhering to other materials also contributes to their dispersal to different substrates (Holz *et al.* 2007).

Spores can be spread by adhering to insects that move through contaminated substrata or by the ingestion and excretion of fungal propagules (Dromph 2003). In nature, many fungi are subject to predation by arthropods, due to their ability to accumulate high amounts of nitrogen and phosphorous (Ruess and Lussenhop 2005). As a response to the lack of nutrients, many fungal species are able to differentiate cells in dormant forms (spores), which are resistant to various types of environmental stresses. This resistance is usually associated with the cell wall, which is more complex than that of most plant cells, allowing the passage through the digestive tract of arthropods while maintaining their viability, and allowing an efficient spreading by invertebrates (Collucio *et al.* 2008).

The presence of entomopathogenic species is linked to arthropod vectored dispersion since the conidia of these species can adhere to the cuticle of a host, and with proper conditions of humidity and temperature develop hyphae that penetrate the cuticle and invade the circulatory system, leading to the host death, and consequent body mummification. This ability to feed on bodies of dead arthropods allows them to grow and colonize new environments (Jurado *et al.* 2008).

5.1.3 The ITS region as a specific fungal marker

The sequencing of the total ITS (Internal Transcribed Spacer) region is a good “molecular tool” for the identification of fungal species. It is a sequence of non-coding ribosomal RNA (rRNA) of a common precursor transcript that has already been confirmed as a powerful

tool for molecular species identification (White *et al.* 1990; Martin *et al.* 2005), as demonstrated in works like those of Schmidt and Moreth (2002), Michaelsen *et al*. (2006) and Schoch *et al*. (2012).

The regions ITS1, the 5.8 S rDNA and ITS2 have benefited of a rapid evolution rate, resulting in a high variation between related species, which makes them specific and therefore useful markers (Korabecna 2007; Bellemain 2010).

5.1.4 Objectives

The main objectives of this study were: to evaluate the role of arthropods as dispersers of fungal propagules and their potential as vectors of contamination between documents; to understand the effects of seasonality on the diversity of fungi associated with arthropods; to assess the relation between fungal species and the corresponding host arthropods.

5.2 Materials and methods

5.2.1 Sampling and fungal culture

Arthropods were collected using sticky base insect traps (www.historyonics.com) that were placed in six different floors and sections from the Archive, at different heights above the ground (ten numbered traps were randomly scattered in each section; η= 80). Traps were checked for arthropods every 2 to 3-months, to assess possible seasonality effects. Arthropods were collected from the traps, using a sterile scalpel blade to separate them from the gluing surface.

Retrieved arthropods were identified to the order level (Brusca and Brusca 2003; Barnard 2011) macerated in sterile water (200µl), and the resulting homogenate was inoculated on PDA (Potato Dextrose Agar) Petri plates with streptomycin (0.5g / L) to prevent bacterial growth.

The different colonies were isolated to axenic cultures and incubated for five to ten days at 28±1ºC, according to their growth rate. The plates were kept for eighteen to twenty days to ensure that slow growing fungi were also isolated. Each new colony was linked to the original trap/site and to the insect that was collected (e.g. A23A- isolate A, from arthropod Thysanura collected in the trap A23).

5.2.2 Molecular and Morphological identification

When the colonies had grown enough for DNA extraction, hyphae were scraped from the agar surface using a sterile scalpel, followed by total DNA extraction using an ABI Prism™ 6100 Nucleic Acid PrepStation, according to the manufacturer standards.

The extracted DNA was subject to amplification of the total ITS region by PCR, using primers ITS4 and ITS1F (White *et al.* 1990; Gardes and Bruns 1996). For a final amplification volume of 25µl, PCR mixes with 12,5µl of Jump Start Taq DNA Polymerase with $MgCl_2$ (Sigma D9307), 0,5µl of each primer (10mM), 10,5µl of ultra-pure water and 1µl of template DNA were used. 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, 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 under a UV light trans-illuminator (Bio Rad Gel Doc XR+). Sequencing of the ITS region was performed with an ABI 3730 Genetic Analyzer, using the Big Dye v.3 Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems), and using the primer ITS4.

Genetic sequences were analyzed using Geneious 5.3.6™ software (www.geneious.com), and were ran against NCBI's BLAST (Basic Local Alignment Search Tool) database in order to assess the similarity with published sequences. In order to confirm the molecular identification, a macroscopic and microscopic analysis of taxonomic traits was also performed (Watanabe 2002).

If the similarity was higher than 95%, the molecular identification was considered a valid match. Molecular identifications with percentages of similarity below this threshold were thoroughly confirmed by morphological identification.

5.2.3 Statistical analysis

Statistical analysis was performed, using the Shannon-Wiener index ($H' = -\Sigma\ Pi \ln(Pi)$) and the species evenness ($E = H' / \ln(S)$), for each floor, arthropod order, and season. Shannon-Wiener index is one of the several diversity indices used to measure diversity in categorical data. It is the analysis of the entropy, treating the species distribution and size of a

population as a probability. This index is used to determine biodiversity values that take into account the number of species, the dominant species and their distribution. Shannon-Wiener index has to be within average values of ($0 < H´ < \ln(S)$) and species evenness between average values of ($0 < E < 1$).

Regarding the Shannon-Wiener index according to arthropod order, the invertebrates caught on the first harvest (preliminary results; in Table 1 indicated as harvest 0) were not taken in account since order identification was not performed.

Table 1 – Identification of fungal isolates obtained after isolation on PDA, per trap location, season, harvest, and order of the captured arthropod (NI- not identified; sp. - isolates identified using only the molecular approach).

Trap	Isolate	Floor	Section	Collection	Season	Arthropod (Order)	Fungal Identification	Similarity	Acession Number
A06	A	2	3	0	Autumn ´10	N.I.	*Biscogniauxia mediterranea*	99%	JQ781705
A06	B	2	3	0	Autumn ´10	N.I.	*Biscogniauxia mediterranea*	99%	JQ781706
A06	C	2	3	0	Autumn ´10	N.I.	*Biscogniauxia mediterranea*	99%	JQ781707
A06	D	2	3	0	Autumn ´10	N.I.	*Biscogniauxia mediterranea*	99%	JQ781708
A06	E	2	3	0	Autumn ´10	N.I.	*Biscogniauxia mediterranea*	99%	JQ781709
A07	A	2	3	0	Autumn ´10	N.I.	*Penicillium miniolutum*	99%	JQ781710
A07	B	2	3	0	Autumn ´10	N.I.	*Coniothyrium sporulosum*	99%	JQ781711
A07	C	2	3	0	Autumn ´10	N.I.	*Penicillium commune*	99%	JQ781712
A07	D	2	3	0	Autumn ´10	N.I.	*Penicillium commune*	99%	JQ781713
A07	E	2	3	0	Autumn ´10	N.I.	*Penicillium commune*	99%	JQ781714
A07	F	2	3	0	Autumn ´10	N.I.	*Penicillium commune*	100%	JQ781715
A07	G	2	3	0	Autumn ´10	N.I.	*Aspergillus nomius*	89%	JQ781716
A10	A	2	3	0	Autumn ´10	N.I.	*Aspergillus nomius*	99%	JQ781719
A10	B	2	3	0	Autumn ´10	N.I.	*Stereum hirsutum*	99%	JQ781720
A12	A	2	2	0	Autumn ´10	N.I.	*Aspergillus nomius*	99%	JQ781726
A12	B	2	2	0	Autumn ´10	N.I.	*Aspergillus nomius*	99%	JQ781727
A16	A	2	2	0	Autumn ´10	N.I.	*Lecanicillium psaliotae*	100%	JQ781732
A22	A	Basement		0	Autumn ´10	N.I.	*Aspergillus versicolor*	99%	JQ781743
A28	B	Basement		0	Autumn ´10	N.I.	*Beauveria Bassiana*	100%	JQ781747
A28	C	Basement		0	Autumn ´10	N.I.	*Penicillium brasilianum*	100%	JQ781748
A28	D	Basement		0	Autumn ´10	N.I.	*Penicillium bilaiae*	100%	JQ781749
A28	E	Basement		0	Autumn ´10	N.I.	*Penicillium bilaiae*	100%	JQ781750
A28	F	Basement		0	Autumn ´10	N.I.	*Penicillium bilaiae*	100%	JQ781751

Trap	Isolate	Floor	Section	Collection	Season	Arthropod (Order)	Fungal Identification	Similarity	Acession Number
A35	A	3	2	0	Autumn ´10	N.I.	*Cladosporium cladosporioides*	99%	JQ781771
A35	B	3	2	0	Autumn ´10	N.I.	*Aspergillus versicolor*	99%	JQ781772
A35	C	3	2	0	Autumn ´10	N.I.	*Penicillium chrysogenum*	100%	JQ781773
A35	E	3	2	0	Autumn ´10	N.I.	*Alternaria arborescens*	97%	JQ781774
A35	F	3	2	0	Autumn ´10	N.I.	*Alternaria sp.*	100%	JQ781775
A40	A	3	2	0	Autumn ´10	N.I.	*Penicillium chrysogenum*	99%	JQ781777
A52	A	4	3	0	Autumn ´10	N.I.	*Penicillium chrysogenum*	100%	JQ781778
A52	B	4	3	0	Autumn ´10	N.I.	*Cladosporium sphaerospermum*	100%	JQ781779
A53	A	4	3	0	Autumn ´10	N.I.	*Penicillium decaturense*	99%	JQ781783
A53	A1	4	3	0	Autumn ´10	N.I.	*Cladosporium cladosporioides*	89%	JQ781784
A53	B	4	3	0	Autumn ´10	N.I.	*Cladosporium cladosporioides*	100%	JQ781785
A60	A	4	3	0	Autumn ´10	N.I.	*Biscogniauxia mediterranea*	99%	JQ781799
A63	A	6	3	0	Autumn ´10	N.I.	*Penicillium chrysogenum*	99%	JQ781800
A63	C	6	3	0	Autumn ´10	N.I.	*Cladosporium cladosporioides*	100%	JQ781801
A63	D	6	3	0	Autumn ´10	N.I.	*Penicillium crustosum*	100%	JQ781802
A63	E	6	3	0	Autumn ´10	N.I.	*Penicillium decaturense*	99%	JQ781803
A63	F	6	3	0	Autumn ´10	N.I.	*Penicillium islandicum*	99%	JQ781804
A65	A	6	3	0	Autumn ´10	N.I.	*Penicillium decaturense*	99%	JQ781811
A65	B	6	3	0	Autumn ´10	N.I.	*Penicillium decaturense*	99%	JQ781812
A65	C	6	3	0	Autumn ´10	N.I.	*Penicillium brevicompactum*	99%	JQ781813
A65	D	6	3	0	Autumn ´10	N.I.	*Penicillium brevicompactum*	97%	JQ781814
A65	E	6	3	0	Autumn ´10	N.I.	*Penicillium sp.*	100%	JQ781815
A69	A	6	3	0	Autumn ´10	N.I.	*Alternaria alternata*	100%	JQ781820
A70	A	6	3	0	Autumn ´10	N.I.	*Penicillium chrysogenum*	100%	JQ781825
A77	A	6	2	0	Autumn ´10	N.I.	*Paecilomyces lilacinus*	99%	JQ781830
A87	A	5	3	0	Autumn ´10	N.I.	*Chaetomium nigricolor*	99%	JQ781840
A87	B	5	3	0	Autumn ´10	N.I.	*Alternaria infectoria*	100%	JQ781841
A87	C	5	3	0	Autumn ´10	N.I.	*Penicillium brevicompactum*	99%	JQ781842
A87	D	5	3	0	Autumn ´10	N.I.	*Penicillium sp.*	99%	JQ781843
A87	E	5	3	0	Autumn ´10	N.I.	*Penicillium crustosum*	97%	JQ781844
A23	A	Basement		I	Winter ´10	Thysanura	*Cladosporium cladosporioides*	99%	JQ781746
A29	A	Basement		I	Winter ´10	Thysanura	*Cladosporium sp.*	100%	JQ781760
A53	C	4	3	I	Winter ´10	Thysanura	*Penicillium olsonii*	99%	JQ781786
A55	C	4	3	I	Winter ´10	Diptera	*Cladosporium pini-ponderosae*	100%	JQ781788
A56	A	4	3	I	Winter ´10	Thysanura	*Penicillium chrysogenum*	100%	JQ781794

Trap	Isolate	Floor	Section	Collection	Season	Arthropod (Order)	Fungal Identification	Similarity	Acession Number
A56	B	4	3	I	Winter ´10	Thysanura	*Cryptosphaeria subcutanea*	99%	JQ781795
A59	A	4	3	I	Winter ´10	Araneae	*Penicillium olsonii*	99%	JQ781796
A59	B	4	3	I	Winter ´10	Araneae	*Penicillium olsonii*	99%	JQ781797
A66	A	6	3	I	Winter ´10	Thysanura	*Penicillium dendriticum*	99%	JQ781816
A69	C	6	3	I	Winter ´10	Thysanura	*Cladosporium sp.*	99%	JQ781821
A85	A	5	3	I	Winter ´10	Diptera	*Aspergillus niger*	99%	JQ781837
A15	A	2	2	II	Spring ´10	Diptera	*Aspergillus nomius*	99%	JQ781730
A15	B	2	2	II	Spring ´10	Diptera	*Aspergillus zhaoqingensis*	99%	JQ781731
A55	E	4	3	II	Spring ´10	Diptera	*Aspergillus parasiticus*	99%	JQ781789
A55	E2	4	3	II	Spring ´10	Diptera	*Aspergillus parasiticus*	99%	JQ781790
A55	F	4	3	II	Spring ´10	Diptera	*Penicillium griseofulvum*	99%	JQ781791
A64	A	6	3	II	Spring ´10	Thysanura	*Penicillium dendriticum*	99%	JQ781805
A64	B	6	3	II	Spring ´10	Thysanura	*Penicillium dipodomyicola*	100%	JQ781806
A66	B	6	3	II	Spring ´10	Araneae	*Penicillium chrysogenum*	100%	JQ781817
A69	C2	6	3	II	Spring ´10	Diptera	*Acremonium furcatum*	87%	JQ781822
A05	A	2	3	III	Summer ´10	Araneae	*Daldinia concentrica*	99%	JQ781703
A05	B	2	3	III	Summer ´10	Coleoptera	*Daldinia concentrica*	98%	JQ781704
A10	C	2	3	III	Summer ´10	Araneae	*Aspergillus flavus*	99%	JQ781721
A22	B	Basement		III	Summer ´10	Araneae	*Cladosporium sp.*	100%	JQ781744
A22	C	Basement		III	Summer ´10	Araneae	*Penicillium griseofulvum*	99%	JQ781745
A28	G	Basement		III	Summer ´10	Thysanura	*Thielavia hyalocarpa*	99%	JQ781752
A29	B	Basement		III	Summer ´10	Thysanura	*Penicillium sp.*	100%	JQ781761
A55	G	Basement		III	Summer ´10	Araneae	*Penicillium griseofulvum*	100%	JQ781792
A59	C	4	3	III	Summer ´10	Araneae	*Neonectria discophora*	85%	JQ781798
A64	B2	6	3	III	Summer ´10	Araneae	*Aspergillus versicolor*	100%	JQ781807
A79	A	6	2	III	Summer ´10	Thysanura	*Penicillium sp.*	99%	JQ781832
A10	D	2	3	IV	Summer ´10	Diptera	*Cladosporium cucumerinum*	99%	JQ781722
A10	E	2	3	IV	Summer ´10	Araneae	*Periconia macrospinosa*	88%	JQ781723
A10	F	2	3	IV	Summer ´10	Araneae	*Penicillium citrinum*	99%	JQ781724
A12	C	2	2	IV	Summer ´10	Diptera	*Epicoccum nigrum*	100%	JQ781728
A12	C2	2	2	IV	Summer ´10	Diptera	*Epicoccum nigrum*	100%	JQ781729
A16	B	2	2	IV	Summer ´10	Diptera	*Phlebiopsis gigantea*	94%	JQ781733
A28	G2	Basement		IV	Summer ´10	Thysanura	*Beauveria Bassiana*	100%	JQ781753
A52	C	4	3	IV	Summer ´10	Lepidoptera	*Penicillium brevicompactum*	98%	JQ781780
A52	C1	4	3	IV	Summer ´10	Lepidoptera	*Aspergillus nomius*	80%	JQ781781
A52	D	4	3	IV	Summer ´10	Araneae	*Phlebiopsis gigantea*	99%	JQ781782
A53	D	4	3	IV	Summer ´10	Araneae	*Absidia anomala*	99%	JQ781787
A55	H	4	3	IV	Summer ´10	Araneae	*Penicillium chrysogenum*	100%	JQ781793
A66	C	6	3	IV	Summer ´10	Thysanura	*Fomes fomentarius*	95%	JQ781818
A66	D	6	3	IV	Summer ´10	Thysanura	*Penicillium chrysogenum*	100%	JQ781819
A69	D	6	3	IV	Summer ´10	Araneae	*Aspergillus candidus*	99%	JQ781823
A69	D1	6	3	IV	Summer ´10	Araneae	*Cladosporium sp.*	99%	JQ781824

Trap	Isolate	Floor	Section	Collection	Season	Arthropod (Order)	Fungal Identification	Similarity	Acession Number
A70	B	6	3	IV	Summer ´10	Thysanura	*Cladosporium cucumerinum*	100%	JQ781826
A70	C	6	3	IV	Summer ´10	Araneae	*Beauveria sp.*	100%	JQ781827
A70	D	6	3	IV	Summer ´10	Araneae	*Penicillium sp.*	100%	JQ781828
A70	E	6	2	IV	Summer ´10	Araneae	*Acremonium strictum*	99%	JQ781829
A77	B	6	2	IV	Summer ´10	Thysanura	*Penicillium chrysogenum*	100%	JQ781831
A85	B	5	3	IV	Summer ´10	Thysanura	*Phlebiopsis gigantea*	99%	JQ781838
A85	C	5	3	IV	Summer ´10	Thysanura	*Penicillium chrysogenum*	100%	JQ781839
A87	F	5	3	IV	Summer ´10	Diptera	*Alternaria alternata*	100%	JQ781845
A03	A	2	3	V	Autumn ´11	Lepidoptera	*Penicillium griseofulvum*	84%	JQ781700
A03	B	2	3	V	Autumn ´11	Lepidoptera	*Cladosporium sp.*	91%	JQ781701
A03	C	2	3	V	Autumn ´11	Lepidoptera	*Penicillium commune*	99%	JQ781702
A10	G	2	3	V	Autumn ´11	Thysanura	*Penicillium commune*	99%	JQ781725
A16	B2	2	2	V	Autumn ´11	Thysanura	*Phlebiopsis gigantea*	94%	JQ781734
A16	C	2	2	V	Autumn ´11	Thysanura	*Penicillium crustosum*	98%	JQ781735
A16	D	2	2	V	Autumn ´11	Thysanura	*Penicillium brevicompactum*	78%	JQ781736
A16	E	2	2	V	Autumn ´11	Thysanura	*Aspergillus nomius*	84%	JQ781737
A20	A	2	2	V	Autumn ´11	Thysanura	*Penicillium raistrikii*	72%	JQ781739
A20	B	2	2	V	Autumn ´11	Thysanura	*Penicillium expansum*	94%	JQ781740
A20	C	2	2	V	Autumn ´11	Thysanura	*Penicillium sp.*	96%	JQ781741
A20	D	2	2	V	Autumn ´11	Thysanura	*Cladosporium sp.*	90%	JQ781742
A28	H	Basement		V	Autumn ´11	Thysanura	*Penicillium commune*	100%	JQ781754
A28	I	Basement		V	Autumn ´11	Thysanura	*Cladosporium sp.*	100%	JQ781755
A28	K	Basement		V	Autumn ´11	Thysanura	*Penicillium brevicompactum*	99%	JQ781756
A28	L	Basement		V	Autumn ´11	Thysanura	*Penicillium commune*	99%	JQ781757
A29	C	Basement		V	Autumn ´11	Thysanura	*Alternaria arborescens*	99%	JQ781762
A29	D	Basement		V	Autumn ´11	Thysanura	*Cladosporium sp.*	100%	JQ781763
A29	E1	Basement		V	Autumn ´11	Thysanura	*Penicillium commune*	99%	JQ781764
A29	E2	Basement		V	Autumn ´11	Thysanura	*Penicillium chrysogenum*	99%	JQ781765
A29	F	Basement		V	Autumn ´11	Thysanura	*Armillaria sp.*	84%	JQ781766
A29	G	Basement		V	Autumn ´11	Thysanura	*Penicillium canescens*	100%	JQ781767
A30	A	Basement		V	Autumn ´11	Lepidoptera	*Penicillium chrysogenum*	99%	JQ781768
A30	B	Basement		V	Autumn ´11	Lepidoptera	*Cladosporium sp.*	100%	JQ781769
A30	C	Basement		V	Autumn ´11	Lepidoptera	*Epicoccum nigrum*	100%	JQ781770
A64	C	4	3	V	Autumn ´11	Thysanura	*Penicillium spinulosum*	99%	JQ781808
A64	D	4	3	V	Autumn ´11	Thysanura	*Penicillium griseofulvum*	99%	JQ781809
A64	E	4	3	V	Autumn ´11	Thysanura	*Penicillium dendriticum*	99%	JQ781810
A83	A	5	3	V	Autumn ´11	Araneae	*Penicillium griseofulvum*	99%	JQ781833
A83	B	5	3	V	Autumn ´11	Araneae	*Penicillium griseofulvum*	99%	JQ781834
A84	A	5	3	V	Autumn ´11	Thysanura	*Penicillium chrysogenum*	99%	JQ781835
A84	B	5	3	V	Autumn ´11	Thysanura	*Cladosporium tenuissimum*	100%	JQ781836
A87	G	5	3	V	Autumn ´11	Araneae	*Engyodontium album*	100%	JQ781846

Trap	Isolate	Floor	Section	Collection	Season	Arthropod (Order)	Fungal Identification	Similarity	Acession Number
A87	H	5	3	V	Autumn ´11	Araneae	*Penicillium chrysogenum*	99%	JQ781847
A07	H	2	3	VI	Winter ´11	Diptera	*Penicillium brevicompactum*	99%	JQ781717
A07	I	2	3	VI	Winter ´11	Diptera	*Cladosporium cladosporioides*	99%	JQ781718
A19	A	2	2	VI	Winter ´11	Diptera	*Myxotrichum deflexum*	99%	JQ781738
A28	M	Basement		VI	Winter ´11	Diptera	*Penicillium brevicompactum*	85%	JQ781758
A28	N	Basement		VI	Winter ´11	Araneae	*Epicoccum nigrum*	100%	JQ781759
A35	G	3	2	VI	Winter ´11	Diptera	*Stemphylium solani*	99%	JQ781776

5.3 Results

Table 1 displays the results of the identification of fungal species isolated from trapped arthropods in the period between autumn 2010 and winter of 2011, as well as the location of the trap where the isolate was obtained, the order of the arthropod caught, the accession number on Genebank (JQ781700 - JQ781847) and the similarity to the sequences deposited in NCBI databases. From a total of 180 arthropods retrieved using traps, 148 fungal isolates corresponding to 25 different fungal genera and 59 different fungal species (Fig. 1) were isolated, identified and kept in culture. Eight out of the 25 obtained genera (*Aspergillus, Beauveria, Chaetomium, Cladosporium, Engyodontium, Lecanicillium, Penicillium and Paecilomyces*) contain species known to be potentially entomopathogenic.

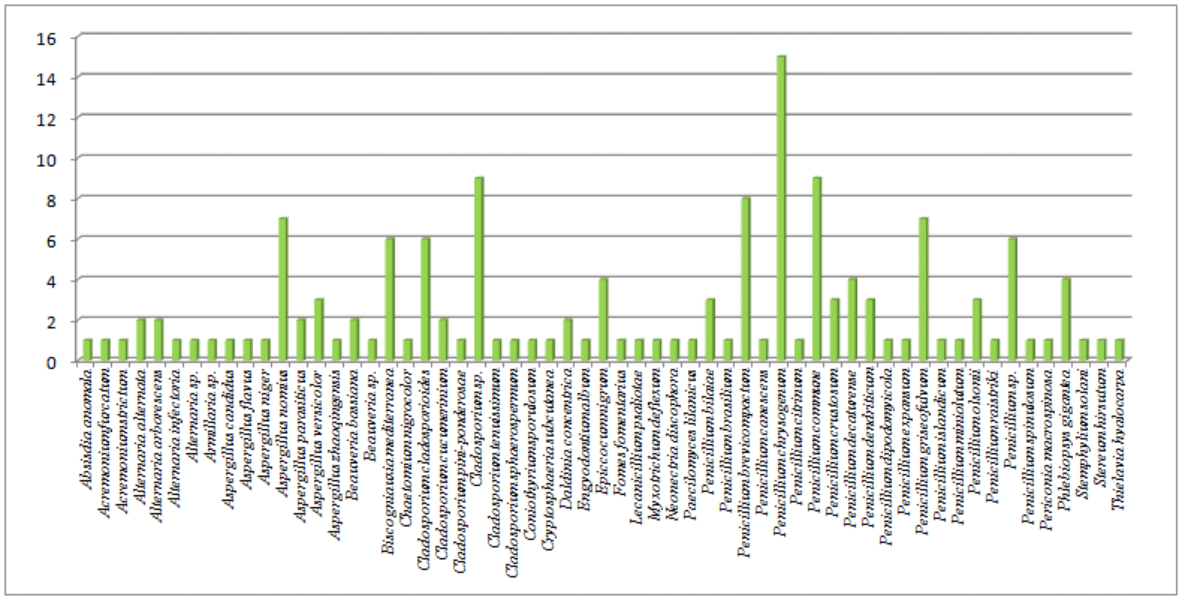

Fig. 1 - Number of isolates by fungal species (sp. - isolates identified using only the molecular approach).

The most common genera were *Penicillium*, *Aspergillus*, *Cladosporium* and *Alternaria* (Fig 2). The most frequent species were *Aspergillus nomius, Biscogniauxia mediterranea, Cladosporium cladosporioides, Penicillium brevicompactum, Penicillium commune, Penicillium chrysogenum* and *Penicillium griseofulvum.*

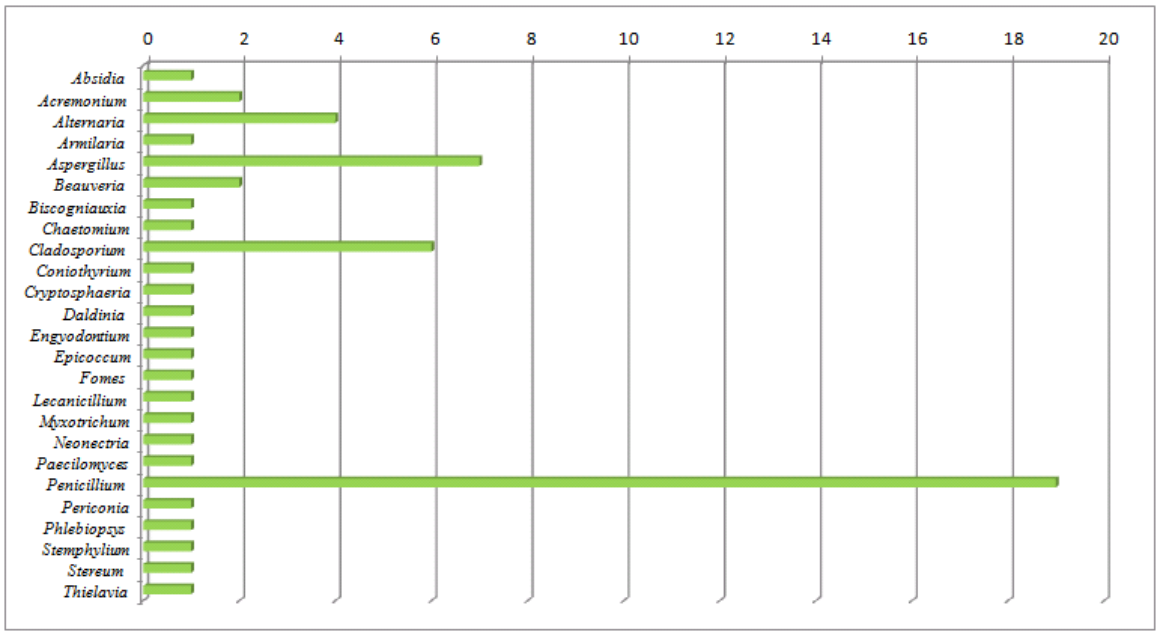

Fig. 2 - Number of species isolated in each genus (sp. - isolates identified using only the molecular approach).

Biodiversity indexes (Shannon-Wiener) were calculated for each floor, arthropod order and season, and are presented in Tables 2 to 4. Fungal diversity was within average values ($0< H' < \ln(59)$ =4.077) and, in general, most species were evenly abundant (0<*E*<1). The highest values of fungal diversity were found in the second floor, associated with the order Thysanura, and during autumn. Particular cases of low or null diversity were found in the third floor and in the order Coleoptera, respectively.

Table 2 – Biodiversity indexes according to each floor.

Floor	**Basement**	**2nd**	**3rd**	**4th**	**5th**	**6th**
Nº of isolated fungi	28	43	7	25	15	30
Nº of species	16	24	6	17	12	19
Shannon Index	2.61	2.91	1.75	2.71	2.39	2.78
Species evenness	0.64	0.71	0.43	0.66	0.59	0.69

The orders Araneae, Diptera , Lepidoptera and Thysanura are associated mainly with fungi belonging to the genera *Absidia*, *Aspergillus*, *Beauveria*, *Cladosporium* and *Penicillium*, while the order Coleoptera is only associated with the species *Daldinia concentrica*.

Table 3 – Biodiversity indexes according to each arthropod order.

Arthropod Order	**Araneae**	**Coleoptera**	**Diptera**	**Lepidoptera**	**Thysanura**
Nº of isolated fungi	25	1	18	8	43
Nº of species	18	1	15	7	25
Shannon Index	2.76	0	2.66	1.91	2.98
Species evenness	0.68	0	0.65	0.47	0.73

A clear influence of the different seasons in the fungal diversity was observed (Table 4). In this way, the genus *Penicillium* was dominant in winter, *Cladosporium* and *Aspergillus* in spring, *Aspergillus, Penicillium* and *Cladosporium* in autumn and finally *Penicillium* in summer.

Table 4 – Biodiversity indexes according to each season.

Season	**Summer**	**Autumn**	**Winter**	**Spring**
Nº of isolated fungi	35	87	17	9
Nº of species	23	36	12	8
Shannon Index	3.0	3.26	2.39	2.04
Species evenness	0.74	0.79	0.58	0.50

Table 5 displays the occurrence of the different species in arthropods, comparing them with the ones previously isolated from documents from the Archive of the University of Coimbra in Chapter 1, in which common species are highlighted).

Table 5 - Comparison of fungal species isolated from arthropods and documents in the Archive of the University of Coimbra (adapted from Mesquita et al. 2009) ('blank' = absent; '+' = Present; sp. - isolates identified using only the molecular approach). Common species are highlighted in bold.

	Support	
Fungal species	**Documents**	**Arthropods**
Absisdia anomala		+
Acremonium furcatum		+
Acremonium strictum		+
Alternaria alternata	**+**	**+**
Alternaria arborescens		+
Alternaria infectoria		+
Alternaria sp.		+
Armillaria sp.		+
Aspergillus candidus		+
Aspergillus flavus		+
Aspergillus fumigatus	+	
Aspergillus nidulans	+	
Aspergillus niger		+
Aspergillus nomius		+
Aspergillus parasiticus		+
Aspergillus versicolor	**+**	**+**
Aspergillus zhaoqingensis		+
Beauveria bassiana		+
Beauveria sp.		+
Biscogniauxia mediterranea		+
Botrytis cinerea	+	
Chaetomium globosum	+	
Chromelosporium carneum	+	
Chaetomium nigrocolor		+
Cladosporium cladosporioides	**+**	**+**
Cladosporium cucumerinium		+
Cladosporium pini-ponderosae		+
Cladosporium sp.		+
Cladosporium tenuissimum		+
Cladosporium sphaerospermum		+
Coprinus sp.	+	
Coniothyrium sporulosum		+
Cryptosphaeria subcutanea		+
Daldinia concentrica		+
Engyodontium album		+

	Support	
Fungal species	**Documents**	**Arthropods**
Epiccocum nigrum	**+**	**+**
Fomes fomentarius		+
Lecanicillium psaliotae		+
Myxotrichum deflexum		+
Neonectria discophora		+
Paecilomyces lilanicus		+
Penicillium bilaiae		+
Penicillium brasilianum		+
Penicillium brevicompactum		+
Penicillium canescens	**+**	**+**
Penicillium chrysogenum	**+**	**+**
Penicillium citrinum		+
Penicillium commune		+
Penicillium crustosum		+
Penicillium decaturense		+
Penicillium dendriticum		+
Penicillium dipodomyicola		+
Penicillium expansum		+
Penicillium griseofulvum		+
Penicillium helicum	+	
Penicillium islandicum		+
Penicillium miniolutum		+
Penicillium olsonii		+
Penicillium raistriki		+
***Penicillium* sp.**	**+**	**+**
Penicillium spinulosum		+
Periconia macrospinosa		+
Phlebia subserialis	+	
Phlebiopsys gigantea	**+**	**+**
Skelotocutis sp.	+	
Stemphylium solani		+
Stereum hirsutum		+
Thanatepharus cucumerinis	+	
Thielavia hyalocarpa		+
Toxicladosporium irritans	+	

5.4 Discussion

When dealing with moulds colonizing heritage collections, and preventing further contamination, it is advised to assess the current arthropod community and to develop adequate countermeasures to control the spreading of fungal propagules and cross-contamination by arthropods (Jurado *et al.* 2008). To our knowledge, these are the first results on the identification of fungi associated with arthropods living in libraries/archives, at least in Portugal.

Regarding the identification of isolated fungi, the molecular analysis using the total ITS region, complemented with morphological analysis, allowed the identification of most isolates with great accuracy. From 180 arthropods captured, 148 fungal isolates, 25 genera and 59 species were identified, with *Alternaria*, *Penicillium*, *Aspergillus* and *Cladosporium* being the most abundant genera. The high frequency of *Aspergillus nomius, Cladosporium cladosporioides, Penicillium brevicompactum, Penicillium commune, Penicillium chrysogenum* and *Penicillium griseofulvum*, isolated from arthropod hosts, is in agreement with other reported works in subterranean environments as catacombs, caves, tombs and hypogean churches and chapels (e.g. Greif and Currah 2007; Jurado *et al.* 2008). These are ubiquitous species and can produce numerous mitospores and conidia, and in addition, some of these fungi are entomopathogenic, and are common contaminants of arthropod cadavers. In a few occasions, observations were made of fungal sporulation and hyphae growing out of arthropod bodies.

Some of the arthropods didn't provide fungal isolates. There is no correlation between obtained results and the order of arthropods, the floor or the season from which they were isolated. This can be explained by the use of generic growth mediums, presence of fungal competition, lack of spore viability and/or low abundance of spores in some of the captured arthropods.

The Shannon indexes for each floor revealed a high fungal diversity in the basement; the second, fourth, fifth and sixth floors and their evenness values suggest a similar distribution of fungal species in each floor. In this way, the fungal diversity, vectored by arthropods, seems not to be affected by spatial location. The exception seems to be the third floor, which presented the lowest biodiversity index. This result can be explained by the fact that the main entrance to the AUC is located in this floor. Moreover, this floor contains a smaller amount of documents and a cleaner environment, with frequent air renewal. On the other

hand, it has more people circulating and overall this is reflected in poorer environmental conditions for fungal development and even to arthropod circulation.

From the analysis of the biodiversity indexes for each arthropod order, we can see that Lepidoptera presented a lower fungal biodiversity while the orders Thysanura, Diptera, Araneae had much higher fungal biodiversity values. We also confirmed an association between entomopathogenic fungi (from the genera *Aspergillus, Beauveria, Cladosporium, Engyodontium, Lecanicillium, Penicillium* and *Paecilomyces*) and the orders of the arthropods they were originally isolated from (Araneae, Coleoptera, Diptera, Lepidoptera and Thysanura). These fungal genera are common components of the mycoflora of these arthropod orders (Greif and Currah 2007). The Araneae, Diptera and Lepidoptera orders were mainly associated with entomopathogenic fungi such as *Absidia*, *Aspergillus*, *Beauveria*, *Cladosporium* and *Penicillium*.

The fact that the order Thysanura presented a high fungal diversity is likely related with the presence of entomopathogenic fungi, but can also be an effect of the behavior of these arthropods, since they move over objects while foraging. They feed on paper and can therefore ingest fungal spores and also contaminate other supports, over a short distance (less than 1, 6 Km), since they are not winged insects (Baverstock 2009). Two non-entomopathogenic species associated with Thysanura were isolated: *Fomes fomentarius* and *Crypthosphaeria subcutanea.* The first is a known plant pathogen and the second is common in bark (Kibby 2003; Romero *et al*. 2003). Moreover, since this is a not sealed building, fungal spores from these species could have been transported inside the archive by other external invertebrates, such as pollinators or herbivores, and became a source of nutrients to Thysanura (Abbot 2002). The order Coleoptera was only associated with *Daldinia concentrica*. It is known that the caterpillars of the Lepidoptera *Harpella forficella* feed on this fungus (Grabe 1942). However, many types of arthropods and other small animals use the stromata of this species as their habitat and therefore can act as vectors of dispersal or by spreading the fungal propagules (Johannesson 2000).

We conclude that five orders of arthropods (Araneae, Coleoptera, Diptera, and Leptidoptera Thysanura) are responsible for the dispersal of propagules in this place, with special emphasis on the orders Araneae, Diptera and Thysanura whose great diversity associated places them as the major orders capable of carrying a greater diversity and density of fungal inoculum.

Regarding seasonality, winter and spring were the seasons with lower fungal biodiversity, whereas summer and autumn presented high biodiversity values. These results are in conformity with other seasonality studies (Medrela-Kuder 2003). The formation of fungal propagules in *Aspergillus*, *Cladosporium* and *Penicillium* species is usually influenced by seasonal variations. Seasonal variations on the frequency of these fungal species can be due to the prevalence of *Aspergillus* spores in winter, of *Penicillium* in winter and spring, and of *Cladosporium* during the whole year, with a higher number of propagules being produced during the summer (Medrela-Kuder 2003). Accordingly, our most common species were: in winter, *Penicillium olsonii*, *Penicillium brevicompactum*, *Cladosporium cladosporioides* and *Cladosporium* sp. (other, unidentified *Cladosporium* species); in spring, *Aspergillus parasiticus*; in autumn, *Aspergillus nomius, Biscogniauxia mediterranea, Penicillium crysogenum*, *Penicillium brevicompactum*, *Penicillium commune* and *Cladosporium* sp.; and in summer, *Penicillium crysogenum*, *Penicillium brevicompactum* and *Phlebiopsys gigantea* differing from the seasonality studies of Medrela-Kuder (2003) only by a not so remarkable presence of *Aspergillus* propagules in winter and a clear predominance of *Penicillium* during all year.

In an attempt to further understand the role of arthropods as possible vectors of fungal dispersion, a comparison was made using the results of a previous work (Mesquita *et al.* 2009) about the fungal diversity colonizing documents from the Archive of the University of Coimbra. About 36% of the species found colonizing documents had also been isolated from the arthropods captured in this study. These species were *Cladosporium cladosporioides*, *Penicillium chrysogenum*, *Aspergillus versicolor*, *Alternaria alternata*, *Epicoccum nigrum*, *Penicillium canescens* and *Phlebiopsis gigantea*. This co-occurrence indicates that at least on this site, these species can be dispersed by mechanisms that use animal vectors.

In addition *Aspergillus versicolor*, *Cladosporium cladosporioides* and *Penicillium cucumerinum* were also isolated from the air (unpublished data).

Our results support the hypothesis that some arthropods can act as vectors of contamination between documents in environments similar to the Archive of the University of Coimbra. Results show that some of the fungal species were consistently present in both arthropods and in the documents, therefore playing an important role in the documents biodeterioration process, considering that these identified fungi can be transported but

also grow on the arthropod bodies (due to the presence of entomopathogenic species) and the confirmation that the spores maintained their viability and were able to grow in PDA medium growth.

With the information retrieved from this study, we now aim to be able to develop safe ways that can contribute in some way, to the preservation of the cultural heritage held in this historical collection. We hope that this piece of knowledge could also help similar institutions in preserving their patrimony.

5.5 Acknowledgements

The authors are grateful to the Archive of the University of Coimbra for the kind collaboration, and to the FCT, for funding the scientific project PTDC/HAH/65262/2006.

General Discussion

The work described in this thesis began as a case study on the presence of biodeteriorating fungi within the Archive of the University of Coimbra (AUC), since according to other published works, fungal organisms were among the most threatening biodeteriorating agents in libraries - this matter was addressed in Chapter 1.

The broad collection of documents stored in the AUC has several different provenances; during the last centuries, it has continuously hosted not only documents from the University of Coimbra, but also many other items and collections from other repositories (either donated or acquired by the institution), some of them have actually been in severe deterioration condition previous to their storage in the AUC. Items from different origins and made of different materials are likely to carry diverse biodeteriorating agents (e.g. fungal organisms) to the interior of the AUC, where many organic materials are available for further exploitation by these organisms. Regardless of the control procedures and thorough monitoring of environmental conditions that are common practices in these institutions, relative humidity and temperature found within a corridor or room are not always representative of the microclimates that are available within certain parts of books or shelves, drawers and other storage compartments. These micro-niches are sometimes capable of providing the necessary water and temperature for some fungal organisms to germinate and develop while feeding on the diverse organic materials that are available, given that they have adequate enzymatic tools to do so. This first step provided us information on the biodeteriorating fungi within the AUC's collection, actually present in documents and items made of different materials: hand-made paper (woven paper), wood-pulp paper and parchment. To have an external comparison, a 17th century parchment in severe deterioration state was thoroughly studied in Chapter 2. A total of eighteen different fungal species were identified from this parchment document alone, as a display of its deeply spread contamination.

By comparing the two studies, coincident species were found, namely *Cladosporium cladosporioides, Penicillium chrysogenum* and *Aspergillus versicolor*; however there were many species present in the analysed parchment that had not previously been found in the parchment documents studied in Chapter 1 and vice-versa. It was somewhat expected that

they would present different populations, since they had different origins and were stored in different repositories.

Overall the diversity of fungal species was high, and this made it difficult to be able to find specific substrate-species relations, however there were some species that were found in a single support type only, suggesting a greater substrate specificity. As previously stated, the diversity of biodeteriorating fungi found in these chapters is comprised not only of species that are able to actively degrade the main components of these items (such as cellulose in paper documents and collagen in parchment documents) but also other opportunistic species that may reach these supports by chance, being able to feed from debris, insect remains, other available substrates (glues, waxes, pigments, etc.), or even sub-products of cellulose or collagen degradation, made available by the activity of other cellulolytic and collagenolytic strains respectively. A good example of this is *Aspergillus versicolor*; it has been isolated from the Archive's documents (Chapter1), the papal bull (Chapter 2), and has also been referred to in other works (Zyska, 1997; Michaelsen et al., 2010). It is only a moderately cellulolytic species, and probably only secondary to the growth of strong cellulolytic species or by feeding from secondary substrates (Michaelsen et al., 2010). Its great plasticity and physiologic versatility allows it to deteriorate other polymeric materials, namely other constituents of books, promoting their mechanical deterioration. This species is generally xerophilic (grows at low water activity (<0.80) with an optimal growth temperature of 30ºC and a minimum of 4ºC. Sometimes, the organisms that start growing on documents are only able to feed from the spore's own reserves, which leads to minor, smaller sized stains, because of their rapid exhaustion (Corte et al., 2003).

It is important to state that the use of malt extract agar as well as potato dextrose agar as alternative culture media provided a different set of species; this suggests that the use of different culture media allows complementary results and the growth of otherwise unobtainable isolates, therefore reaching a broader spectrum of organisms. The species requirements for an effective growth, and inter-species competition, sometimes prevent the growth of slower growing strains. Indeed, it is very difficult, if not impossible, to assure that all present organisms are screened during such analyses, or even with the use of culture-independent techniques such as the amplification and DGGE analysis of all present strains. Nevertheless it was crucial to have an insight on the variety of contaminating organisms in order to develop and test adequate control procedures. Having found human

pathogenic strains among the isolates (such as *Toxicocladosporium irritans, Aspergillus flavus, Aspergillus versicolor, Cladosporium cladosporioides, Penicillium brevicompactum* and *Penicillium chrysogenum*) emphasises the need to develop methodologies for the treatment of these collections, since users, librarians and restorers frequently handle them. These results are in accordance with several other studies, in which many of these species are stated (Corte et al., 2003; Rakotonirainy et al., 2007; Michaelsen et al., 2010). There are also references to other species, which is rather expected given the great variety of potential biodeteriogens among fungi. Many of these species are ubiquitous, part of the mycoflora of soil, or usually present in the air (Corte et al., 2003), and they can reveal biodeteriogenic roles within determined and adequate environmental conditions (Arai, 2000).

Collecting all this information about the most frequent contaminating fungal groups present in our test pieces, and comparing these results with the many publications on this subject, suggested that most common and important genera (in what concerns to control treatments) had already been found in our samples; species such as *Cladosporium cladosporioides* and *Penicillium chrysogenum*, (together with other species to allow results comparison), could therefore be the target of decontamination procedures, such as radiation, in the assessment of its effectiveness in the disinfection of contaminating fungal organisms.

Cladosporium cladosporioides is a ubiquitous fungal species. As a biodeteriogen, it was found and isolated from documents in the works described in Chapters 1 and 2, as well as in several other publications that address this biodeterioration topic, in different places and materials (Botelho et al., 1988; Blank and Corrigan, 1995; Szczepanowska and Cavaliere, 2000; Abrusci et al., 2005; Da Silva et al., 2006; Michaelsen et al., 2006; Michaelsen et al., 2012; Vivar et al., 2012).

Cladosporium cladosporioides was therefore selected as test species for the work described in Chapter 3. The dark pigments that are produced and accumulated in its mycelium, protect this organism from environmental extremes (Da Silva et al., 2006). Zyska (1997) reports it as one of the most frequent moulds, isolated from different support materials: paper, parchment, textiles, wax seals, art of archives, and others. Furthermore, it has a high radio resistance, making it ideal for the purpose of this test, since radiation doses that are

able to inactivate it will likely also inactivate other species that are present. This task provided information on the radiation resistance as well as the dose needed to inactivate and stop the growth of *Cladosporium cladosporioides* spores. Two different methods were used in this work, providing different and complementary information regarding the irradiation process and its inactivating potential: CFU analysis allowed the quantification of total spore viability (pre-germination), according to the obtained survival curves; and the growth analysis provided qualitative data concerning medium term effects in sub-lethal doses (e.g. biomass, the germination/growth delay and colony diameter). Radiation doses of 8.2 kGy, up to 15.4 kGy were applied to the samples, having caused a significant decrease in all tested parameters: spore viability, colony growth and biomass and the number of viable counts in the CFU analyses. In fact, the dose of 15.4 kGy sterilized all samples, in the sense that no growth or biomass increase was observed after irradiation. Our results for this species, in terms of radio resistance, are in agreement with those previously reported for *Cladosporium cladosporioides* (Botelho et al., 1988).

The authors Da Silva et al. (2006) determined the dose of 16 kGy as that generally required for fungal inactivation, stating that there was no need to go beyond those values in order to inactivate common and somewhat radio resistant contaminants, such as *Aspergillus niger* and *Cladosporium cladosporioides.* Similarly, our results point out that it is possible to disinfect documents colonized by fungi using radiation doses lower than 15 kGy. This dose has been tested and described to be safe to paper supports in terms of physical resistance and colour changes (Gonzalez et al., 2002; Rizzo et al., 2002; Rochetti et al., 2002; Magaudda, 2004). Regarding the resistance of parchment to radiation, a collaboration work with Nunes et al. (2012) revealed that radiation doses up to 30 kGy (much higher than those required for fungal inactivation) did not cause significant changes to the texture or other mechanical and optical (colour) properties on the tested parchment documents, suggesting that the previously stated dose of 15 kGy can also be safely applied to parchment.

This task provided us with information on the radiation dose required for the disinfection of *Cladosporium cladosporioides* (and consequently of other less resistant fungi), and on the effects of gamma radiation in parameters such as the CFU count and growth (both weight and colony diameter) after irradiation with different doses. However there were some clear

limitations to this procedure; it delivered little information on the early stages of fungal growth, it didn´t refer to individual spores and it required the growth of colonies for several days within an incubator and several daily measurements for a 21-day period.

In this way, we chose to explore a different and more straightforward procedure that would allow the assessment of the radiation effects on parameters such as spore growth, viability and metabolic activity of individual cells, using the high throughput process that is flow cytometry, as described in Chapter 4. The power of flow cytometry resides in the fact that it can perform single cell analysis of several parameters simultaneously, on hundreds of particles per second. Parameters such as the size and complexity of spores are based on their optical characteristics, and therefore there is no need for any kind of stain to be applied. On the other hand, the use of specific stains provides information on particular aspects; in this case the fluorescence intensity of PI in unviable cells was 5 to 7x higher than in viable cells, allowing the assessment of the viability of individual spores as effect of a sterilization treatment, without the need to re-culture the cells in cell plates; additionally, the use of Dihydroethidium allowed the assessment of each cell's metabolic activity status, as an indicator of the actual cell condition after irradiation with different doses. The DHE fluorescence intensity was much higher in growing spores, while it just presented a little variation in dead cells, decreasing over time. The integration of all these results provided very good insights on the effects of the radiation treatment during the first hours of spore germination, and overall, this experiment opened the door for the development of new procedures to assess other cellular parameters if other stains are used.

Our results from Chapters 3 and 4, together with the referred collaborations with Nunes et al. (2012, 2013a), support the D_{min} of 5 kGy for the effective disinfection treatment of contaminated paper and parchment documents. This dose will likely inactivate all present organisms, or otherwise reduce them to very low counts and is, because of that, an effective choice as a control procedure, since even 15 kGy is still considerably lower than the D_{max} for both paper and parchment.

After disinfection, it is important to prevent the re-contamination as much as possible, regardless of the disinfection methodology used. Once the treated documents return to their corresponding repositories, recontamination can occur through different ways, such as: cross contamination inadvertently done by users; the settling of airborne propagules; or

the action of vector organisms (such as arthropods) that roam through shelves and documents, potentially spreading spores of different fungal organisms throughout these collections.

To assess the air bioburden within the archive, and its possible relation with the results on the fungal diversity found in documents, a study on the air contamination inside the archive was performed in a parallel collaboration (Nunes et al., 2013a); according to this publication, the registered monthly average RH levels (2006-2011) were between 38.2% and 48.9%, while temperatures ranged from 12-29ºC, which are within the 'safe' storage conditions in what regards to initial contamination needs. However, the already referred micro-niches, as well as the fact that some of the colonies are already active, allows fungal proliferation in these supports, even when these 'safe' conditions are met. Nevertheless, keeping documents within these 'safe' conditions is still the most effective storage method to limit further degradation and preventing the occurrence of new contamination.

On the work of Nunes et al. (2013a), from the seven identified fungal genera, the most frequent were *Penicillium* (6,11%), *Fusarium* (2.04%) and *Aspergillus* (1.64%), and known pathogenic and toxigenic species that had been isolated from our documents were also retrieved (e.g. *Aspergillus versicolor, Aspergillus fumigatus*). These results are in agreement with the studies from Borrego et al. (2008, 2010) and Guiamet et al. (2011), who analysed the microbial communities of indoor air in different historical archives in Cuba.

The role of arthropods as vectors for the spreading of fungal propagules was studied and described in Chapter 5. A total of 148 fungal isolates were retrieved from 180 arthropods that were captured in 80 traps placed within the Archive of the University of Coimbra. The most frequent species were *Aspergillus nomius, Biscogniauxia mediterranea, Cladosporium cladosporioides, Penicillium brevicompactum, Penicillium commune, Penicillium chrysogenum* and *Penicillium griseofulvum.* These are mostly ubiquitous species that produce numerous mitospores and conidia; in addition, some of these fungi are entomopathogenic, and are common contaminants of arthropod cadavers. No direct correlation was found between the obtained isolates and the order of arthropods they were retrieved from. This might be related to several factors, such as: the number of analyses might not have been sufficient in order to be able to establish a relation; the use of generic growth mediums which difficult the isolation of slow growing, or substrate specific strains; the presence of fungal competition, that may prevent the isolation of less

competitive strains; the lack of spore viability, since sometimes the spores carried by these organisms may not be able to grow; but also to the low abundance of spores in some of the captured arthropods. Nevertheless, an association between entomopathogenic fungi (from the genera *Aspergillus, Beauveria, Cladosporium, Engyodontium, Lecanicillium, Penicillium* and *Paecilomyces*) and the orders of the arthropods they were originally isolated from (Araneae, Coleoptera, Diptera, Lepidoptera and Thysanura) was confirmed. These fungal genera are common components of the mycoflora of these arthropod orders (Greif and Currah, 2007). The Araneae, Diptera and Lepidoptera orders were mainly associated with specific genera of entomopathogenic fungi such as *Absidia*, *Aspergillus*, *Beauveria*, *Cladosporium* and *Penicillium*.

These results support that five orders of arthropods (Araneae, Coleoptera, Diptera, and Leptidoptera Thysanura) are involved in the dispersal of fungal propagules in the AUC, with special emphasis on the orders Araneae, Diptera and Thysanura, whose great diversity places them as the major orders that are capable of carrying a greater diversity and density of fungal propagules.

When comparing the results from Chapter 5 to the fungal diversity found in documents from the AUC, as described in Chapter 1, we find that about 36% of the species found colonizing documents were also isolated from the arthropods captured in this study. These main examples are *Cladosporium cladosporioides*, *Penicillium chrysogenum*, *Aspergillus versicolor*, *Alternaria alternata*, *Epicoccum nigrum*, *Penicillium canescens* and *Phlebiopsis gigantea*. This suggests that at least on this site, these species can be dispersed by mechanisms that use animals as vectors.

Additionally, this work also provided information on the seasonality effects on the prevalence of certain genera in the different seasons. In this way, the genus *Penicillium* was dominant in winter, *Cladosporium* and *Aspergillus* in spring, *Aspergillus, Penicillium* and *Cladosporium* in autumn and finally *Penicillium* in summer. Winter and spring were the seasons with lower fungal biodiversity, whereas summer and autumn presented high biodiversity values. These results are in conformity with other seasonality studies (Medrela-Kuder, 2003).

Conclusions

Conclusions

The work described in this thesis was complemented with three other collaborations with Nunes et al. (2012, 2013a, 2013b), for a more complete vision of the process of biodeterioration of cultural heritage documents and how to control it. Put together, we assessed the contaminating organisms present in different supports (although this thesis focuses only on fungal organisms, the works of Nunes et al. (2013a, 2013b) complement our results with bacterial population data); we also determined the recommended treatment doses that are strong enough to inactivate infecting organisms (D_{min}) while still being safe (D_{max}) for both parchment (Nunes et al., 2012) and paper supports (Adamo et al., 1998; Adamo et al., 2001); and we studied the roles of two relevant vectors of propagule spreading - air and arthropods, using the Archive of the University as a case study.

Additionally, a new methodology was developed that allows a faster determination of the different cellular-level effects of an inactivation treatment during the first hours of fungal spore germination and growth, in a high throughput single-cell analysis technique using flow cytometry.

The information provided on this thesis is an added contribute to the better understanding of the biodeterioration processes in paper and parchment supports, and will assist other researchers and conservators in fighting the tangible issue of the biodeterioration of cultural heritage items.

Regarding the initial objectives of this work, most of them were successfully accomplished: contaminating organisms retrieved from several heritage documents (made of different materials) were isolated and identified using morphological and molecular methods. Several different species were found, many of which had already been described in the literature, but other less common species were also retrieved from our samples. We were unable to establish a relation between the different taxonomical groups and the different support types, and now we do understand that it is very difficult, if not impossible, to determine the total fungal diversity within a place such as the AUC, specially since this diversity can include non-specific, opportunistic fungal species as well as other more relevant biodeteriogens. We did however observe that some of the isolated species were

found only in a given support type, since they are substrate specific, and thus unable to thrive in other supports.

Considering the effects of gamma radiation on the fungal organisms, several parameters were assessed in different species, at different growth stages, and using different methodologies. Additionally, a new procedure was developed with the use of flow cytometry. It was clear that gamma radiation impacted strongly on the fungal growth rate, viability and metabolic activity, and the different approaches allowed determining adequate treatment doses (D_{min}), fortunately below the D_{max} for both paper and parchment.

The last objective addressed fungal propagule vectoring by arthropods. Many species that were found in documents were present in the captured arthropods, suggesting that they do act as agents of fungal dispersion within indoor environments, and therefore advising their control. Again, it was not possible to determine a relation between specific fungi and arthropod *taxa*, probably due to the fact that not enough samples were retrieved, considering the high fungal diversity obtained. Seasonality effects were also identified, with different genera being prevalent according to the different seasons.

Future prospects

This interdisciplinary work started with the identification of contaminating fungal *taxa*, then followed by the application of a sterilization procedure using gamma rays. The effects of this treatment were evaluated, by looking at different biological parameters; initially, more general traits such as growth and biomass were assessed. We then found it would be relevant to investigate further, by analysing at the cellular level, the viability, spore growth and metabolic condition of irradiated spores.

In our opinion, it would be of great interest to go even further, and study the effects of these treatments at the molecular level, namely in the domains of genomics and proteomics. Michaelsen et al. (2012) started by performing a series of genetic analyses of organisms subject to different conservation treatments (one of which was gamma radiation). Samples treated with gamma rays showed DGGE patterns with numerous unspecific bands, one month after treatment, and those patterns indicated a mixed population of both intact DNA from surviving fungi but also DNA artifacts, as a consequence

of DNA fragmentation due to the irradiation procedure. However, these strains were kept in culture for one year and no more visible effects of gamma radiation on the DNA of pure strains were observed. The surviving fungal population recovered with time, and only intact DNA was detected.

The field of proteomics is a very complex one, but can provide very interesting information if correctly addressed. The effects of gamma radiation on the proteins of irradiated fungal organisms, as well as the comparison with strains obtained from the growth of resistant spores, could provide information on the groups of proteins that can, in someway, be responsible for a higher resistance to gamma radiation. We do know that radiation resistance is attained by different organisms with the use of different strategies, from radiation shielding to prevent damage, to rapid DNA repair systems. Preliminary studies have been developed, by performing SDS-PAGE analysis of different irradiated colonies, and differences in band patterns have been found, varying according to the absorbed dose (unpublished data), therefore showing that changes at the proteomic level do occur after gamma radiation. A next step could consist in performing 2-D analysis, and subsequent isolation and identification of proteins that display different levels of expression after irradiation, and assess their role in radiation resistance.

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