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Fungal Communities in Archives: Assessment Strategies and Impact on Paper Conservation and Human Health

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Fungal Communities in Archives: Assessment Strategies and Impact on Paper Conservation and Human Health Fungal

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Dedicated to my family

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The faculty of art is to change events; the faculty of science is to foresee them.

— Henry Thomas Buckle

'The Influence of Women on the Progress of Knowledge,' a discourse delivered at the Royal Institution (19 Mar 1858) reprinted from Fraser's Magazine (Apr 1858) in The Miscellaneous and Posthumous Works of Henry Thomas Buckle (1872), Vol. 1, 4.

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Abstract

Fungi are ubiquitous and, as such, are current contaminants in archives and librairies. Provided the adequate relative humidity, temperature and water activity, however, they can stop being innocent inhabitants and become dangerous threats to the organic materials that hold our written heritage. Their impact, however, is not restricted to the assets of an archive or library. New concerns arise from their incredible ability to thrive as human health can also be affected. In a contaminated environment, staff and attendees share the same space with fungal communities and the human body can suffer from this interaction in the most diverse ways. So, indoor air quality studies in archives and libraries should always comprehend not only the study of fungal communities but also their analysis under two perspectives: documents safekeeping and human health protection.

In this study it was important to determine the conditions provided by some of our heritage holder's institutions and develop the best strategy to identify their fungal flora. This strategy encompassed both air and surfaces and both traditional culturing methods and molecular biology protocols. Knowing the environment in Portuguese institutions is essential for the development of guidelines and establishment of recommendations. Only by using this knowledge can a safer environment be created and the purpose of keeping our heritage while maintaining our health is fully attained.

Keywords: Fungi, archives, indoor air quality, health, conservation

Resumo

Os fungos existem em todos os ambientes e, como tal, não surpreende a sua presença em arquivos e bibliotecas. No entanto, quando existem as condições *certas* de humidade relativa, temperatura e actividade da água, os fungos deixam de ser simples ocupantes e transformam-se numa perigosa ameaça ao nosso património. O seu impacto, no entanto, não se limita ao acervo de um arquivo ou biblioteca. Da sua incrível tenacidade nascem novas preocupações, desta vez relacionadas com a saúde humana. Num ambiente contaminado, tanto funcionários como visitantes podem sofrer com esta interacção. É por este motivo que os estudos de qualidade do ar interior nestas instituições deverão sempre incluir não só o estudo das comunidades fúngicas como a sua análise sob ambas as perspectivas: a salvaguarda da nossa herança escrita e protecção da saúde humana.

Neste estudo foi importante conhecer as condições fornecidas por alguns dos nossos Arquivos e desenvolver a melhor estratégia para a identificação da microflora fúngica. Enquadradas nesta estratégia estiveram amostras de ar e de superficies tendo sido usados tanto os métodos tradicionais de cultura como protocolos de biologia molecular. Conhecer o ambiente é essencial para o desenvolvimento de directrizes e recomendações. Só utilizando este conhecimento é que será possível criar ambientes mais seguros onde a salvaguarda do património pode caminhar lado a lado com a manutenção da nossa saúde enquanto cuidadores.

Palavras-chave: Fungos, arquivos, qualidade do ar interior, saúde, conservação

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Abbreviations

ADE – Arquivo Distrital de Évora

AFCE – Automated Fluorescent Capillary Electrophoresis

AHU – Arquivo Histórico Ultramarino

CFU – Colony forming unit

DG18 – Dichloran-Glycerol media

DGGE – Denaturing Gradient Gel Electrophoresis

DHPLC – Denaturing High Performance Liquid Chromatography

HVAC – Heating, Ventilation and Air Conditioning

IAQ – Indoor Air Quality

IHRU – Instituto de Habitação e Reabilitação Urbana

MEA – Malt Extract Agar media

PCR – Polymerase Chain Reaction

PM – Particulate matter

rDNA – Ribossomal DNA

RH – Relative Humidity

TT – Torre do Tombo

VOCs – Volatile Organic Compounds

Aw – Water activity

1. Introduction

Fungal microorganisms are ubiquitous. Composed of thousands of genera and species, the Eumycota Kingdom (true fungi) is particularly relevant by its ever changing ability to adapt to harsh environments. Its presence can be noticed in a geyser's hot temperature and in the coldness of our refrigerators and its persistence and natural appetence for survival is remarkable.

Their structure is very diverse: they can be unicellular, multicellular, or dimorphic, which means the fungi is unicellular or multicellular depending on environmental conditions. Fungi's morphological and sexual instability has had serious consequences in fungal nomenclature which is now an extremely complex and evolving discipline. The advent of DNA analysis has proved invaluable in the classification of fungi and their inter-relationships. Within the Eumycota, there are two divisions: Zygomycota (the common mildew or black bread mould caused by *Mucor* sp. or *Rhizopus* sp., for instance) and Dykaryomycota, which comprehends both Ascomycotina (including the Ascomycotina Class, where most of the fungi found in archives are represented; anamorphs and teleomorphs are presented for this class depending on the type of reproduction - asexual or sexual, respectively) and Basidiomycotina (mushrooms, not relevant in fungal biodeterioration in archives) (Florian, 2002).

Fungal cell walls are rigid and contain complex polysaccharides called chitin and glucans. Instead of cholesterol, fungi use ergosterol in their cell membranes.

Fungi in the morphological vegetative stage consist of a tangle of slender, thread-like hyphae, whereas the reproductive stage is usually more obvious (Fig. 1.1). The hyphae acts like a true root for a better adherence to the substrate while the asexual reproductive structures like spores allow wide distribution and increase fungal resistance to adversity.

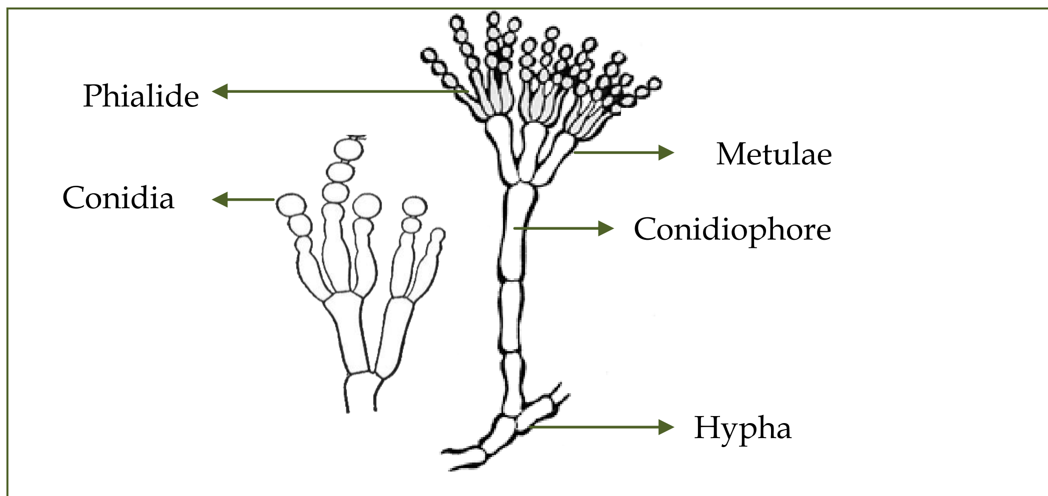

Figure 1.1 - Special areas were sampled for fungal presence analysis. A sterile cotton swab was used for the effect.

(Image retrieved from: http://www.virtualmuseum.ca/sgc-cms/expositions-exhibitions/champignons-mushrooms/Images/Fungus/Illustrations/Lg/penicillium_md.gif)

As saprophyte heterotrophs they use dead or decomposing organic matter as a source of carbon and have the ability to deteriorate a wide variety of materials: from wood and leather to synthetic and stone materials (Gallo et al., 2003). When they interact with artefacts in general they can be responsible for their biodeterioration which brings structural, chemical and aesthetic changes to the materials (Zyska, 1997; Arai, 2000; Sterflinger, 2010). Biodeterioration or biological decay is defined as "any undesirable change in the properties of a material caused by living organisms" (Hueck, 1965).

Fungi and the Biodeterioration of Paper

Although incredibly beneficial in some areas, the world is more accustomed to see fungi as threats to our health and it is not wrong to think so. However, it is not just human health that is in jeopardy when close contact is established with some species or fungi or human presence is maintained in heavily contaminated environments (Pinheiro et al., 2015a, in press).

Archives and libraries are the custodians of our written heritage and most of it is recorded in the complex matrix we call paper. Invented in China around 100 AD, it is of vegetal origin and mostly composed of cellulose. In the case of rag paper, used up until the XIX century, the composition is almost entirely pure cellulose but modern paper (made of wood pulp) contains not only cellulose but also lignin, hemicellulose, pectin, waxes, tannins and proteins (Gallo et al., 2003).

Cellulose fibres are linear polymers of D-glucose monomers linked by glycoside bonds. Hemicellulose is composed of several glucose monomers (D-glucose, D-mannose, D-xylose, D-arabinose, etc...) and its fibbers are considered short. The occurrence of paper biodeterioration is dependent not only on the presence of organisms but also on the materials that constitute the paper. The susceptibility of a given material to colonization by organisms is based on its intrinsic properties and is defined by Guilitte (1995) in a concept called bioreceptivity. This author subdivided this concept in three types: primary bioreceptivity, as the intrinsic potential of a material to suffer biological colonisation; secondary bioreceptivity, as the ability of an altered material, changed over time by physical and chemical agents, to be colonised; and tertiary bioreceptivity, as the potential of biological colonisation of material altered by human hand (e.g. after a conservation treatment).

The differences in paper composition affect their bioreceptivity as hemicellulose´s disorganized structure is more prone to attack than the structure assumed by the aligned long microfibrils that constitute pure cellulose (Gallo et al., 2003; Klemm et al., 2005).

Because modern wood pulp paper is of low quality, several substances are added during production. For an opaque appearance, fillers such as clays and chalks are used while for reducing the ink spread and even out the paper surface starch, gelatine or rosin is used (Cappitelli and Sorlini, 2005). Due to the quality of its fibbers, paper made of pure cellulose is less attacked but the substances added to the paper manufacturing may increase its bioreceptivity, playing an important role in the biodeterioration by fungi (Nitterus, 2000). If we add dust particles caught between the paper fibbers during production and handling and the organic input brought by human manipulation, it is safe to say microorganism have a wide variety of organic materials to sustain them.

Several studies have focused on effects microfungi (artificial, paraphyletic group, distinguished from macrofungi only by the absence of a large, multicellular fruiting body) can have on our written heritage which can include pigment's and ink's discoloration (Florian and Manning, 2000; Pinzari et al., 2006; Mesquita et al., 2009), chemical stress (Canhoto et al., 2004; Konkol, 2010), physical and mechanical stress (Ponce-Jimenez et al., 2002a, 2002b; Caneva et al., 2003; Konkol, 2010).

The fungal hyphae penetrate and act mechanically on the paper fibbers leaving them weakened and prone to further damage (Ponce-Jimenez et al., 2002a; Sterflinger, 2010). Fungal spores and early growth on paper are not immediately visible to the naked eye, as damage can already have happened when the hyphae become visible (Figure 1.2).

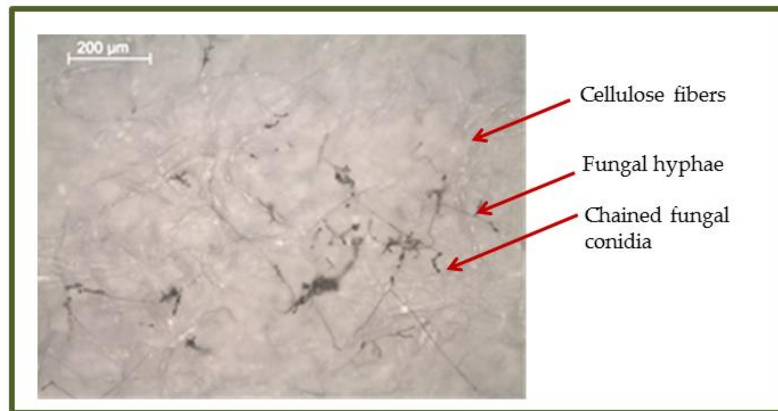

Figure 1.2 – All different life stages of fungi can be observed on paper: spores, germinated young hyphae or adult mycelium and spore forming structures. None of these were visible to the naked eye. Image obtained using an optical microscope Leica DMI 5000 M (50x to 1000x), a courtesy of CENIMAT.

Some microfungi can, in certain conditions, produce enzymes known as cellulolytic which can have a strong impact on paper-based heritage (Fabbri et al., 1997; da Silva et al. 2006; Adamo et al., 2003). These enzymes promote the cellulose chain's tear and while this reaction takes place more acidic metabolites are being excreted and the pH is lowered (Ponce-Jimenez et al., 2002b). Cellulose, however, is not the only means of survival for fungi as glucanases, laccases, phenolases, keratinases and mono-oxygenases can also be produced (Sterflinger, 2010) to degrade other molecular food sources. The release of all these extracellular enzymes or pigments acts chemically on the substrate.

Fungi can also be responsible for a deteriorative modification of paper with the appearance of brownish and reddish stains known as foxing (Arai, 2000; Florian and Manning, 2000; Rakotonirainy et al., 2007; Michaelsen, 2010a).

Actually, in the case of foxing spots, there is no active growth of the fungus and these stained areas do not appear mouldy at all. It is now assumed that the foxed areas are a result of a dyeing fungal structure and the chromatic changes induced in the paper are a result of these structures' ageing process (Rakotonirainy et al., 2007). These particular stains might migrate through successive pages, causing irreversible damage

and the number of stained areas increases with time and can ultimately deem the document unreadable (Rakotonirainy et al., 2007). Foxing is not fully understood in its nature as it could result either of the activity of fungi and/or the oxidation of heavy metals deposits (Cappitelli and Sorlini, 2005). Nevertheless, it is thought that the majority of foxing stains are caused by fungi (Arai 2000; Corte et al., 2003; Rakotonirainy et al., 2007).

As shown, mould growth on paper is defined as an active and destructive process which can cause structural damage and also chromatic changes. Nutrients, moisture, and temperature are the most important factors that influence fungal growth (Khan et al., 2012). The requirement for moisture depends on the fungal genus or species and is expressed in terms of water activity (a_w) also known as equilibrated relative humidity (see Chapter 6 for further details). This corresponds to the free non-bound water available to the microorganisms and it ranges from 0 to 1. Higher a_w materials tend to support more microorganisms: bacteria require a substrate with at least 0.90 and fungi at least 0.65 (Rockland and Beuchat, 1987) or 0.64 (Samson et al, 2010).

Micro-climates with very high a_w can be generated in a room with an otherwise low relative humidity (RH). For this reason, a measurement of indoor RH alone can be a poor predictor of mould problems (Nielsen et al., 2003) and merely decreasing might just not be enough. The microclimates created in books or even between the paper fibbers can be distinct from the relative humidity values in the room where they stand (Sterflinger, 2010), sometimes even after a very long time.

So, we know fungi can be dangerous…But how so? And how many are enough to cause damage?

To answer these questions we have to look at each fungus up close and examine its particularities. Does it produce pigments? Does it have the means to deteriorate cellulose, for instance? Several studies have focused on these issues and although we cannot yet say we have our enemy figured out, we are getting closer.

Genera *Cladosporium, Fusarium, Alternaria, Stachybotrys* and *Chaetomium* display a strong cellulolytic activity and produce a series of metabolites (protease, amylase, etc…) (Andersen et al., 2000, 2002; Borrego et al., 2010). *Aspergillus* sp. and *Trichoderma* sp. are also well known efficient producers of cellulose degrading enzymes (Jayant et al., 2011). Most of the studies on fungal cellulase activity have been limited to species of *Aspergillus, Fusarium, Trichoderma, Myrothecium*, and *Penicillium* (Chadeganipour et al., 2013) so it is possible that the list of fungi able to produce this enzymatic complex is actually much longer.

Aspergillus penicillioides and *Eurotium* sp. (*herbariorum* and others) have been associated with foxing (Arai, 2000; Florian and Manning, 2000). *Emericella nidulans* is known to produce a brownish pigment while the metabolite produced by *A. versicolor* is of an orange tone (Michaelsen et al., 2009).

No globally accepted international standard for archives has yet been established (Cappitelli et al., 2010) either regarding the species or amount of fungi that can be considered dangerous when present in an archival environment. However, some countries have designed their own national guidelines (MIBAC, 2001) and some countries have set them informally (Brokerhof et al., 2007; Harkawy et al., 2011) (see Table 1.1).

Several authors have proposed limits in Poland where the proposed indoor limit value is the same as the limits stipulated for outside air. Karbowska-Berent et al. (2011) based their values after studying the microbial quality on selected archives and libraries and concluded that the higher concentrations of airborne fungi can signal the existence of a moisture problem or internal microbial sources (Harkawy et al., 2011).

Table 1.1 - Limits proposed for air fungal contamination in archives and libraries. Each formed colony is called a colony forming unit (CFU).

Source	Country	Limit (CFU/m^3)
Cieplik (1997)	Poland	• 150 for a mixture of several species • 50 for particular species • 500 for common airborne fungal contaminants
Flieder and Capderou (1999)	France	100
MIBAC	Italy	150
Dutch National archives (Brokerhoff et al., 2007)	Netherlands	• 0-25, no expected problems • 25-100, possible presence of source, further testing needed • 100-1000, source present, mould often observed on objects • 1000, active mould growth
Parchas (2008)	France	120
Proposed guidelines (Harkawy et al, 2011)	Poland	5000
Karbowska-Berent et al. (2011)	Poland	200

The CFU (colony forming units) limits presented by the Dutch National archives (Brokerhoff et al., 2007) refer to mixed flora and in case of just one species present, then 25-100 CFU/m^3 already translates the presence of a source. As in other settings, there are no proposed limits for surface contamination (CFU/m^2). It could be very interesting to think about this since it is the surface of books and their condition that we intend to see preserved.

We have seen that in archives and libraries the presence of fungi can be detrimental to the documents and books safekept in these premises. But this principle is also true to the health of the individuals that work there or visit these locations. This du-

ality makes it of the upmost importance to know the environment where books are kept and people consult them or tend to their care.

Fungi and Human Health

Excerpts of this section are adapted from: Fungi in archives, a Double Concern, In: Environmental Mycology in Public Health: An Overview on Fungi and Mycotoxins for Risk Assessment and Management (Pinheiro 2015a, in press)

Fungal communities interact with paper based heritage. But what is the impact of a fungal contaminated environment on the health of staff and visitors?

Fungi are considered as a serious threat to public health (Khan et al., 2012). When in contact with the human skin, fungi can be responsible for the development of dermatological problems such as *tinea unguis*, scalp disorders and dermal lesions with varying severity degrees (Horner et al, 1995; Sousa et al, 2001). And fungi do not need to establish close contact to induce health problems. Spores can be inhaled and, when conditions are met, be responsible for lung and respiratory impairment. In cases of reduced immunity, this scenario can result in death (Horner et al., 1995). Both spores and hyphae are potential allergens and their sole presence can trigger a histaminic response (Horne et al., 1995; Khan et al., 2012). Sometimes difficult to diagnose, this affection can last for weeks and bring about costs related to work absence and a general decrease in life quality (Horner et al, 1995).

The mechanisms responsible for triggering mould induced illness include: type I allergy, non-IgE mediated specific histamine release and inflammation, changes in lymphocyte composition, generalized immunosuppression and toxic reactions. All of these can be caused by fungal proteins, structural elements, microbial volatile organic compounds (MVOCs) or mycotoxins and other secondary metabolites released from fungal spores and colony fragments after inhalation (Nielsen et al., 2003). Mycotoxins are low-molecular-weight natural products which constitute a toxigenically and chem-

ically heterogeneous assemblage that are grouped together because they can cause disease and death in human beings and other vertebrates (Bennett and Klich, 2003).

The subject of the health impact of fungal growth on the health of workers and attendees of libraries and archives has been mentioned for almost a century but, despite its relevance, there is still a lack of information concerning the fungal population in libraries and archives (either quantitatively or qualitatively) and its relation to health issues.

One of the first records on this concern dates back to the 1915 August 23 issue of Every Week where Rose Murray, one of the six women referred to in the article "Women Who Hold Down Unusual Jobs" is described as "*the only woman in the world who holds the position of physician and surgeon to 'sick' books […]. She is the 'doctor' for all the volumes in the New York Free Public Library. There is a very lively element of danger in her position, because books, like people, derive their sickness largely from germs and microbes. That is why Miss Murray goes about her work dressed just like a surgeon at an operation. Her equipment consists of a huge apron and a veil of cheesecloth*." The image that illustrates the text shows a woman nearly all enveloped in a white gown, with only her eyes, hands and the bottom of her dress showing (Abbey Newsletter, 1994). Was she right in her choice to heavily protect herself?

According to some authors, the most common malaises reported by staff working in libraries, archives or book containing premises are dermatitis, rhinitis, allergies and asthma (Valentin, 2007). Allergic broncopulmonary aspergillosis and hypersensitivity pneumonitis (Valentin, 2007) are probably the most serious. In a museum environment, Wiszniewska et al. (2009) concluded that 30% of museum employees were sensitized to at least one of the fungal allergens tested and that the prevalence of allergic symptoms among the subjects was relatively high and frequently related to specific sensitization. The most frequent symptoms reported by the examined subjects were: conjunctivitis (68.5%), rhinitis (66%), skin symptoms (54%), chronic cough (26%) and dyspnoea (28%) (Wiszniewska et al., 2009).

In 1997, Zyska performed an extensive review of the fungi encountered and identified in archives. Some of the fungi encountered are common air contaminants, such as *Penicillium* sp. or *Cladosporium* sp., but others can be considered detrimental to human health – as *Stachybotrys chartarum* or *Aspergillus fumigatus*.

High level exposure to airborne viable fungi (10^6 CFU/m^3) was determined as the cause in a case of organic dust toxic syndrome in a museum staff handling mouldy books (Kolstad et al., 2002). According to Zielińska-Jankiewicz et al. (2008), some of the archive workers who participated in a survey conducted by Schata in 1995 reported various skin, eye and respiratory symptoms which could have been associated with occupational exposure to moulds. It was estimated that about one third of archive workers might have developed allergy to moulds, which is about twice as high as in the general population (Zielińska-Jankiewicz et al., 2008). The workers taking part in the survey performed by Krake et al. in 1999, reported respiratory and sinus-related symptoms which could have been associated with workplace exposure to moulds (Zielińska-Jankiewicz et al., 2008). In this study, mycological microflora belonging mostly to the *Penicillium*, *Cladosporium, Aspergillus, Alternaria,* and *Tritirachium* species was detected in levels that ranged from 200 to 450 CFU/m^3, depending on the facility, and for the *Tritirachium* species it approximated 800 CFU/m^3.

Health-risk levels suggested in literature vary greatly from author to author and through the years. Table 1.2 presents some of the available maximum fungal concentrations (CFU/m^3) proposals for indoor air (considering multiple building types).

The extreme variability in the mentioned values may be interpreted as a pointer to the need of identifying the species present in a given environment.

Table 1.2 – Proposed maximum concentrations (CFU/m³) limits and considerations for indoor air fungal contamination (Indoor Air Quality)

Proposed Limit	Maximum fungal concentration indoors
Holmberg (1984) (as cited by Flannigan, 2001)	2200 CFU/m³
Ohgke et al. (1987) Hurts et al. (1997)	More than 100 CFU/m³ are a sign of internal contamination
Reynolds, Streifel and McJilton (1990)	500 CFU/m³
Commission of the European Communities (1993) (as cited by Zielińska-Jankiewicz et al., 2008).	2000 CFU/m³ (considered very high by the Commission)
Yang, Hung and Lewis (1993); Etkin (1994)	200 CFU/m³.
Klánová (2000)	2000 CFU/m³ (higher should be considered a health threat)
World Health Organization (as cited by Goyer et al., 2001)	150 CFU/m³

In Portugal, the ordinance 353-A/2013, December 4th, came to substitute NT-SCE-02 for the regulation of the climatic certification of offices and service's buildings. The former "legislation" (NT stands for technical note and complements the 2006 Decree-Law) was more rigorous than the present legislation since, for these types of buildings, all the criteria had to be met at the time of inspection (see Chapter 5 for further details). The Ordinance 353-A/2013 stipulates reference and conformity conditions and now the indoor environment is considered safe when the indoor/outdoor (I/O) fungal ratio is lower than one: higher than one ratios are not expected for fungi (Micalli et al., 2003) since contamination comes normally from the atmosphere but study on indoor fungal contamination is only taken further when this condition is not met. When it is not, then there should be no visible fungal growth; fungal load should never exceed the 500 CFU/m³; the presence of a mixture of relatively uncommon species should not exceed 150 CFU/m³ and one uncommon species (*Acremonium* sp., *Chrysonil-*

ia sp., *Tricothecium* sp., *Curvularia* sp., *Nigrospora* sp.) should not exceed 50 CFU/m^3. In the former legislation uncommon species were defined as not being "*Cladosporium* sp., *Alternaria* sp. and *Penicillium* sp".

As before, pathogenic (disease causing) species such as *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Blastomyces dermatitidis* and *Coccidioides immitis* are not tolerated. The presence of potentially toxinogenic (toxin emitting) fungi like *Stachybotrys chartarum (S. atra)*, *Fusarium moniliforme, Fusarium culmorum*, *Trichoderma viride*, *Aspergillus versicolor, A.flavus, A. ochraceus, A. fumigatus* and *A. niger,* in higher than 12 CFU/m^3 is a sign of an environment of low(er) quality. By the former rules no minimum was set for these species.

Some authors (Ren, Jankun e Leaderer, 1999) consider air sampling to be the best method to determine fungal contamination and only air samples are accounted for in these guidelines. Highlight in them is the need for fungal identification and not just a mere quantification. The established relation between the minimum amount of spores that can cause serious allergic reactions and the fungal species has been reported by Valentin et al., 2007, as between 100/m^3 for *Alternaria alternata* and 3000/m^3 for *Cladosporium herbarum* which reinforces the idea of identification rather than quantification only (Micalli et al., 2003).

In most studies on indoor fungal contamination four main measures are applied:

- total viable mould counts,
- total mould counts (viable and nonviable),
- specific mould species (qualitative or quantitative)
- and β-(1,3)-d-glucan level (beta 1-3 glucans are structural elements of the fungus cell wall considered potent inflammatory agents (Valentin, 2003) and responsible for hypersensitivity reactions (Salkinoja-Salonen et al., 2003).

For the first three options, several methods can be chosen: gravitational deposition (Durham sampler, Tauber trap or simple Petri dishes); impact (of which the An-

dersen sampler is the most popular), suction, filtration, electrostatic precipitation, thermal precipitation and impingement (de Nuntiis et al., 2003) (see Chapter 3 for further details).

The resulting samples can be analysed using the traditional culturing methods of incubation and microscopic analysis or by molecular biology techniques, capable of going further in terms of fungal identification and of sorting out the non-viable fraction. Whenever possible, the best option is to perform both analyses.

For the determination of the atmospheric allergen load (β-(1, 3)-d-glucan level included) immunological techniques such as EIA (enzyme Immunoassay), ELISA (Enzyme-linked Immunoassay) are frequently used (Favalli et al., 2003).

1.1 Research problems

- Since the last review regarding fungi in archives was performed in 1997 and refers to fungi identified in museums and archives before the advent of modern identification techniques, is it safe to say we know the fungal communities present in our archives and libraries?

- In fungal aerobiology studies what methodologies should be used? Classic culturing methods, molecular biology protocols or both? Given the existing choices in both fields, which particular methodologies should be pursued and optimized?

- Knowing the fungal flora present in archives and librairies is extremely important to assess the risk and to devise mechanisms to eliminate/diminish it. What is the present situation in Portuguese archives?

- Are the fungal communities found in selected Portuguese archives in agreement with international results?

- What can we expect from the fungal flora identified in the selected archives? What is its impact on document conservation and health?

- There are no existing guidelines on fungal contamination for Archives. Can the collected data be a valuable tool in addressing this issue?
- Fungal community studies are a pivotal part of any indoor air quality study. Do archives and libraries comply with national legislation (for health purposes) and proposed limits (for conservation)?

1.2 Objectives

The main objective of this thesis is to assess the fungal communities present in archives and study their impact on both paper conservation and human health. To achieve these goals, it was necessary:

- To review existing literature on fungal contamination in archives and methodologies on fungal identification.
- To design a study to assess fungal contamination in archives. The selected methodology should include all steps of the experimental work: sampling, sample treatment and fungal identification.
- To assess fungal contamination in selected Portuguese archives. This evaluation should include air samples, surface samples and areas affected by fungal growth and/or pigmentation.
- To compare the resulting fungal assessment with the results obtained abroad.
- To evaluate the characteristics presented by the identified fungal flora and estimate their influence on both the staff's and attendees health and the document's conservation.
- To assess the indoor air quality in Portuguese archives, both from the paper conservation and the human health point of view.

- To investigate the influence of water activity on fungal development and fungal load on documents. Also to study the relationship between the water activity on documents and relative humidity from the air.

- To test the application of the molecular biology protocol developed outside the archives.

1.3 Thesis outline

It is essential to determine the fungal profile of a given setting to truly understand the impact fungi can have on both document's conservation and human health preservation. Indoor air quality studies do not omit this fact and include both identification and quantification of fungal species/genera (Decree Law 78 /2006, April 4th and Ordinance 353-A/2013, December 4th). It was the purpose of this study to address indoor air quality in archives and libraries with a special focus on the characterization of fungal flora communities and its relation to documents biodeterioration and human health.

In 1997, almost 20 years ago, Zyska reviewed the fungi encountered and identified in archives and these results refer to analysis on books, documents, air and dust samples in a time where fungal identification was only achieved by conventional culturing methods. An updated review is imperative and presented in Chapter 2.

After having the knowledge about fungi identified in archives all over the world, it is important to find out the Portuguese reality regarding this subject. Therefore, four Portuguese archives were selected for study. Methodologies to perform this study are presented in Chapter 3 and include classical culturing methods and modern molecular biology protocols especially relevant to determine the non-viable fraction of the biological contaminants. Within the latter, denaturing high performance liquid chromatography (DHPLC), a very recent technology, first created to detect gene mutation has already been applied to the study of microbial community but only bacterial ones. study of microbial communities. In this study, a new methodology was developed and firstly applied to both fungal and yeasts mixed samples to identify its components.

Once presented the methodology, the results from a study on airborne and settled fungi carried out in four Portuguese settings are presented. Considerations were taken from their presence, from both the document's safekeeping and the human health perspective (Chapter 4).

Fungal communities are intrinsically related to water activity and other parameters such as particulate matter. This last one is a key component in any indoor air quality evaluation - of which there are no records for Portuguese archives. Biological, physical and chemical parameters must be kept within limits in order to obtain a certification for air quality. Certification parameters were analysed in two Portuguese archives in order to assess both the indoor air quality for people attending or working in these premises and for the valuable written heritage that must be kept for future generations (Chapter 5).

The study on fungal contamination showed a wide and varied fungal community. Water activity is determinant to evaluate its potential for deteriogenic and toxinogenic activity. An entire food industry relies on the principle of water balance between substrate and the environment and this should also be considered when it is cultural heritage we're discussing. This relevant issue is presented and discussed in Chapter 6.

Desirably, the breakthroughs achieved in a PhD project gain value when they can also serve other scientific areas. As mentioned, this was a project meant to join together the areas of health and written heritage conservation. Chapter 3 introduced the DHPLC and its utility in the identification of fungi present in paper samples and the developed methodology was applied successfully both in the field of clinical diagnosis and fungal contamination in a gilded wood church structure. Both methodology applications are presented in Chapter 7.

The main results and conclusions of this study and the further research that needs to be done in this area are presented in Chapter 8.

2. Fungi in archives: state of the art

A review of the literature

The last formal review on the area of fungi in archives and libraries was performed by Zyska in 1997 (almost 20 years ago) and recollects data from very diverse origins. In fact, it is not just focused on books but on panoply of materials one can find in museums, archives or libraries. It does have the great advantage (sometimes forgotten by some of its recent followers) of presenting data from both air and surface samples which can be essential to truly know the fungal communities. Some spores, given their characteristics, tend to aggregate and are not easily airborne which makes it difficult to find them in air samples although they can be present in the environment (Duchaine and Mérieux, 2001). This could be deemed of lesser importance if this was not the case for *Stachybotrys chartarum*, one the fungi most associated with Sick Building Syndrome (Bennett and Klich, 2003).

Because no other method was sufficiently evolved at the time, the identifications gathered by Zyska include only classical culturing methods which leave out the trendy and modern molecular biology protocols. And these do bring out in the open some fungal species which were either unidentifiable (*mycelia sterilia* or sterile micelia) or simply did not show any growth.

Between 1997 and 2013 several authors have embraced the task of characterizing the fungal flora present in archives. A compilation of these studies is presented in the next pages. Tables 2.1 to 2.6 refer to fungi identified:

- in air samples (Tables 2.1, 2.2, 2.3 and 2.5)
- surface samples (shelves, tables and floor) (Table 2.4 and 2.5)
- archival document cases (Table 2.4 and 2.5)
- document samples (Table 2.6)

2.1 Air studies

Due to the difficulty of achieving species identification - for reasons as simple as the overwhelming variety of species within a fungal genus - many studies stop at this taxonomic level. *Penicillium* species, for instance, are impossible to identify using just *simple* culturing methods and/or relying on just one growth media.

Several countries have performed indoor air studies in other settings. While in Scotland the most prevalent genus is *Cladosporium* (Stevens, 2004), in Great Britain the most frequently isolated is *Penicillium*. In the Netherlands, North America, Canada, Australia, Germany, Lithuania, Taiwan and Turkey, *Penicillium* and *Aspergillus* join *Cladosporium* as the most common indoor fungi genera (Stevens, 2004).

With slightly different results and mentioning some species, the most common indoor species for Singh (2001) were *Cladosporium herbarum*, *Alternaria alternata*, *Eurotium herbariorum*, *Penicillium* sp., *Aspergillus* sp. (namely *Aspergillus versicolor*), *Aureobasidium pullulans, Mucor* sp., *Phoma* sp. and *Wallemia* sp.

In Portugal, indoor air quality studies have found *Cladosporium, Aspergillus* and *Alternaria* as the most common genera (Viegas et al., 2012). The outdoor air is one of the main sources of fungi and in Lisbon's outdoor environment the most predominant genera are *Cladosporium, Penicillium, Aspergillus and Alternaria* (Viegas et al, 2012).

Zyska (1997) and Gallo et al. (2003, data recollected in 1998 for the first version of the book) mention some of the most common fungi in archives and librairies. When to these two reviews we add the identified fungal genera found in field studies (Gambale et al. (1993); Rakotonirainy et al. (1999); Jain (2000); Bueno et al. (2003); Aira (2007); Valentin (2007); Ruga et al. (2008); Wlazlo et al. (2008); Zielińska-Jankiewicz et al. (2008); Borrego et al. (2010); Harkawy et al. (2011); Borrego et al. (2012); Lugauskas and Krikštaponis (2004) and Nunes et al. (2013)) the frequency of each fungal genera can be presented as it is in Table 2.1:

Table 2.1- Fungal genera found in the air of archives grouped according to the number of studies in which they were identified.

Presence in the selected studies	Fungal genera
0% a)	*Acrothecium* sp., *Chaetomella* sp., *Coprinus* sp., *Gymnoascus* sp., *Pellicularia* sp., *Ramichloridium* sp. *Rhizoctonia* sp., *Serpula* sp., *Thielavia* sp., *Torula* sp.
About 10% of the studies	*Acremoniella* sp., *Basipetospora* sp., *Bispora* sp., *Ceratsporum* sp., *Cryptococcus* sp., *Dactylella, Doratomyces* sp., *Dreschelera* sp., *Emericella* sp., *Gonytrichum* sp., *Harpographium* sp., *Heterocephalum* sp., *Microsporum* sp.,*Monascus* sp., *Monosporium* sp., *Mortiriella* sp., *Pestalotia* sp.,*Prohytroma* sp., *Rhizomucor* sp., *Scedosporium sp.*, *Scolecobasidium* sp., *Sepedonium* sp., *Sporodiniella* sp., *Sporotrichum* sp., *Sporothrix* sp., *Syncephalastrum* sp., *Trycophyton* sp., *Wallemia* sp.
15 to 30% of the studies	*Arthrinium* sp., *Aureobasidium* sp., *Candida* sp., *Chrysonilia* sp., *Chrysosporium* sp., *Epicoccum* sp., *Eurotium* sp.,*Gliocladium* sp., *Helminthosporium* sp., *Humicola* sp., *Mucor* sp., *Mycelia sterilia, Myrothecium* sp., *Neurospora* sp., *Nigrospora* sp., *Oidiodendrum* sp., *Phoma* sp., *Rhodotorula* sp., *Stachybotrys* sp., *Stemphylium* sp., *Talaromyces* sp., *Trichocladium* sp., *Trichothecium* sp., *Ulocladium* sp., *Verticillium* sp.
30 to 50% of the studies	*Acremonium* sp., *Botrytis* sp., *Curvularia* sp., *Paecilomyces* sp., *Rhizopus* sp., *Scopulariopsis* sp.,*Trichoderma* sp.
More than 50%	*Alternaria* sp., *Aspergillus* sp., *Chaetomium* sp., *Cladosporium* sp., *Fusarium* sp., *Geotrichum* sp., *Penicillium* sp.

a)These genera are cited as common in the two reviews analysed (Zyska, 1997 and Gallo et al., 2003). They have not been identified in the air of archives/librairies since then.

Several fungi are mentioned as common but, since Zyska's review, they have not been found in recent studies. It is the case of *Acrothecium* sp., *Chaetomella* sp., *Coprinus* sp., *Gymnoascus* sp., *Pellicularia* sp., *Ramichloridium* sp., *Rhizoctonia* sp., *Serpula* sp. or *Thielavia* sp. Since the 1997 review was based on studies from the early 20th century, and given the ever-changing and complex nomenclature for Fungi it is possible that these genera were either wrongly attributed or now belong to other taxons. Gallo et al. (2003) adds *Torula* sp. to this list.

The most common genera found in air samples retrieved from archives are *Alternaria, Aspergillus, Chaetomium, Cladosporium, Fusarium, Geotrichum* and *Penicillium*. Some of these are also the most common fungi found outdoors worldwide. Interestingly, *Chaetomium* sp., *Fusarium* sp. *and Geotrichum* sp. are special contaminants in archives and all have biodeterioration potential.

Related to Table 2.1, Table 2.2 presents a review of the most common species found in the air of archives/librairies according to the author's on which Table 2.1 was based. Despite the mentioned difficulties, the identification to a species level – whenever possible – is important since some relevant metabolites/mycotoxins/pigments are species-specific. In the left column are the fungal genera/species and the reviews which presented them as common in air samples in archives. In the right column are the studies where these fungi were actually identified through experimental work. The blank entries in the right column refer to fungi not mentioned recently in field studies.

Table 2.2 - Fungal genera/species identified in air samples in archives.

Fungal genera/species	Isolated and identified by:
Acremoniella sp. Sacc	Bueno et al. (2003)
Acremonium sp. Link (b)(d)	Gambale et al. (1993); Bueno et al. (2003); Lugauskas and Krikštaponis (2004); Wlazlo et al. (2008); Nunes et al. (2013);
Acremonium charticola (Lindau) W. Gams	Wlazlo et al. (2008); Zielińska-Jankiewicz et al. (2008)
Acremonium kiliense Grütz	Lugauskas and Krikštaponis (2004)
Acremonium murorum (Corda) W. Gams	Lugauskas and Krikštaponis (2004); Zielińska-Jankiewicz et al. (2008)
Acremonium roseum Petch	Lugauskas and Krikštaponis (2004)
Acremonium strictum W. Gams	Lugauskas and Krikštaponis (2004); Wlazlo et al. (2008); Zielińska-Jankiewicz et al. (2008); Harkawy et al.(2011)
Acrothecium sp. (Corda) Preuss (d)	
Alternaria sp. Nees (b)(d)	Gambale et al. (1993); Bueno et al. (2003); Aira (2007); Valentin (2007); Ruga et al. (2008); Wlazlo et al. (2008); Nunes et al. (2013)
Alternaria alternata (Fr.) Keissl. (a)(d)	Lugauskas and Krikštaponis (2004); Wlazlo et al. (2008); Zielińska-Jankiewicz et al. (2008); Borrego et al. (2012)
Alternaria tenuíssima (Nees) Wiltshire	Lugauskas and Krikštaponis (2004)
Arthrinium phaeospermum (Corda) M.B. Ellis	Lugauskas and Krikštaponis (2004); Aira (2007)
Aspergillus sp. P. Micheli ex Haller (b)(d)	Gambale et al. (1993); Bueno et al. (2003); Lugauskas and Krikštaponis (2004); Aira (2007); Valentin (2007); Ruga et al. (2008); Wlazlo et al. (2008); Borrego et al. (2012)

Fungal genera/species	Isolated and identified by:
Aspergillus candidus Link	Wlazlo et al. (2008)
Aspergillus chevalieri L. Mangin	Lugauskas and Krikštaponis (2004)
Aspergillus clavatus Desm.	Lugauskas and Krikštaponis (2004); Wlazlo et al. (2008); Borrego et al. (2012)
Aspergillus flavipes (Bainier & Sartory) Thom & Church	Lugauskas and Krikštaponis (2004)
Aspergillus flavus Link (c)	Lugauskas and Krikštaponis (2004); Borrego et al. (2012); Wlazlo et al. (2008)
Aspergillus foetidus (N. Nakata) Thom et Raper	Lugauskas and Krikštaponis (2004)
Aspergillus fumigatus Fresen.	Lugauskas and Krikštaponis (2004); Aira (2007); Wlazlo et al. (2008); Zielińska-Jankiewicz et al. (2008); Nunes at al (2013)
Aspergillus glaucus Link	Wlazlo et al. (2008)
Aspergillus melleus Yukawa	Wlazlo et al. (2008)
Aspergillus niger Tiegh. (c)	Lugauskas and Krikštaponis (2004); Aira (2007); Wlazlo et al. (2008); Valentin (2007); Zielińska-Jankiewicz et al. (2008); Harkawy et al. (2011); Borrego et al. (2012)
Aspergillus ochraceus K. Wilh	Wlazlo et al. (2008)
Aspergillus oryzae (Ahlb.) Cohn	Lugauskas and Krikštaponis (2004)
Aspergillus ostianus Wehmer	Wlazlo et al. (2008)
Aspergillus penicillioides Sp.eg.	Wlazlo et al. (2008)
Aspergillus puniceus Kwon-Chung et Fennell	Lugauskas and Krikštaponis (2004)
Aspergillus repens (Corda) Sacc. (a)	Lugauskas and Krikštaponis (2004); Wlazlo et al. (2008)
Aspergillus restrictus G. Sm	Zielińska-Jankiewicz et al. (2008)
Aspergillus sydowii (Bainier & Sartory) Thom & Church	Lugauskas and Krikštaponis (2004)
Aspergillus terreus Thom	Lugauskas and Krikštaponis (2004); Borrego et al. (2012);
Aspergillus tricolor Frisvad, Seifert, Samson&Mills	Wlazlo et al. (2008)
*Aspergillus ustus (*Bainier) Thom & Church (c)	Lugauskas and Krikštaponis (2004); Zielińska-Jankiewicz et al. (2008)
Aspergillus versicolor (Vuill.) Tirab (a)(d)	Lugauskas and Krikštaponis (2004); Wlazlo et al. (2008); Zielińska-Jankiewicz et al. (2008); Harkawy et al.(2011); Borrego et al. (2012);
Aspergillus wentii Wehmer	Lugauskas and Krikštaponis (2004)
*Aureobasidium sp.*Viala & G. Boyer (b)(d)	Gambale et al. (1993); Ruga et al. (2008)
Aureobasidium pullulans (De Bary) G. Arnaud ex Cif., Ribaldi & Corte (a)(d)	Lugauskas and Krikštaponis (2004); Zielińska-Jankiewicz et al. (2008)
Basipetospora sp.G.T. Cole & W.B. Kendr.	Bueno et al. (2003)
Bispora sp. Corda	Jain (2000)
Botrytis sp. P. Micheli ex Haller	Bueno et al. (2003); Ruga et al. (2008)
Botrytis cinerea (Pers.) (a)	Lugauskas and Krikštaponis (2004); Valentin (2007); Aira (2007); Zielińska-Jankiewicz et al. (2008)
Candida sp. Berkhout	Lugauskas and Krikštaponis (2004); Wlazlo et al. 2008
Candida albicans (C.P. Robin) Berkhout	Lugauskas and Krikštaponis (2004)
Candida famata (F.C. Harrison) S.A. Mey. & Yarrow	Harkawy et al. (2011); Wlazlo et al. (2008)
Ceratosporium sp. Schwein.	Bueno et al. (2003)
Chaetomella sp. Fuckel (d)	

Fungal genera/species	Isolated and identified by:
Chaetomium sp. Kunze (b)(d)	Bueno et al. (2003); Lugauskas and Krikštaponis (2004); Ruga et al. (2008); Valentin (2007); Zielińska-Jankiewicz et al. (2008); Harkawy et al. (2011)
Chaetomium bostrychodes Zopf	Wlazlo et al. (2008)
Chaetomium elongatum Czerepanova	Wlazlo et al. (2008); Harkawy et al.(2011);
Chaetomium globosum Kunze ex Fr. (c)(d)	Lugauskas and Krikštaponis (2004); Zielińska-Jankiewicz et al. (2008); Nunes et al. (2013);
Chaetomium indicum Corda	Aira (2007)
Chaetomium nigricolor L.M. Ames	Nunes et al. (2013)
Cryptococcus sp.Vuill.	Wlazlo et al. (2008)
*Cryptococcus laurentii (*Kuff.) C.E. Skinner	Wlazlo et al. (2008)
Chrysonilia sp. Arx (b)	Valentin (2007)
Chrysonilia sitophila (Mont.) Arx	Wlazlo et al. (2008)
*Chrysosporium sp.*Corda	Bueno et al.(2003); Lugauskas and Krikštaponis (2004)
Chrysosporium merdarium (Link) J.W. Carmich.	Lugauskas and Krikštaponis (2004)
Cladosporium sp.Link (b)(d)	Gambale et al. (1993); Bueno et al. (2003); Aira (2007), Valentin (2007); Ruga et al. (2008); Wlazlo et al. (2008); Borrego et al. (2012)
Cladosporium cladosporioides (Fresen.) G.A. de Vries	Lugauskas and Krikštaponis (2004); Wlazlo et al. (2008); Zielińska-Jankiewicz et al. (2008); Borrego et al. (2012)
Cladosporium elatum (Harz) Nannf.	Borrego et al. (2012)
Cladosporium elongatum Persoon	Wlazlo et al. (2008)
Cladosporium fulvum Cooke	Valentin (2007)
Cladosporium herbarum (Pers.) Link (a)(c)(d)	Jain (2000); Lugauskas and Krikštaponis (2004); Valentin (2007); Zielińska-Jankiewicz et al. (2008); Borrego et al. (2012)
Cladosporium sphaerospermum Penz.	Lugauskas and Krikštaponis (2004); Borrego et al. (2012)
Coprinus sp. Pers. (d)	
Curvularia sp. Boedijn (d)	Gambale et al. (1993); Bueno et al. (2003); Borrego et al. (2012)
Curvularia lunata (Wakker) Boedijn	Lugauskas and Krikštaponis (2004); Valentin (2007)
Dactylella sp. Grove	Bueno et al. (2003)
Doratomyces sp.Corda (d)	
Doratomyces stemonitis(Pers.) F.J. Morton & G. Sm	Lugauskas and Krikštaponis (2004)
Drechslera sp.S. Ito	Bueno et al. (2003)
Emericella nidulans (Eidam) Vuill *(anamorph: A. nidulans)*	Wlazlo et al. (2008)
Epicoccum sp. Link (d)	Gambale et al. (1993); Bueno et al. (2003); Ruga et al. (2008)
Epicoccum purpurascens Ehrenb.	Zielińska-Jankiewicz et al. (2008)
?Eurotium amstelodamii L. Mangin	Wlazlo et al. (2008)
?Eurotium repens de Bary, *(anamorph: Aspergillus pseudoglaucus)*	Zielińska-Jankiewicz et al. (2008)
Fusarium sp.Link (b)(d)	Gambale et al. (1993); Bueno et al. (2003); Valentin (2007); Ruga et al. (2008); Wlazlo et al. (2008); Zielińska-Jankiewicz et al. (2008); Borrego et al. (2012)
Fusarium roseum Link	Valentin (2007)
Fusarium oxysporum Schltdl. (e)	
Fusarium semitectum Berk. & Ravenel	Valentin (2007)
Fusarium solani (Mart.) Sacc.	Borrego et al. (2012); Nunes et al. (2013)
*Geotrichum sp.*Link	Gambale et al. (1993); Ruga et al. (2008); Borrego et al. (2010)

Fungal genera/species	Isolated and identified by:
Geotrichum candidum Link	Bueno et al. (2003); Lugauskas and Krikštaponis (2004); Valentin (2007);Wlazlo et al. (2008); Harkawy et al. (2011)
Gliocladium sp. Corda (d)	Valentin (2007)
Gliocladium roseum Bainier	Lugauskas and Krikštaponis (2004)
*Gliocladium virens*J.H. Mill., Giddens & A.A. Foster	Lugauskas and Krikštaponis (2004)
Gonytrichum sp. Nees & T. Nees	Bueno et al. (2003)
Harpographium sp.Sacc.	Bueno et al. (2003)
Helminthosporium sp. Link (b)	Jain (2000); Gambale et al. (1993); Bueno et al. (2003);
Hetereocephalum sp.Thaxt.	Wlazlo et al. (2008)
Heterocephalum auranticum Thaxt.	Wlazlo et al. (2008)
Humicola sp. Traaen (d)	Bueno et al. (2003); Ruga et al. (2008)
Humicola fuscoatra Traaen	Zielińska-Jankiewicz et al. (2008)
Microsporum canis (E. Bodin) E. Bodin	Lugauskas and Krikštaponis (2004)
Microsporum gypseum (E. Bodin) Guiart & Grigoraki	Lugauskas and Krikštaponis (2004)
Monascus sp.Tiegh.	Gambale et al. (1993)
Monosporium silvaticum Oudem.	Wlazlo et al. (2008)
Mortiriella exigua Linnem.	Lugauskas and Krikštaponis (2004)
Mortierella hyalina(Harz) W. Gams	Lugauskas and Krikštaponis (2004)
Mortierella polycephala Coem.	Lugauskas and Krikštaponis (2004)
Mortierella pusilla Oudem.	Lugauskas and Krikštaponis (2004)
Mucor sp. Fresen.(b)	Lugauskas and Krikštaponis (2004); Valentin (2007)
Mucor mucedo L.	Lugauskas and Krikštaponis (2004)
Mucor piriformis Scop.	Lugauskas and Krikštaponis (2004)
Mucor plumbeus Bom	Wlazlo et al. (2008)
Mucor racemosus Bull.	Lugauskas and Krikštaponis (2004); Wlazlo et al. (2008)
Mucor strictus Hagem	Lugauskas and Krikštaponis (2004)
Mycelia sterilia	Gambale et al. (1993); Lugauskas and Krikštaponis (2004); Borrrego et al. (2010)
Myrothecium roridum Tode	Valentin (2007); Ruga et al.(2008)
Neurospora sp.Shear & B.O. Dodge	Gambale et al. (1993); Bueno et al. (2003); Borrego et al. (2010);
Nigrospora sp. Zimm.	Gambale et al. (1993); Bueno et al. (2003)
Oidiodendron sp. Robak	Wlazlo et al. (2008)
Oidiodendron citrinum G.L. Barron	Wlazlo et al. (2008)
Oidiodendron echinulatum G.L. Barron	Lugauskas and Krikštaponis (2004)
Oidiodendron flavum Barron	Wlazlo et al. (2008)
Oidiodendron rhodogenum Robak	Wlazlo et al. (2008)
Oidiodendron truncatum Barron	Wlazlo et al. (2008)
Paecilomyces sp. Bainier (b)(d)	Bueno et al. (2003);Valentin (2007); Wlazlo et al. (2008)
Paecilomyces lilacinus (Thom) Samson	Nunes et al. (2013)
Paecilomyces variotti Bainier (c)	Lugauskas and Krikštaponis (2004); Wlazlo et al. (2008); Zielińska-Jankiewicz et al. (2008)
Pellicularia sp. Cooke (d)	
Penicillium sp. Link (b)(d)	Gambale et al. (1993); Bueno et al. (2003); Lugauskas and Krikštaponis (2004); Aira (2007); Valentin (2007); Ruga et al. (2008); Wlazlo et al. (2008); Zielińska-Jankiewicz et al. (2008); Harkawy et al. (2011); Nunes et al. (2013);
Penicillium aurantiogriseum Dierckx (d)	Wlazlo et al. (2008)

Fungal genera/species	Isolated and identified by:
Penicillium brevicompactum Dierckx (a)	Wlazlo et al. (2008)
Penicillium chermesinum Biourge	Lugauskas and Krikštaponis (2004)
Penicillium chrysogenum Thom (d)	Lugauskas and Krikštaponis (2004); Wlazlo et al. (2008); Zielińska-Jankiewicz et al. (2008); Borrego et al. (2012);
Penicillium citrinum Thom	Wlazlo et al. (2008); Zielińska-Jankiewicz et al. (2008); Borrego et al. (2012)
Penicillium commune Thom	Valentin (2007); Wlazlo et al. (2008); Borrego et al. (2012);
Penicillium corylophilum Dierckx	Lugauskas and Krikštaponis (2004); Zielińska-Jankiewicz et al. (2008)
Penicillium cyclopium Westling	Zielińska-Jankiewicz et al. (2008)
Penicillium expansum Link (d)	Lugauskas and Krikštaponis (2004)
Penicillium glabrum (Wehmer) Westling	Wlazlo et al. (2008)
Penicillium griseofulvum Dierckx	Borrego et al. (2012)
Penicillium oxalicum Currie and Thom	Lugauskas and Krikštaponis (2004)
Penicillium spinulosum Thom	Lugauskas and Krikštaponis (2004); Zielińska-Jankiewicz et al. (2008)
Penicillium sublateritum Biourge	Wlazlo et al. (2008)
Penicillium verrucosum Dierckx	Lugauskas and Krikštaponis (2004); Wlazlo et al. (2008)
Penicillium waksmanii K. M. Zalessky	Wlazlo et al. (2008)
Pestalotia sp. De Not. (d)	Valentin (2007)
Phoma sp. Sacc.(b)(d)	Gambale et al. (1993)
Phoma betae A.B. Frank	Valentin (2007)
Phoma levelei Boerem et Bollen	Lugauskas and Krikštaponis (2004)
Prohytroma sp.	Wlazlo et al. (2008)
Ramichloridium sp. Stahel ex de Hoog (d)	
Rhizoctonia sp. DC(d)	
Rhizomucor pusillus (Lindt) Schipper	Lugauskas and Krikštaponis (2004)
Rhizopus sp. Ehrenb.(b)(d)	Gambale et al. (1993); Valentin (2007)
Rhizopus arrhizus A. Fisch.	Wlazlo et al. (2008)
Rhizopus nigricans Ehrenb.	Zielińska-Jankiewicz et al. (2008)
Rhizopus stolonifer (Ehrenb.) Vuill.	Jain (2000); Aira (2007); Wlazlo et al. (2008)
Rhodotorula sp. F.C. Harrison	Gambale et al. (1993); Lugauskas and Krikštaponis (2004)
Rhodotorula glutinis (Fresen.) F.C. Harrison	Wlazlo et al. (2008)
Rhodotorula mucilaginosa (A. Jörg.) F.C. Harrison	Wlazlo et al. (2008)
Scolecobasidium sp. E.V. Abbott	Lugauskas and Krikštaponis (2004)
Scopulariopsis sp. Bainier (b)(d)	Bueno et al. (2003); Ruga et al. (2008); Valentin (2007); Borrego et al. (2012)
Scopulariopsis brevicaulis (Sacc.) Bainier	Wlazlo et al. (2008)
Scopulariopsis fusca Zach	Wlazlo et al. (2008)
Sepedonium sp. Link (d)	Bueno et al. (2003)
Serpula sp. (Pers.) Gray (d)	
Sporodiniella umbellata Boedijn ex H.C. Evans & Samson	Lugauskas and Krikštaponis (2004)
Sporotrichum sp.Link (d)	
Sporotrichum olivaceum (Link) Fr.	Lugauskas and Krikštaponis (2004)
Sporothrix sp. Hektoen & C.F. Perkins	Bueno et al. (2003)
Stachybotrys sp. Corda (b,d)	

Fungal genera/species	Isolated and identified by:
Stachybotrys chartarum (Ehrenb.) S. Hughes	Lugauskas and Krikštaponis (2004); Valentin (2007)
Stemphylium Wallr. (b,d)	Bueno et al. (2003); Ruga et al. (2008); Valentin (2007)
Syncephalastrum sp. J. Schröt.	Borrego et al. (2012)
Talaromyces funiculosus (c)	Lugauskas and Krikštaponis (2004); Wlazlo et al. (2008); Zielińska-Jankiewicz et al. (2008)
Talaromyces helicus (Raper and Fennel)	Zielińska-Jankiewicz et al. (2008)
Talaromyces purpurogenus Stoll	Lugauskas and Krikštaponis (2004); Aira (2007); Wlazlo et al. (2008)
Talaromyces rugulosus (Thom) Samson, Yilmaz, Frisvad and Seifert)	Zielińska-Jankiewicz et al. (2008)
Thielavia sp. Zopf (d)	
Torula sp Pers. (b)(d)	
Trichocladium sp. Harz (d)	Bueno et al. (2003); Valentin (2007)
Trichoderma sp. Pers (b)(d)	Gambale et al. (1993); Lugauskas and Krikštaponis (2004); Valentin (2007); Wlazlo et al. (2008)
Trichoderma viride (c)	Lugauskas and Krikštaponis (2004); Valentin (2007); Wlazlo et al. (2008); Zielińska-Jankiewicz et al. (2008)
Tricophyton sp. Malmsten	Ruga et al. (2008)
Trichothecium sp.Link (b,d)	
Trichothecium laxicephalum (Kamyschko) M.A. Litv.	Wlazlo et al. (2008)
Trichothecium roseum (Pers.) Link	Valentin (2007); Wlazlo et al. (2008); Zielińska-Jankiewicz et al. (2008)
Ulocladium sp. Preuss	Bueno et al. (2003); Valentin (2007); Wlazlo et al. (2008)
Ulocladium chartarum (Preuss) E.G. Simmons	Zielińska-Jankiewicz et al. (2008)
Verticillium sp. Nees(b,d)	Zielińska-Jankiewicz et al. (2008)
Verticillium album Licop.	Lugauskas and Krikštaponis (2004)
Wallemia sebi (Fr.) v. Arx (a)	Wlazlo et al. (2008)

Notes: The entries marked with a "?" before the name are still under taxonomic evaluation. * Nunes et al. (2013) applied both conventional and molecular biology methods to perform the identification.

(a) Included in the Lugauskas and Krikštaponis, 2004 revision; (b) Included in the Gallo et al., 2003 revision; (c) Included in the Rakotonirainy et al. 1999 revision; (d) Included in the Zyska, 1997 revision; (e) Included in the Valentin et al., 2003, revision.

Nomenclature changes have deemed obsolete some of the fungal names mentioned in the consulted sources (Tables 2.1 and 2.2):

- *Cephalotrichum* sp. (Zyska, 1997) is now *Doratomyces* sp.,
- *Cephalosporium* sp. (Gallo et al., 2003) is now *Acremonium* sp., *Cephalosporium acremonium* and *C. charticola* (Wlazlo et al., 2008) are now *Acremonium strictum* and A. *charticola*, respectively,
- *Chloridium* sp. (Zyska, 1997) is now *Ramichloridium* sp.

- *Hormiscium* sp. (Gallo et al., 2003) is now *Torula* sp. In the *Torula* genera some species are now classified as belonging to the *Exophiala*, *Candida*, *Rhodotorula*, *Cladophialophora* or *Cryptococcus* genera.
- *Mucor globosus* (Lugauskas and Krikštaponis, 2004) is now known as *M. racemosus*
- *Rhizopus oryzae* (Wlazlo et al., 2008) as *R. arrhizus*.

In the *Penicillium* genus a taxonomic revolution is underway. Due to the large variety of species in this genus and the existing havoc in fungal nomenclature some of the species are being genetically analysed and re-named accordingly. Also the species from the subgenus biverticillium are now being grouped under the *Talaromyces* genera. This brings some changes to the names of the species identified by some of the mentioned authors (*www.aspergilluspenicillium.org*). As such:

- *P. frequentans* (Wlazlo et al., 2008) and *P. terlikowskii* (also Wlazlo et al., 2008) are now known as *P. glabrum*.
- *P. funiculosum* (Rakotonirainy et al., 1999; Lugauskas and Krikštaponis, 2004; Wlazlo et al.; 2008; Zielińska-Jankiewicz et al., 2008) is now known as *Talaromyces funiculosus*,
- *P. griseoazureum* (Wlazlo et al., 2008) is now *P. waksmanii*
- *P.rugulosum* (Zielińska-Jankiewicz et al., 2008) is now *Talaromyces rugulosus.*

The studies on which Tables 2.1 and 2.2 are based are all qualitative in nature but some of them also include fungal counts. Different sampling methods produce different results and comparison between them is sometimes very difficult. The CFU/m^3 varies greatly between these quantitative studies and some of the results are presented in Table 2.3.

Table 2.3 – Air contamination studies performed in archives and librairies.

Study	Location		Method	CFU/m³
Lugauskas and Krikštaponis (2004)	Library of the Lithuanian Academy	a)Rare books department (several areas); b)Old periodicals department.	slit-to-agar single stage impactor and gravitation settle plates	Min-Max: a)300-500; b)100-400
	Martynas Mazˇvydas National Library of Lithuania		slit-to-agar single stage impactor	Averages: 300-800.
Borrego et al (2012)	a)Historical Archive of Museum of La Plata (HAMLP), b)Archive of Historical and Cartographic Research Department from the Geodesy Direction (AHCRD), c)Archive of Notaries of Buenos Aires Province (AN)		Petri dishes	Average: a)120; b)1271 and c)7667
Jain (2000)	Library of the University of Rajasthan (Jaipur, India)		Andersen two stage impactor	Min-Max: 40 - 450
Ruga et al (2008)	Doctorate Library, Perugia University (Italy)	Library	Personal Volumetric Air Sampler Spore Trap and gravitation settle plates	Average: 7000 spores/m³
Zielińska-Jankiewicz et al. (2008)	Library and Archive Storage facilities	**Library:** a)storage room I b)storage room II c)rare books department d)bookbinding workshop **Archive:** e)restoration and maintenance unit f)torage room I g)storage room II	Impact method	Min-Max (average) a)160–1700 (570) b)20 –780 (290) c)60 –800 (180) d)260–1800 (830) Archive e)260 –2900 (2300) f)60–1200 (630) g)50–940 (290)

Study	Location		Method	CFU/m^3
Cappitelli et al. (2009)	Historical Archive of Ca' Granda	8 locations in the Capitolo d'Estate (main hall) and 13 locations in the basement	MAS-100 microbial air sampler (single-stage sieve impactor)	Min-Max: main hall :475-800 (morning); 680-1500 (afternoon) Basement: 230-1600 (morning)

Lugauskas and Krikštaponis (2004) used a slit-to-agar single stage impactor but the use of Petri dishes (Bueno et al. 2003; Borrego et al., 2010, 2012) in quantitative studies can also be used and translated into CFU/m^3 based on the exposure time and plate area (data only made available by Borrego et al. 2010, 2012). Valentin (2007) presents a qualitative and quantitative study but the CFU/m^3 fungi data are joined with the bacteria data. Cappitelli et al. (2009) study was only quantitative and, as such, the results were not included in either Tables 2.1 or 2.2.

2.2 Surface Studies

There are very few studies assessing fungal contamination in surfaces but, as already mentioned, depending on the type on the spore, it can be very beneficial to perform them.

Maggi et al. (2000) conjugated surface samples of both document bindings and metal shelves and also performed a quantitative analysis (per $24cm^2$) in each of the tested surfaces. Maggi's results, along with Krysińska-Traczyk's (1994) results from the study on dust from contaminated books (see Zielińska-Jankiewicz et al., 2008, as the original is in polish) and the surface results from Zielińska-Jankiewicz et al., 2008, are all presented in Table 2.4. The organic surfaces considered include books, paper, documents (Zielinska-Jankiewicz et al, 2008; Krysińska-Traczyk, 1994) and book bindings (Maggi, 2000) while the inorganic surfaces relate to the floor and metallic shelves.

Table 2.4 - Fungal genus/species found on dust and/or surface samples (books, book bindings or shelves/floor).

Fungi	Organic surfaces	Inorganic surfaces
Acremonium sp. Link	Maggi et al. (2000)	
Alternaria sp. Nees	Maggi et al. (2000)	Maggi et al. (2000)
Alternaria alternata (Fr.) Keissl.	Maggi et al. (2000)	Maggi et al. (2000)
Arthrinium phaeospermum (Corda) M.B. Ellis	Maggi et al. (2000)	
Aspergillus sp. P. Micheli ex Haller	Maggi et al. (2000)	Maggi et al. (2000);
Aspergillus flavus Link	Maggi et al. (2000)	
Aspergillus fumigatus Fresem	Maggi et al. (2000); Zielińska-Jankiewicz et al. (2008)	
Aspergillus japonicus Saito	Maggi et al. (2000)	
Aspergillus niger Tiegh	Krysińska-Traczyk (1994); Maggi et al. (2000)	Maggi et al. (2000);
Aspergillus ochraceus K. Wilh	Maggi et al. (2000)	Maggi et al. (2000)
Aspergillus sydowii (Bainier & Sar-tory) Thom & Church		Maggi et al. (2000)
Aspergillus versicolor (Vuill.) Tirab	Maggi et al. (2000)	Maggi et al. (2000)
Botrytis cinerea Pers.	Maggi et al. (2000)	Maggi et al. (2000)
Chaetomium sp. Kunze	Maggi et al. (2000)	Maggi et al. (2000)
Chaetomium globosum Kunze ex Fr.	Maggi et al. (2000)	Maggi et al. (2000)
*Cladosporium sp.*Link	Maggi et al. (2000)	Maggi et al. (2000)
Cladosporium cladosporioides (Fresen.) G.A. de Vries	Maggi et al. (2000);	Maggi et al. (2000)
Cladosporium cucumerinum Ellis & Arthur	Maggi et al. (2000)	Maggi et al. (2000)
Cladosporium herbarum (Pers.) Link	Krysińska-Traczyk (1994); Maggi et al. (2000); Zielińska-Jankiewicz et al. (2008)	Maggi et al. (2000)
Cladosporium macrocarpum Preuss	Maggi et al. (2000)	
Cladosporium sphaerospermum Penz.	Maggi et al. (2000)	Maggi et al. (2000)
Emericella nidulans (Eidam) Vuill *(anamorph: A. nidulans)*	Maggi et al. (2000)	Maggi et al. (2000)
Geotrichum candidum Link	Krysińska-Traczyk (1994)	
Mucor racemosus Bull.	Krysińska-Traczyk (1994)	
Mycelia sterilia	Maggi et al. (2000)	Maggi et al. (2000)
Paecilomyces variotti Bainier	Maggi et al. (2000); Zielińska-Jankiewicz et al. (2008)	Maggi et al. (2000)

Fungi	Organic surfaces	Inorganic surfaces
***Penicillium sp.* Link**	Krysińska-Traczyk (1994); Maggi et al. (2000); Zielińska-Jankiewicz et al. (2008)	Maggi et al. (2000)
***Penicillium brevicompactum* Dierckx**	Maggi et al. (2000)	Maggi et al. (2000)
***Penicillium chermesinum* Biourge**	Maggi et al. (2000)	
***Penicillium chrysogenum* Thom**	Maggi et al. (2000); Zielińska-Jankiewicz et al. (2008)	Maggi et al. (2000)
***Penicillium citreonigrum*Dierckx**		Maggi et al. (2000)
Penicillium citrinum Thom	Maggi et al. (2000)	
***Penicillium coprophylum* Berk. and M. A. Curtis**		Maggi et al. (2000)
***Penicillium corylophilum* Dierckx**	Maggi et al. (2000); Zielińska-Jankiewicz et al. (2008)	
***Penicillium cyclopium* Westling**	Maggi et al. (2000)	Maggi et al. (2000)
***Penicillium digitatum* (Pers.: Fr.) Sacc**	Maggi et al. (2000)	
***Penicillium griseofulveum* Dierckx**	Maggi et al. (2000)	Maggi et al. (2000)
***Penicillium italicum* Wehmer**	Maggi et al. (2000)	Maggi et al. (2000)
***Penicillium lividum* Westling**	Maggi et al. (2000)	Maggi et al. (2000)
***Penicillium myczynski* K. M. Zalessky**	Maggi et al. (2000)	Maggi et al. (2000)
***Penicillium oxalicum* Currie and Thom**	Maggi et al. (2000)	
***Penicillium rugulosum* Thom**		Maggi et al. (2000)
***Penicillium viridicatum* Westling**	Maggi et al. (2000)	Maggi et al. (2000)
***Penicillium waksmanii* K. M. Zalessky**	Maggi et al. (2000)	Maggi et al. (2000)
***Periconia cookei* E.W. Mason & M.B. Ellis**	Maggi et al. (2000)	
***Pithomyces sp.*Berk. & Broome**		Maggi et al. (2000)
Rhizopus nigricans	Zielińska-Jankiewicz et al. (2008)	
***Torula herbarum* (Pers.) Link**	Maggi et al. (2000)	Maggi et al. (2000)
***Trichoderma sp.*Pers**	Maggi et al. (2000)	Maggi et al. (2000)
***Trichoderma aureoviride* Rifai**		Maggi et al. (2000)
***Trichoderma hamatum* (Bonord.) Bainier**	Maggi et al. (2000)	Maggi et al. (2000)
***Trichoderma harzianum* Rifai**	Maggi et al. (2000)	Maggi et al. (2000)
***Trichoderma viride* Pers.**	Krysińska-Traczyk (1994)	

Since there are no limits on fungal load in surfaces we cannot yet say if the environment was heavily contaminated in the locations where the three studies presented in Table 2.4 were performed. It is interesting, however, to point out that 22% of the fungal flora identified in Maggi et al (2000) was only found in book bindings (organic material) while only 10% was found solely on inorganic shelves.

Some studies do couple air and surface samples. This was the case of Harkawy et al. (2011), Chadeganipour et al (2013), Valentin (2007) and Zielińska-Jankiewicz et al. (2008). Unfortunately only two of the mentioned studies can be used to compare the microbial taxa isolated (see Table 2.5). Valentin et al (2007) only characterizes qualitatively the air samples and Chadeganipour et al (2013) presents the data for the two types of samples concomitantly.

Table 2.5 - Fungi isolated from settled dust and air.

Fungi	Organic surfaces (books, document cases, wooden shelves)	Air samples
***Acremonium charticola* (Lindau) W. Gams**		Zielińska-Jankiewicz et al. (2008)
Acremonium murorum (Corda) W. Gams	Zielińska-Jankiewicz et al. (2008)	Zielińska-Jankiewicz et al. (2008)
***Acremonium strictum* W. Gams**		Harkawy et al. (2011); Zielińska-Jankiewicz et al. (2008);
***Alternaria alternata* (Fr.) Keissl**		Zielińska-Jankiewicz et al. (2008)
***Aspergillus* sp. P. Micheli ex Haller**	Harkawy (2011)	
***Aspergillus fumigatus* Fresem**	Zielińska-Jankiewicz et al. (2008)	Zielińska-Jankiewicz et al. (2008)
***Aspergillus niger* Tiegh**	Harkawy (2011)	Harkawy et al. (2011) Zielińska-Jankiewicz et al. (2008);
Aspergillus restrictus G. Sm		Zielińska-Jankiewicz et al. (2008)
Aspergillus sydowii (Bainier & Sartory) Thom & Church		Zielińska-Jankiewicz et al. (2008)
Aspergillus ustus (Bainier) Thom & Church	Zielińska-Jankiewicz et al. (2008)	Zielińska-Jankiewicz et al. (2008)
***Aspergillus versicolor* (Vuill.) Tirab**		Harkawy et al. (2011) Zielińska-Jankiewicz et al. (2008)
***Aureobasidium pullulans* (De Bary) G. Arnaud ex Cif., Ribaldi & Corte**		Zielińska-Jankiewicz et al. (2008)

Fungi	Organic surfaces (books, document cases, wooden shelves)	Air samples
Botrytis cinerea Pers.		Zielińska-Jankiewicz et al. (2008)
***Candida famata* (F.C. Harrison) S.A. Mey. & Yarrow**	Harkawy (2011)	Harkawy et al. (2011)
***Chaetomium sp.* Kunze**		Harkawy et al. (2011) Zielińska-Jankiewicz et al. (2008)¸
***Chaetomium globosum* Kunze ex Fr.**		Zielińska-Jankiewicz et al. (2008)
***Chaetomium elongatum* Czerepanova**		Harkawy et al. (2011)
***Cladosporium cladosporioides* (Fresen.) G.A. de Vries**	Zielińska-Jankiewicz et al. (2008)	Zielińska-Jankiewicz et al. (2008
***Cladosporium herbarum* (Pers.) Link**	Zielińska-Jankiewicz et al. (2008)	Zielińska-Jankiewicz et al. (2008)
Epicoccum purpurascens (formerly known as nigrum)		Zielińska-Jankiewicz et al. (2008)
Eurotium repens* de Bary *(anamorph: Aspergillus pseudo-glaucus)		Zielińska-Jankiewicz et al. (2008)
***Fusarium sp.* Link**		Zielińska-Jankiewicz et al. (2008)
***Geotrichum candidum* Link**		Harkawy et al. (2011)
***Humicola sp.* Traaen**		Zielińska-Jankiewicz et al. (2008)
***Humicola fuscoatra* Traaen**		Zielińska-Jankiewicz et al. (2008)
***Paecilomyces variotti* Bainier**	Zielińska-Jankiewicz et al. (2008)	Zielińska-Jankiewicz et al. (2008)
***Penicillium sp.* Link**	Harkawy (2011) Zielińska-Jankiewicz et al. (2008	Harkawy et al. (2011) Zielińska-Jankiewicz et al. (2008)
***Penicillium chrysogenum* Thom**	Zielińska-Jankiewicz et al. (2008)	Zielińska-Jankiewicz et al. (2008)
Penicillium citrinum Thom		Zielińska-Jankiewicz et al. (2008)
***Penicillium corylophilum* Dierckx**	Zielińska-Jankiewicz et al. (2008)	Zielińska-Jankiewicz et al. (2008)
***Penicillium cyclopium* Westling**		Zielińska-Jankiewicz et al. (2008)
Penicillium funiculosum		Zielińska-Jankiewicz et al. (2008)
***Penicillium rugulosum* Thom**		Zielińska-Jankiewicz et al. (2008)
Penicillium spinulosum Thom		Zielińska-Jankiewicz et al. (2008)
***Penicillium verrucosum* Dierckx**	Harkawyy (2011)	

Fungi	Organic surfaces (books, document cases, wooden shelves)	Air samples
Rhizopus nigricans Ehrenb.	Zielińska-Jankiewicz et al. (2008)	Zielińska-Jankiewicz et al. (2008)
Talaromyces helicus (Raper and Fennel)		Zielińska-Jankiewicz et al. (2008)
***Trichoderma viride* Pers.**		Zielińska-Jankiewicz et al. (2008)
***Trichothecium roseum* (Pers.) Link**		Zielińska-Jankiewicz et al. (2008)
Ulocladium chartarum (Preuss) E.G. Simmons		Zielińska-Jankiewicz et al. (2008)
***Verticillium sp.* Nees**		Zielińska-Jankiewicz et al. (2008)

When comparing the results from air and surface samples it is relevant to point out that important data would be missed if air samples would not have been taken (*Chaetomium* sp., *Aspergillus versicolor* or *Geotrichum candidum*, for instance, would not have been identified). It is not so easy to mention fungi that would go unnoticed if surface samples had not been taken but the vast majority of fungi identified in the presented studies (Table 2.5) are very easily airborne.

In fact, in some cases, no difference is observed when exposure assessment is based on air or dust samples (Kolstad et al. 2002) but it can be very useful to couple both methods, since some fungi, like *Stachybotrys sp.*, can be difficult to isolate from an air sample even when present in the studied environment (Buttner et al., 1993; Duchaine and Mérieux, 2001; Pinheiro et al., 2011b).

Sampling surfaces is also the best choice when looking for dermatophytes. These are fungi capable of degrading the keratin existing in soil, hence the name keratinophylic (Viegas, 2012). These fungi can contaminate and invade living tissues causing dermatomycosis. Some dermatophytes have kept the affinity for animal tissues while others infect only humans (Viegas, 2012). None of the three genera (*Microsporum*, *Trichophyton* and *Epidermophyton*) were found in previous studies but *T. rubrum* and other keratinophylic fungi were isolated in a University library (Jain et al., 2012). Mesquita et al. (2009) identified, in a paper sample, *Toxicocladosporium irritans*, a fungus that pro-

duces ample amounts of volatile metabolites and causes a skin rash within minutes of opening an inoculated dish.

Though not included in Table 2.5, Valentin (2007) compares air and surface analyses and concludes that cellulose objects require longer exposure time in an improved climate to achieve a significant decrease in microbial growth. This adds to the importance of surface samples on cellulosic materials as these are a more reliable measuring tool in the event of altered environmental conditions.

In both Harkawy (2011) and Zielińska-Jankiewicz et al. (2008) studies the air samples resulted in a higher diversity than the surface samples but this is not always the case in other settings (gymnasiums, elderly care centres, for instance) where the number of different species/genera in surfaces doubles the one found in the air samples (Viegas et al., 2011; Viegas et al., 2014). Since the studies are very scarce in archives and librairies it is difficult to confirm this trend.

2.3 Case Studies in Documents

A great part of the investigation on fungi and paper relies on document analysis, when a small area is sampled to better understand the reasons behind any alteration (usually chromatic). Foxed areas are of particular interest since, as already mentioned, questions are still raised regarding the onset and evolution of these marks.

Table 2.6 summarizes the review on fungal findings in paper based documents and books (small areas, qualitative-only studies since the sampled area is not determined). In the left column are the fungal genera/species and the reviews which present them as common in altered documental areas in archives. In the right column are the studies where these fungi were actually identified through experimental work. The absence of any entry in the right column means this fungus has not been recently isolated in altered document samples.

Table 2.6 – Fungal genera/species identified in paper samples (cellulose-based samples, documents and books).

The fungi, in these cases, are isolated from small, altered areas and are not quantifiable. The fungi mentioned only in the first column have not been identified in field studies, just mentioned in reviews**.**

Fungi	Altered Cellulose-based samples: Case studies
***Acremonium* sp. Link (d)(e)**	Lourenço (2005); da Silva (2006),
***Acremonium murorum* (Corda) W. Gams**	Zielińska-Jankiewicz et al. (2008)
***Acrothecium* sp. (Corda) Preuss (e)**	
***Alternaria* sp. Nees (a)(e)**	Rojas et al. (2009); Borrego et al. (2012)
***Alternaria alternata* (Fr.) Keissl. (c)(d)(e)**	Bacilkova (2006); Mesquita et al. (2009)
***Alternaria solani* Sorauer (d)**	
***Alternaria tenuíssima* (Nees) Wiltshire (c)**	
***Arthrinium urticae* M.B. Ellis**	Corte et al. (2003)
***Aspergillus* sp. P. Micheli ex Haller (e)**	Gambale et al. (1993); Florian and Manning (2000); Corte et al. (2003); Zotti et al. (2007); Rojas et al. (2009); Borrego et al. (2012).
***Aspergillus candidus* Link (e)**	Bacilkova (2006)
***Aspergillus carneus* Blochwitz**	Zotti et al. (2008)
***Aspergillus clavatus* Desm. (a)**	
***Aspergillus flavus* Link (a)(c)(e)**	Nyuksha (1990); Nol et al. (2001); Borrego et al. (2012); Zotti et al. (2008)
***Aspergillus fumigatus* Fresen (c)(d)(e)**	Nol et al. (2001); Lourenço (2005); Mesquita et al. (2009); Zielińska-Jankiewicz et al. (2008)
***Aspergillus glaucus* Link (a)(d)**	
***Aspergillus japonicus* Saito**	da Silva (2006); Rakotonirainy et al. (2007)
***Aspergillus melleus* Yukawa**	Zotti et al. (2011)
***Aspergillus nidulans* (Eidam) G. Winter (d)**	Lourenço (2005); Mesquita et al. (2009); Michaelsen et al. (2009)
***Aspergillus niger* Tiegh. (c; d, a, e)**	Nol et al. (2001); Bacilkova (2006); da Silva (2006); Lourenço (2005); Michaelsen et al. (2009); Borrego et al. (2012)
***Aspergillus ochraceus* K. Wilh (a)(e)**	Lourenço (2005)
***Aspergillus oryzae* (Ahlb.) Cohn**	Rakotonirainy et al. (2007)
***Aspergillus penicillioides* Speg.**	Arai (2000); Michaelsen et al (2010b)
***Aspergillus repens* (Corda) Sacc (a)**	
***Aspergillus ruber* (Jos. König et al.) Thom & Church (a)**	
***Aspergillus sclerotiorum* G. A. Huber**	Zotti et al. (2011)
***Aspergillus sydowii* (Bainier & Sartory) Thom & Church**	Lourenço (2005); Zielińska-Jankiewicz et al. (2008)
***Aspergillus tamarii* Kita (d)**	Nol et al. (2001)
***Aspergillus terreus* Thom (c)(e)**	

Fungi	Altered Cellulose-based samples: Case studies
Aspergillus ustus **(Bainier) Thom & Church**	Rakotonirainy et al. (2007); Zielińska-Jankiewicz et al. (2008)
Aspergillus versicolor **(Vuill.) Tirab (c)(e)**	Lourenço (2005); Mesquita et al. (2009); Michaelsen et al. (2009) Michaelsen et al (2010b)
Aspergillus wentii **Wehmer**	Bacilkova (2006)
Aureobasidium sp.**Viala & G. Boyer (e)**	
Aureobasidium pullulans **(De Bary) G. Arnaud ex Cif., Ribaldi & Corte (e)**	Zotti et al. (2007)
Bjerkandera adusta **(Willd.) P. Karst.**	Rakotonirainy et al. (2007)
Botryotinia fuckeliana **(de Bary) Whetzel *(teleomorph of Botrytis cinerea)***	Michaelsen et al. (2009)
Botrytis **sp. P. Micheli ex Haller (e)**	Lourenço (2005)
Botrytis cinerea **Pers (e)**	Mesquita et al. (2009)
Botryotrichum **sp.Sacc. & Marchal (e)**	
Botryotrichum atrogriseum **J.F.H. Beyma (e)**	
Candida guilliermondii **(Castell.) Langeron & Guerra (a)**	
Chaetomium **sp. Kunze (e)**	Corte et al. (2003);Lourenço (2005); Rojas et al. (2009)
Chaetomium bostrychodes **Zopf (e)**	
Chaetomium elatum **Kunze (c)(e)**	
Chaetomium globosum **Kunze ex Fr. (c)(d)(e)**	Corte et al. (2003);Lourenço (2005); Rakotonirainy et al. (2007); Mesquita et al. (2009)
Chaetomium gracile **Udagawa**	Corte et al. (2003)
Chaetomium indicum **Corda (a)(c)(e)**	Lourenço (2005)
Chaetomium murorum **Corda (e)**	
Chromelosporium carneum **(Pers.) Henne-bert**	Mesquita et al. (2009)
Chrysosporium **sp.Corda (e)**	
Cladobotryum **sp.Nees (e)**	
Cladosporium **sp.Link**	Gambale et al. (1993); Corte et al. (2003); Lourenço (2005); Bacilkova (2006); Michaelsen et al. (2009); Rojas et al. (2009); Borrego et al. (2012)
Cladosporium cladosporioides **(Fresen.) G.A. de Vries (d)**	da Silva et al. (2006); Zotti et al. (2007); Zielińska-Jankiewicz et al. (2008); Mesquita et al. (2009); Michaelsen et al. (2009)
Cladosporium elatum **(Harz) Nannf. (a)**	
Cladosporium herbarum **(Pers.) Link (d)(e)**	da Silva et al. (2006); Zielińska-Jankiewicz et al. (2008); Michaelsen et al. (2009)
Cladosporium resinae **(Lindau) G.A. de Vries**	Bacilkova (2006)

Fungi	Altered Cellulose-based samples: Case studies
***Cladosporium sphaerospermum* Penz.**	Corte et al. (2003); di Bonaventura et al. (2003; not conclusive); Zotti et al. (2011)
***Coprinus* sp.Pers.**	Mesquita et al. (2009)
***Cunninghamella* sp. Matr.**	Corte et al. (2003)
***Cunninghamella elegans* Lendn.**	Corte et al. (2003)
***Curvularia* sp. Boedijn (e)**	
***Curvularia lunata* (Wakker) Boedijn (d)**	
***Debaryomyces hansenii*(Zopf) Lodder & Kreger**	Michaelsen et al. (2009)
***Doratomyces* sp. Corda (d)(e)**	
***Doratomyces stemonitis*(Pers.) F.J. Morton & G. Sm**	Zotti et al. (2007)
***Eladia saccula* (E. Dale) G. Sm.**	Rojas et al. (2009)
***Epicoccum* sp. Link (e)**	Corte et al. (2003); Lourenço (2005)
***Epicoccum purpurascens* Ehrenb. (e)**	Corte et al. (2003)
***Eurotium* sp. Link**	Florian and Manning (2000); Corte et al. (2003)
***?Eurotium amstelodami* L. Mangin (a)(b)**	
***Eurotium chevalieri* L. Mangin *(anamorph: Aspergillus chevalieri)* (b)**	
Eurotium halophilicum	Michaelsen et al (2010a,b)
Eurotium herbariorum* (Weber ex F.H. Wigg.) Link *(anamorph: Aspergillus glaucus* Link*)	Arai (2000)
***Fusarium* sp. Link (a)**	da Silva et al. (2006);
***Fusarium oxysporum* Schltdl. (d)**	
***Fusarium semitectum* Berk. & Ravenel (d)**	
***Fusicladium* sp. Bonord.**	Corte et al. (2003)
***Geomyces pannorum* (Link) Sigler & J.W. Carmich**	Zotti et al. (2007)
***Geosmithia putterilli* (Thom) Pitt**	Zotti et al. (2007)
***Geotrichum* sp. Link (d) (e)**	
Gloeotinia temulenta (Phialea temulenta,* Prill. & Delacr.*)	Rakotonirainy et al. (2007)
***Gliocladium catenulatum* J.C. Gilman & E.V. Abbott (d)**	
***Gliocladium roseum* Bainier**	Nol et al. (2001)
***Gymnoascus* sp. Baran (e)**	

Fungi	Altered Cellulose-based samples: Case studies
Helicostylum **sp. Corda (e)**	
Humicola grisea **Traaen (d)**	
Kockovaella **sp. Nakase, I. Banno & Y. Yamada**	di Bonaventura et al. (2003, not conclusive)
Melanospora **sp. Corda (e)**	
Memnoniella **sp. Höhn (d)**	
Microsporum **sp.Gruby (d)**	
Mucor **sp. Fresen (a)(e)**	Lourenço (2005); Michaelsen et al. (2009)
Mucor racemosus **Bull (c)(d)(e)**	
Mycelia sterilia	da Silva et al. (2006); Zotti et al. (2008)
Myrothecium verrucaria **(Alb. & Schwein.) Ditmar (d)**	
Neurospora **sp. Shear & B.O. Dodge (d)**	
Neurospora sitophyla **Shear & B.O. Dodge (*anamorph: Chrysonilia sitophyla* (e)**	
Oidiodendrium **sp. Robak**	Corte et al. (2003)
Oidiodendron citrinum **G.L. Barron**	Corte et al. (2003)
Paecilomyces **sp. Bainier (e)**	Lourenço (2005)
Paecilomyces variotti **Bainier (a)(d)**	Zielińska-Jankiewicz et al. (2008); Zotti et al. (2008)
Penicillium **sp. Link (e)**	Gambale et al. (1993); Florian and Manning (2000); Corte et al. (2003); da Silva et al. (2006); Zielińska-Jankiewicz et al. (2008); Mesquita et al. (2009); Rojas et al. (2009); Borrego et al. (2012)
Penicillium albidum **Sopp**	Bacilkova (2006)
Penicillium bilaiae **Chalab.**	Lourenço et al. (2005)
Penicillium brevicompactum **Dierckx (c)(d)**	Corte et al. (2003); Lourenço et al. (2005)
Penicillium chrysogenum **Thom (c)(d)**	Corte et al. (2003); Bacilkova (2006); Zielińska-Jankiewicz et al. (2008); Mesquita et al. (2009); Borrego et al. (2012); Michaelsen et al. (2009);
Penicillium citreonigrum **Dierckx**	da Silva et al. (2006)
Penicillium citrinum **Thom (a)**	Corte et al. (2003); Lourenço (2005); da Silva et al. (2006); Rakotonirainy et al. (2007)
Penicillium commune **Thom (d)**	Michaelsen et al. (2009); Borrego et al. (2012)
Penicillium corylophilum **Dierckx**	Lourenço et al. (2005); Zielińska-Jankiewicz et al. (2008)
Penicillium decumbens **Thom**	Corte et al. (2003); Lourenço et al. (2005)
Penicillium expansum **Link (a)**	Corte et al. (2003); Lourenço et al. (2005)
Penicillium fellutanum **Biourge (a)**	da Silva et al. (2006)
Penicillium glabrum **(Wehmer) Westling (d)(a)**	Lourenço et al. (2005)

Fungi	Altered Cellulose-based samples: Case studies
***Penicillium griseofulveum* Dierckx**	Lourenço et al. (2005)
***Penicillium oxalicum* Currie and Thom**	Lourenço et al. (2005)
***Penicillium paxilii* Bainier**	Zotti et al. (2007)
***Penicillium restrictum* J. C. Gilman and E. V. Abbott**	da Silva et al. (2006); Zotti et al. (2007)
***Penicillium spinulosum* Thom (a)**	Lourenço et al. (2005); Zotti et al. (2007)
***Penicillium steckii* K. M. Zalessky**	Zotti et al. (2007)
***Penicillium turbatum* Westling**	Zotti et al. (2007)
***Pestalotia oxyanthi* Thüm. (d)**	
***Peziza* sp. Fr.**	Corte et al. (2003)
***Peziza ostracoderma* Korf**	Corte et al. (2003)
***Phialophora* sp. Medlar**	Lourenço et al. (2005)
***Phlebia subserialis* (Bourdot & Galzin) Donk**	Mesquita et al. (2009)
***Phoma* sp. Sacc. (e)**	Corte et al. (2003); Lourenço et al. (2005)
***Phoma pigmentivora* Massee (d)**	
***Phoma pomorum* Thüm.**	Corte et al. (2003)
***Polyporus brumalis* (Pers.) Fr.**	Rakotonirainy et al. (2007)
***Ramichloridium* Stahel ex de Hoog (e)**	
***Rhizopus* sp. Ehrenb. (e)**	Lourenço et al. (2005)
***Rhizopus arrhizus* A. Fisch. (a)**	Michaelsen et al. (2009)
***Rhizopus nigricans Ehrenb.* (d)**	Zielińska-Jankiewicz et al. (2008)
***Rhizopus stolonifer* (Ehrenb.) Vuill. (c)**	
Rhodotorula sp. FC Harrison (e)	
Rhodotorula aurantiaca	Michaelsen et al ,(2010b)
***Saccharicola bicolor* (D. Hawksw., W.J. Kaiser & Ndimande) D. Hawksw. & O.E. Erikss.**	Rakotonirainy et al. (2007)
***Scopulariopsis* sp. Bainier**	Borrego et al. (2012)
***Scopulariopsis nigricans* (d)**	
***Scopulariopsis brevicaulis* (Sacc.) Bainier**	Bacilkova (2006)
***Sordaria* sp.Ces. & De Not. (e)**	
***Sporotrichum pruinosum* J.C. Gilman & E.V. Abbott (d)**	
***Stachybotrys chartarum* (Ehrenb.) S. Hughes (c)(a)(d)**	
***Stemphylium* Wallr. (a)(e)**	
***Stemphylium botryosum* Wallr. (c)(d)**	
***Stemphylium vesicarium* (Wallr.) E.G. Simmons (d)**	

Fungi	Altered Cellulose-based samples: Case studies
***Talaromyces funiculosus* Thom (a)(c)**	Nol et al. (2001)
***Talaromyces helicus* (Raper and Fennel)**	Mesquita et al. (2009); Borrego et al. (2012)
***Talaromyces minioluteus* (Dierckx) Samson, Yilmaz, Frisvad and Seifert**	Rakotonirainy et al. (2007)
***Talaromyces pinophilus* (Hedgcock)**	Michaelsen et al. (2009)
***Talaromyces purpurogenus* Stoll (a)**	Zotti et al. (2011)
***Talaromyces ruber* Stoll. (a)(c)**	
***Thielavia* sp. Zopf**	di Bonaventura et al. (2003, not conclusive)
***Toxicocladosporium irritans* Crous & U. Braun**	Mesquita et al. (2009)
***Trichoderma* sp. Pers (e)**	Gambale et al. (1993); Corte et al. (2003); Lourenço (2005); Michaelsen et al. (2009)
***Trichoderma citrinoviride* Bissett**	Rakotonirainy et al. (2007)
***Trichoderma harzianum* Rifai (a)**	
***Trichoderma koningii* Oudem.**	Rakotonirainy et al. (2007)
***Trichoderma pseudokoningii* Rifai**	Zotti et al. (2007)
***Trichoderma viride* Pers. (a)(c)(d)**	Lourenço (2005); Bacilkova (2006)
***Trichosporum* sp. Vuill.**	da Silva et al. (2006)
***Trichothecium roseum* (Pers.) Link (e)**	
***Ulocladium* sp. Preuss (a)(e)**	Corte et al. (2003); Rakotonirainy et al. (2007)
***Ulocladium botrytis* Preuss**	Corte et al. (2003)
***Ulocladium chartrum (Preuss)* E.G. Simmons**	Rakotonirainy et al. (2007)
***Ulocladium consortiale* (Thüm.) E.G. Simmons (d)**	
***Ulocladium cucurbitae* (Letendre & Roum.) E.G. Simmons**	Rakotonirainy et al. (2007)
***Verticillium* sp. (e)**	
***Verticillium nigriscens* Pethybr. (d)**	
(Other) Yeasts	Corte et al. (2003); Michaelsen et al.(2009)

(a) Included in the review by Sterflinger et al., 2010. (b) Included in the review by Pinzari et al., 2004. (c) Included in the review by Gallo et al. (2003). (d) Included in the review by Valentin et al. (2003). (e) Included in the review by Zyska (1997).

Zyska (1997), Gallo et al. (2003), Valentin et al. (2003), Pinzari et al., (2004) and Sterflinger (2010) mention some of the most common fungi found in documents and books while all the others (Nol et al. (2001), di Bonaventura (2003), Corte et al. (2003), Bacilkova (2006), da Silva et al. (2006), Rakotonirainy et al. (2007), Zielińska-Jankiewicz

et al. (2008), Mesquita et al. (2009), Michaelsen et al. (2009), and Zotti et al. (2007, 2008, 2011) report experimental work.

Besides the already mentioned *Cephalotrichum* and *Chloridium* (Zyska, 1997), *Cephalosporium* (Valentin et al., 2003), *Rhizopus oryzae (*Sterflinger 2010), *P. frequentans* (Sterflinger, 2010; Valentin et al., 2003,) *and P. funiculosum (*Gallo et al. 2003, Sterflinger et al. 2010 and Nol et al., 2001*),* some species have meanwhile seen a change in their taxonomic name:

- *Epicoccum nigrum* (Zyska 1997) is now *E. purpurascens;*
- *Fusarium roseum* (Valentin et al., 2003) is now *F. semitectum*;
- *Sporotrichum pulvurulentum (*Valentin et al., 2003) is now *S. pruinosum*

In the *Penicillium* genera (*www.aspergilluspenicillium.org*)

- *P. helicum* (Mesquita et al., 2009) is now *Talaromyces helicus*;
- *P. minioluteum* (Rakotonirainy et al., 2007) is now *Talaromyces minioluteus*,
- *P. notatum* (Bacilkova 2006; Gallo et al., 2003; Valentin et al., 2003) is now *P. chrysogenum*;
- P. *pilophylum* (Michaelsen et al., 2009, 2010d) is now *Talaromyces pinophilus;*
- *P. purpurogenum* (Sterflinger, 2010) is now *Talaromyces purpurogenus*;
- *P. rubrum* (Sterflinger, 2010) is now *Talaromyces ruber*
- *P. variabile (*Sterflinger, 2010; Lourenço et al., 2005 and Corte et al., 2003*)* is now *P. expansum.*

Many of the fungal identifications gathered by Zyska (1997) and Gallo et al. (2003), original data recollected for the 1998 edition) have no recent entries which may be justified by the reasons presented earlier: nomenclature changes and identification methodologies.

Molecular biology methods have already been applied in samples removed from documents. It is the case of di Bonaventura et al. (2003), Michaelsen et al. (2009), Rakotonorainy et al. (2007) and Mesquita et al. (2009). Molecular analysis has allowed the identification of species that had never been mentioned or found before: *Botryotinia fuckeliana* (de Bary) Whetzel, *Chromelosporium carneum* (Pers.) Hennebert, *Coprinus sp.* Pers, *Debaryomyces hansenii* (Zopf) Lodder & Kreger, *Kockovaella* sp. Nakase, I. Banno & Y. Yamada (not conclusive), *Phlebia subserialis* (Bourdot & Galzin) Donk, *Talaromyces*

pinophilus (Hedgcock), *Thielavia* sp. Zopf, *Toxicocladosporium irritans* Crous & U. Braun, *Saccharicola bicolor*, *Gleotinia temulata* Prill. & Delacr. and *Bjerkandera adusta* Willd.) P. Karst.

2.4 Fungal contamination and paper conservation

Although not internationally established, some authors have proposed a few threshold values for archives and other heritage safekeeping settings (Table 1.1). In Figure 2.1, we can see how some of the quantitative studies presented in Table 2.3 have performed in accordance to these proposed limits (the depicted limits were presented earlier in Table 1.1).

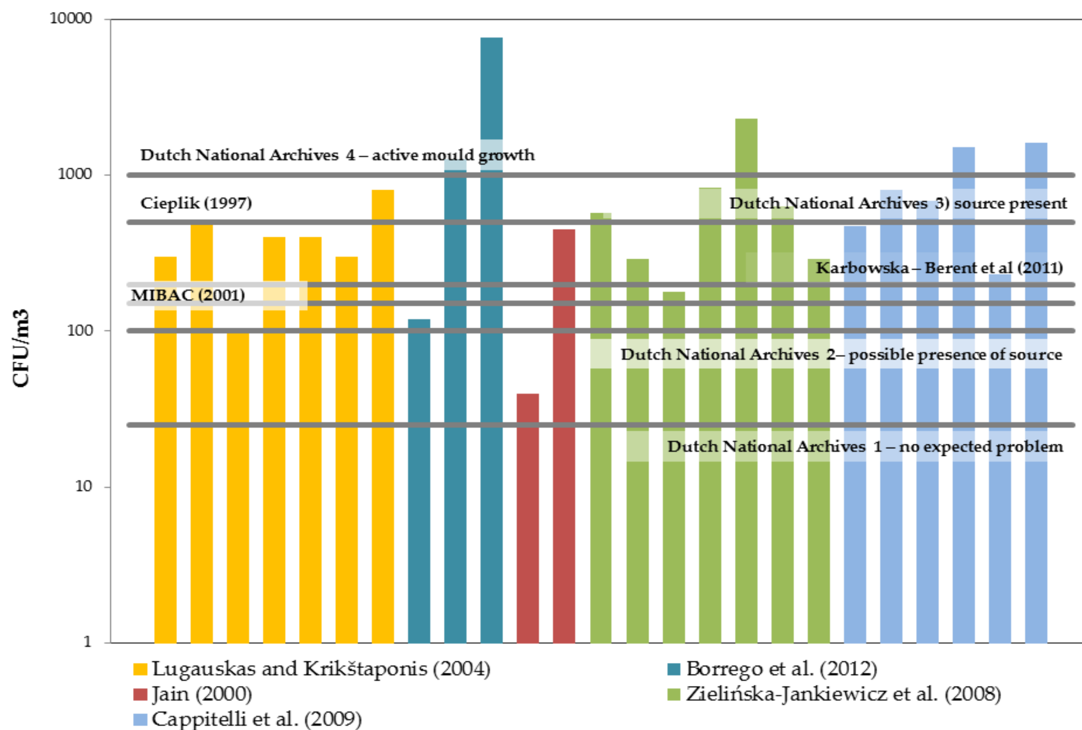

Figure 2.1 - Quantitative air analysis studies performed in archives and their relationship to proposed limits for conservation.
Please mind the logarithmic scale.

All of the quantitative studies indicate contaminated environments, according to the lowest level established by the Dutch National Archives guidelines. In fact, only in the studies performed by Jain (2000) and Lugauskas and Kristaponis (2004) were all the

results lower than the upper limit stipulated by the Dutch (1000 CFU/m^3). Considered the proposed guidelines, it seems that *normal* (and attainable) values should be between 100 and 500 CFU/m^3, and there is no reason to consider higher values as allowable. As happens with indoor air quality guidelines the existence of an array of values to be taken as limits points to the need of further studies, namely the identification of the contaminating species. Many of the fungi most commonly encountered in archives and librairies are capable of degrading cellulose: *Aspergillus* sp., *Chaetomium* sp., *Alternaria* sp., and *Penicillium* sp. are included in this group (Konkol, 2010) and their presence should be monitored for this reason.

Identifying the microfungal agents responsible for paper biodeterioration has not, however, been an easy task and has led to confusion and never-ending lists of possible culprits.

The identification of a fungal genus or species on a document does not necessarily mean this fungus is causing foxing. Michaelsen (2010a, Chapter I, page 17), proposes a vast list of fungi as detrimental to paper based materials as foxing causing fungus but care must be taken as some of the species mentioned are found "on" paper but no relationship is assumed between these fungi and foxing.

From Michaelsen´s list, the studies performed by Florian and Manning (2000), Nol et al. (2001), Corte et al. (2003), Rakotonirainy et al. (2007), Zotti et al. (2008) and Mesquita et al. (2009) can be selected as they, in fact, represent an association between an undesirable change in a document (not necessarily foxing as Mesquita et al. (2009) also selected documents presenting microfungal structures with other observable texture changes) and a fungal genus or species.

Pasquariello and Maggi (2003) mention *Trichoderma, Chaetomium, Paecilomyces, Cladosporium, Alternaria* and *Fusarium* genera as promoters of paper biodeterioration. For Caneva et al. (2003), it is necessary to pay particular attention to fungi of the following genera: *Alternaria* , *Aspergillus, Chaetomium, Mucor, Myrothecium, Penicillium, Rhizopus, Stachybotrys, Trichoderma, Trichothecium* and *Ulocladium* as their presence has been positively associated with effects such as erosion, spots, pigmentation and chang-

es in the mechanical characteristics of paper. Gallo et al. (2003) propose a wider list of dangerous fungi since all of the mentioned in this study (see Table 2.1 for air sample contaminants) plus *Gymnoascus* sp. are considered detrimental for paper. Care must be taken, however, when reading Table 2.7 since the probable cause for the biodeterioration might be the identified fungi but these assumptions should not be made without a battery of chemical tests to *prove* this association.

Besides correlating altered document samples with fungal elements (historical or actual field work correlation), Table 2.7 also presents the most common enzymes and metabolites produced by biodeterioration inducing fungi. In line with previous data, in the left column are included the authors for whom there is an historical association between biodeterioration and a fungus while in the middle column are the studies involving the identification of fungi in an altered document sample.

Table 2.7 – Enzymes and Metabolites produced by some of the genera/species associated with paper biodeterioration.

Fungi	Studies associating fungi with paper biodeterioration	Enzymes and Metabolites
***Acremonium sp*. Link (b)**	Lourenço et al., 2005; da Silva, 2006	
***Alternaria sp. Nees* (a)(c)**	Borrego et al., 2012	Protease, amylase, cellulase; pigment (Borrego et al., 2012); cellulase (Harkawy et al. 2011; Menezes, 2009);
Alternaria solani (e)		intracellular black pigment (Szczepanowska and Lovett Jr. 1992)
Alternaria alternata (Fr.) Keissl	Mesquita et al., 2009	Protease, amylase, cellulase
***Arthrinium sp*.Kunze**	Corte et al. (2003)	
***Arthrinium urticae* M.B. Ellis**	Corte et al. (2003)	
***Aspergillus sp*. P. Micheli ex Haller (a)(b)**	Borrego et al., 2012; Corte et al., 2003; Florian and Manning, 2000; Gambale et al., 1993; Zotti et al., 2007	Protease, amylase (www. doctorfungus.org, Samson et al., 2004; Rojas et al., 2009); cellulase (Menezes, 2009; Jayant et al., 2011; Bueno et al., 2003; Pangallo et al., 2009; Harkawy et al., 2011)
***Aspergillus carneus* Blochwitz**	Zotti et al. , 2008	Cellulase, pigment (Nol et al., 2001)

Fungi	Studies associating fungi with paper bio-deterioration	Enzymes and Metabolites
***Aspergillus flavus* Link**	Borrego et al., 2012; Nyuksha 1990; Zotti et al. 2008; Nol et al., 2001	Cellulase, protease, amylase (Borrego et al., 2012); pigment, cellulase (Nol et al., 2001; Nittérus et al., 2000)
***Aspergillus fumigatus* Fresem**	Mesquita et al., 2009; Lourenço et al., 2005; Nol et al., 2001	protease, amylase, cellulase; cellulase, pigment (Nol et al., 2001)
***Aspergillus glaucus* Link**		protease (Samson et al., 2004)
***Aspergillus japonicus* Saito**	da Silva, 2006; Rakotonirainy et al., 2007	
***Aspergillus melleus* Yukawa**	Zotti et al., 2011	
***Aspergillus nidulans* (Eidam) G. Winter**	Mesquita et al.,2009; Lourenço, 2005; Michaelsen et al., 2009	protease, amylase; melanin (Florian, 2002)
***Aspergillus niger* Tiegh.,**	Borrego et al., 2012; Lourenço, 2005; Michaelsen et al., 2009; Nol et al., 2001; da Silva, 2006	amylase, cellulase, protease; cellulase, protease, amylase and pigment (Borrego et al., 2012); cellulase (Smith, 1977); cellulase, pigment (Nol et al., 2001; Nittérus, 2000)
***Aspergillus ochraceus* K. Wilh**	Lourenço et al., 2005	
Aspergillus oryzae	Rakotonirainy et al., 2007	
***Aspergillus penicillioides* Speg.**	Arai, 2000;	
***Aspergillus sclerotiorum* G. A. Huber**	Zotti et al., 2011	
***Aspergillus sydowii* (Bainier & Sartory) Thom & Church**	Lourenço et al., 2005	
***Aspergillus tamarii* Kita**	Nol et al., 2001	cellulase, pigment (Nol et al., 2001);
***Aspergillus terreus* Thom (b)(d)**		cellulase (Pangallo et al., 2009; Rautela and Cowling, 1966);cellulase, hemicellulase, amylolytic and gelatinolytic activity (Michaelsen et al, 2010b); pigment, cellulase (Nol et al., 2001)
***Aspergillus ustus* (Bainier) Thom & Church**	Rakotonirainy et al., 2007	Cellulase (Pangallo et al., 2009)
***Aspergillus versicolor* (Vuill.) Tirab**	Mesquita et al., 2009; Lourenço et al., 2005; Michaelsen et al., 2009	Pink-orange pigment (versicolorine) (Michalsen et al., 2009); cellulase (Pangallo et al., 2009)
***Aureobasidium sp.Viala & G. Boyer* (d)(b)**		Lipase,protease,cellulase (Harkawy et al., 2011)
Aureobasidium pullulans (De Bary) G. Arnaud ex Cif., Ribaldi & Corte	Zotti et al., 2007;	Decomposes pectins, dextrins, oligossacharides and starches (Zotti et al., 2007); produces cellulases and melanin (Michaelsen et al, 2010b)

Fungi	Studies associating fungi with paper bio-deterioration	Enzymes and Metabolites
Bjerkandera adusta **(Willd.) P. Karst.**	Rakotonirainy et al., 2007	
Bullera globispora **B.N. Johri & Bandoni**	(Michaelsen et al, 2010a)	
Botryotinia fuckeliana (de Bary) Whetzel (teleomorph of Botrytis cinerea)	Michaelsen et al., 2009	
Botrytis sp.P. Micheli ex Haller	Lourenço et al., 2005	Lipase; protease (Harkawy et al., 2011); cellulase (Harkawy et al., 2011)
Botrytis cinerea Pers.	Mesquita et al., 2009	Cellulase
C. tropicalis (Castellani) Berkhout	Michaelsen et al, 2010a	
Chaetomium sp. Kunze **(b)(c)**	Corte et al., 2003; Lourenço et al., 2005	Lipase,protease,cellulase (Harkawy et al. 2011); amylase (Rojas et al., 2009); chaetomidin (Szczepanowska et al., 1992);
Chaetomium elatum Kunze		Cellulase (Pangallo et al., 2009)
Chaetomium globosum Kunze ex Fr. (e)	Corte et al., 2003; Mesquita et al., 2009; Lourenço et al., 2005; Rakotonirainy et al., 2007	Cellulase (Samson et al., 2004; Pangallo et al., 2009; Corte et al., 2003; Smith, 1977; Rautela and Cowling, 1966; Nitterus, 2000a); yellow - greyish-brown pigment (Szczepanowska and Lovett Jr., 1992); pigment (Nitterus, 2000a)
Chaetomium gracile **Udagawa**	Corte et al., 2003	
Chaetomium indicum Corda	Lourenço et al., 2005	Rautela and Cowling, 1966
Chrysonilia sp. Arx **(b)**		
Chromelosporium carneum **(Pers.) Hennebert**	Mesquita et al., 2009	
*Cladosporium sp.***Link (b)(c)**	Borrego et al., 2012; Corte et al., 2003; Gambale et al., 1993;Lourenço et al., 2005; Michaelsen et al., 2009	Lipase, protease (Harkawy et al. 2011); cellulase (Menezes;Ruga et al, 2008; Pangallo et al., 2009; Harkawy et al. 2011)
Cladosporium cladosporioides (Fresen.) G.A. de Vries	Zotti et al., 2007; Mesquita et al., 2009; Michaelsen et al., 2009, da Silva et al., 2006	protease, amylase; cellulase (Pangallo et al., 2009)
Cladosporium herbarum (Pers.) Link	Michaelsen et al., 2009, da Silva et al. (2006)	protease, amylase; cellulase (Rautela and Cowling, 1966)
Cladosporium sphaerospermum Penz.	Corte et al., 2003; di Bonaventura et al., 2003; not conclusive); Zotti et al., 2011	
Coprinus sp.Pers.	Mesquita et al., 2009	
Cunninghamella sp. Matr.sp	Corte et al., 2003	

Fungi	Studies associating fungi with paper bio-deterioration	Enzymes and Metabolites
***Cunninghamella elegans* Lendn.**	Corte et al., 2003	
Debaryomyces hansenii(Zopf) Lodder & Kreger	Michaelsen et al., 2009	
***Doratomyces sp.Corda* (b)**		
Doratomyces stemonitis(Pers.) F.J. Morton & G. Sm	Zotti et al., 2007	
Eladia saccula (E. Dale) G. Sm.	Rojas et al. (2009)	protease (Rojas et al. 2009)
***Epicoccum sp. Link* (d)**	Corte et al., 2003; Lourenço et al., 2005	Lipase, protease (Harkawy et al. 2011); cellulase (Menezes)
***Epicoccum purpurascens* Ehrenb**	Corte et al., 2003	protease, amylase; cellulase (Corte et al., 2003)
Eurotium sp. Link	Corte et al., 2003; Florian and Manning (2000)	
Eurotium herbariorum (Weber ex F.H. Wigg.) Link (anamorph: Aspergillus glaucus Link)	Arai, 2000	
***Fusarium sp.Link* (b)(c)**	da Silva et al., 2006	Cellulase (Menezes, 2009; Bueno et al., 2003); fusarubin (red pigment) (Szczepanowska et al., 1992)
Fusarium oxysporum (e)		fusarubin (red pigment) Szczepanowska and Lovett Jr., 1992
Fusicladium sp. Bonord.	Corte et al., 2003	
Geomyces pannorum(Link) Sigler & J.W. Carmich	Zotti et al., 2007	Decomposes cellulose and paper (Zotti et al., 2007)
Geosmithia putterilli(Thom) Pitt	Zotti et al., 2007	
Geotrichum sp.Link		Protease (*www.doctorfungus.org*)
Gloeotinia temulenta	Rakotonirainy et al., 2007	
Gliocladium roseum Bainier	Nol et al., 2001	cellulase, pigment (Nol et al., 2001);
***Gymnoascus sp. Baran.* (b)**		
***Helminthosporium sp. Link* (b)**		
Humicola sp.Traaen	Lourenço et al., 2005	cellulase (Bueno et al. 2003)
Humicola grisea Traaen		Cellulase
Kockovaella sp.Nakase, I. Banno & Y. Yamada	di Bonaventura et al., 2003 (not conclusive)	

Fungi	Studies associating fungi with paper biodeterioration	Enzymes and Metabolites
***Mucor sp.* Fresen. (a)(b)**	Lourenço et al., 2005; Michaelsen et al., 2009	Lipase, protease (Harkawy et al., 2011)
***Myrothecium sp.* (a)**		
***Myrothecium verrucaria* (Alb. & Schwein.) Ditmar**		Cellulase (Smith, 1977; Rautela and Cowling, 1966)
***Oidiodendrium sp.*Robak**	Corte et al., 2003	
***Oidiodendron citrinum*G.L. Barron**	Corte et al., 2003	
***Paecilomyces sp.*Bainier (b)(c)**	Lourenço et al., 2005	Lipase (Harkawy et al. 2011)
***Paecilomyces variotti* Bainier**	Zotti et al., 2008	
***Penicillium sp.* Link (a)(b)**	Borrego et al., 2012; Florian and Manning, 2000; Corte et al., 2003;Gambale et al., 1993; Mesquita et al., 2009; da Silva et al., 2006	Cellulase (Bueno et al., 2003; Pangallo et al., 2009; Harkawy et al., 2011); amylase, protease (Rojas et al., 2009)
***Penicillium bilaiae* Chalab.**	Lourenço et al., 2005	
***Penicillium brevicompactum* Dierckx**	Corte et al., 2003; Lourenço et al., 2005	Botryodiploidin (Samson et al., 2010)
***Penicillium canescens* Sopp**	Corte et al., 2003; Mesquita et al., 2009	
***Penicillium chrysogenum* Thom (e)**	Borrego et al., 2012; Corte et al., 2003; Mesquita et al., 2009; Michaelsen et al., 2009; Bacilkova, 2006	Cellulase (Pangallo et al., 2009); cellulase, protease, amylase (Borrego et al., 2012); xanthocillins and citronen (yellow green pigments, Szczepanowska and Lovett Jr.. 1992); cellulase (Jayant et al., 2011); chrysogine (Samson et al., 2010)
***Penicillium citreonigrum* Dierckx**	da Silva et al. (2006)	Citroviridin A (Samson et al., 2010)
***Penicillium citrinum* Thom**	Corte et al., 2003; Lourenço et al., 2005; da Silva et al.,2006; Rakotonirainy et al., 2007	Citrinin
***Penicillium commune* Thom**	Michaelsen et al., 2009; Borrego et al., 2012	Cellulase, protease, amylase and pigment (Borrego et al., 2012)
***Penicillium corylophilum* Dierckx**	Lourenço et al., 2005	
***Penicillium decumbens* Thom**	Corte et al., 2003; Lourenço et al., 2005	
***Penicillium expansum* Link**	Lourenço et al., 2005; Corte et al., 2003	Cellulase (Pangallo et al., 2009); Chaetoglobosin A and C (Samson et al., 2010); Citrinin (Samson et al.,2010)
***Penicillium fellutanum* Biourge**	da Silva et al. 2006	
Penicillium glabrum	Lourenço et al., 2005	Cellulase (Pangallo et al., 2009); Citromy-

Fungi	Studies associating fungi with paper bio-deterioration	Enzymes and Metabolites
***(Wehmer)* Westling**		cetin (Samson et al., 2010)
***Penicillium griseofulveum-* Dierckx**	Lourenço et al., 2005	
***Penicillium oxalicum* Currie and Thom**	Lourenço et al., 2005	
***Penicillium paxilii* Bainier**	Zotti et al., 2007	
***Penicillium restrictum* J. C. Gilman and E. V. Abbott**	da Silva et al., 2006; Zotti et al., 2007	
***Penicillium simplicissium* (Oudem.)**	Lourenço et al., 2005	
***Penicillium solitum* Westling**	Lourenço et al., 2005	
***Penicillium spinulosum* Thom**	Lourenço et al., 2005, Zotti et al., 2007,	Reduces: pectine, cellulose (Zotti et al., 2007)
***Penicillium steckii* K. M. Zalessky**	Zotti et al., 2007	
***Penicillium turbatum* Westling**	Zotti et al., 2007	
***Peziza sp.* Fr.**	Corte et al., 2003	
***Peziza ostracoderma* Korf**	Corte et al., 2003	Cellulase (Corte et al., 2003)
***Phialophora sp.* Medlar**	Lourenço et al., 2005	
***Phlebia subserialis* (Bourdot & Galzin) Donk**	Mesquita et al., 2009	
***Phoma sp.* Sacc. (b)**	Corte et al., 2003; Lourenço et al.,2005	Cellulase (Harkawy et al. 2011)
***Phoma pomorum* Thüm.**	Corte et al., 2003	Cellulase (Corte et al., 2003)
***Polyporus brumalis* (Pers.) Fr.**	Rakotonirainy et al., 2007	
***Rhizopus sp.* Ehrenb. (a)(b)**	Lourenço et al., 2005	
***Rhizopus arrhizus* A. Fisch.**	Michaelsen et al., 2009	
Rhodotorula sp. FC Harrison (e)		
Rhodotorula aurantiaca	Michaelsen et al (2010b)	
***Saccharicola bicolor* (D. Hawksw., W.J. Kaiser & Ndimande) D. Hawksw. & O.E. Erikss.**	Rakotonirainy et al., 2007	
***Scopulariopsis sp.* Bainier (b)**	Borrego et al., 2012	
***Skeletocutis sp.*Kotlába & Pouzar**	Mesquita et al., 2009	
***Sporobolomyces roseus* Kluyver & C.B. Niel**	Michaelsen et al (2010a)	
***Stachybotrys sp.* Corda**		Pigments (greenish, yellowish, Andersen

Fungi	Studies associating fungi with paper bio-deterioration	Enzymes and Metabolites
(a)(b)		et al., 2002)
***Stemphylium* Wallr. (b)**		Cellulase (Harkawy et al., 2011)
***Talaromyces funiculosus* Thom Samson, Yilmaz, Frisvad and Seifert**	Nol et al., 2001	Cellulase, pigment (Nol et al., 2001);
***Talaromyces helicus*(Raper and Fennel)**	Borrego et al., 2012; Mesquita et al., 2009	Cellulase, protease, amylase (Borrego et al., 2012)
***Talaromyces minioluteus* (Dierckx) Samson, Yilmaz, Frisvad and Seifert**	Rakotonirainy et al., 2007	
***Talaromyces pinophilus* Hedgcock**	Michaelsen et al., 2009	Cellulase (Michaelsen et al., 2009, 2010d)
***Talaromyces purpurogenus* Stoll**	Zotti et al., 2011	
***Thielavia sp.* Zopf**	di Bonaventura et al., 2003 (not conclusive)	
***Torula sp.* Pers. (b)**		
***Toxicocladosporium irritans* Crous & U. Braun**	Mesquita et al., 2009	
***Trichoderma sp.*Pers (a)(b)(c)**	Corte et al., 2003; Gambale et al., 1993; Lourenço et al., 2005; Michaelsen et al., 2009	Lipase, protease (Harkawy et al., 2011); cellulase (Menezes, 2009; Jayant et al., 2011; Corte et al., 2003; Rautela and Cowling, 1966; Harkawy et al., 2011)
***Trichoderma citrinoviride* Bissett**	Rakotonirainy et al., 2007	
***Trichoderma koningii* Oudem.**	Rakotonirainy et al., 2007	
***Trichoderma pseudokoningii* Rifai**	Zotti et al., 2007	Degrades cellulose, xylan, oligossacharides, tannins and proteins (Zotti et al., 2007)
***Trichoderma viride* Pers.**	Lourenço et al., 2005	Cellulase (Pangallo et al., 2009; Smith, 1977; Rautela and Cowling, 1966); pigment, cellulase (Nitterus, 2000)
***Trichosporum* Vuill.sp.**	da Silva et al., 2006	
***Trichothecium sp.*Link (a)(b)**		Cellulase (Harkawy et al., 2011)
***Ulocladium sp.*Preuss (a)**	Corte et al., 2003; Rakotonirainy et al., 2007	Cellulase (Harkawy et al., 2011)
***Ulocladium botrytis* Preuss**	Corte et al., 2003	Cellulase, Corte et al., 2003
***Ulocladium chartarum* (Preuss) E.G. Simmons**	Rakotonirainy et al., 2007	
***Ulocladium cucurbitae* (Letendre & Roum.) E.G. Simmons**	Rakotonirainy et al., 2007	

Fungi	Studies associating fungi with paper bio-deterioration	Enzymes and Metabolites
***Verticillium sp.* Nees (b)**		Lipase, protease (Harkawy et al. 2011)
Unidentified Yeasts	Corte et al., 2003; Michaelsen et al., 2009;Zotti et al., 2008	

** *Penicillium variabile* Wehmer is now *Penicillium expansum* (a) Caneva et al. (2003); (b) Gallo et al. (2003); (c) Pasquariello and Maggi (2003) (d) Zyska, 1997 (e) Szczepanowska and Lovett Jr. (1992).

From the 136 fungal species/genera included in Table 2.7, 41 have the metabolic ability to induce paper deterioration when and if conditions are met as they can produce cellulases.

Many secondary metabolites are produced by the fungi presented in the above Table. Most of these function as toxins against plants, bacteria, and other fungi. They can cause a toxic response at low doses and are referred to as mycotoxins (Nielsen et al., 2003) and are better discussed regarding human health.

2.5 Fungal Contamination and Human Health

We already saw (Chapter 1) how diverse fungal concentration limits can be for air samples. Table 2.1 shows only the most common genera but in Table 2.2 it is possible to verify that the potentially pathogenic/toxinogenic species (Valentin et al., 2003, NTE-SCE-02) *Alternaria alternata, Aspergillus glaucus, A. niger, A. fumigatus* and *A. versicolor, Chaetomium globosum, Cladosporium herbarum, Penicillium brevicompactum* and *Stachybotrys atra* have all been identified. Valentin et al. 2010 and the Ordinance Law 353-A/2013 also consider potentially toxinogenic *A. flavus, A. ochraceus* and *A. terreus, Trichoderma viride* and *Rhizopus nigricans*, also present in the performed studies.

In terms of fungal load, a widely used parameter to express indoor air quality, the comparison between the proposed limits (see Table 1.2) and the data obtained by some of the air studies included in Table 2.3 is presented next (Figure 2.2).

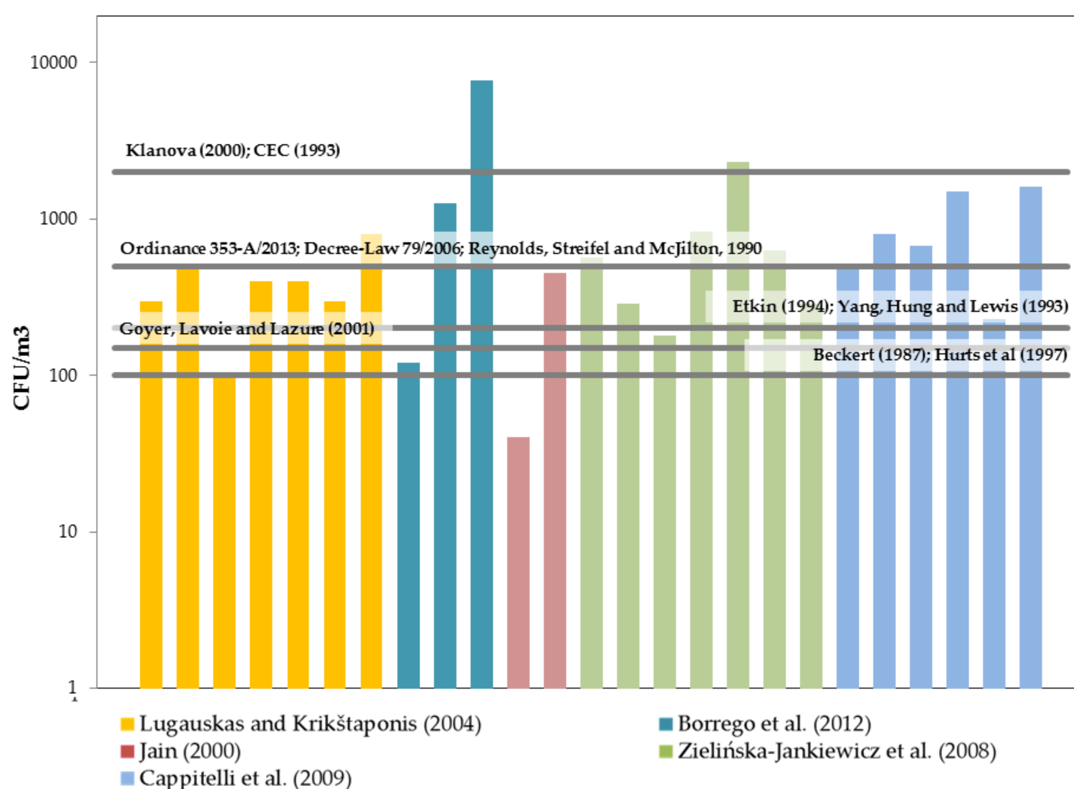

Figure 2.2 – Quantitative air analysis results from the available studies. These results are compared to proposed limits for good air quality.
Please mind the logarithmic scale.

Most of the presented limits are stricter than the ones Portuguese legislation establishes. Most of the studies included in Figure 2.2 show readings higher than most presented limits which come to reinforce the need for further studies in archives and studies involving the workers in these institutions. Only Jain's (2000) study delivered results below the Portuguese Ordinance (500 CFU/m^3) limit.

In archives/libraries some studies have been performed using just the surface's analysis. In Maggi et al. (2000) the most represented genera were Aspergillus (*Aspergillus fumigatus* included), *Cladosporium* sp., *Penicillium* sp., *Chaetomium* sp. and *Alternaria* sp. An average of 10 CFU were determined per sampled area (24cm^2).

The study performed by Zielinska-Jankiewicz et al. (2008) (already presented in Tables 2.4 and 2.5) included both air and surface samples. *Cladosporium* and *Penicillium* were the most prevalent genera. Twelve species were regarded as potentially pathogenic for humans: 8 of them displayed allergic properties and 11 displayed toxic prop-

erties. Quantitatively, the levels reached from 2.3 x 10^3 CFU/m^3 from air samples and 8-10 CFU/100 cm^2 from surface samples (Zielinska-Jankiewicz, 2008).

At the Jasna Góra monastery library Harkawy et al. (2011) concluded that the maximum viable fungal aerosol concentrations did not exceed the 100 CFU/m^3 and that *Aspergillus niger* and *A. versicolor* were present in the sampled air. The settled dust showed a different microbial structure with only five fungal genera/species encountered and a different prevalence distribution. The maximum concentration obtained was 10000CFU/m2.

All fungi (and living organisms) excrete metabolites: volatile, as happens with *T.irritans*, or not. Many of the mentioned fungi found in the air and surfaces in archives also produce mycotoxins.

From the results obtained by their surveys, Lugauskas and Kristaponis (2004) stress that the allergenic properties of the fungi detected in librairies, and their potential to produce, accumulate and release volatile toxic secondary metabolites into the library surroundings might pose a health risk to library workers.

Both genera *Chaetomium* and *Stachybotrys* have produced various secondary metabolites in pure culture. C. *globosum* can produce chaetoglobosins and chetomin whereas other *Chaetomium* species can, in certain occasions, produce sterigmatocystin, a potent carcinogen. *S. chartarum* can liberate trichothecenes and atranones, considered responsible for mycotoxicoses in humans. The mycotoxins produced by this fungus can cause skin irritation while the inhalation of the spores.

Aspergillus species, abundant in the air samples presented in Table 2.1, are also mycotoxins producers. *A. versicolor* produces sterimagtocystin and nidulotoxin (Samson et al., 2004, 2010) while *A. terreus* produces citrinin (Bennett and Klich, 2003; Samson et al., 2004) and *A. ochraceus* and *A. niger* produce ochratoxins (Bennett and Klich, 2003; Samson et al., 2004).

In vitro experiments show that metabolite and mycotoxins production is influenced by the media used the incubation temperature and water activity. This also explains why mycotoxins are not always detected even in the presence of mycotoxin producing fungi. Moulds are likely to generate different metabolites when they grow on building materials and toxin production is affected by the biologic neighbours in mixed cultures (Nielsen et al., 2003). Secondary metabolites and mycotoxins are spe-

cies-specific which makes it very important to identify isolates to the species levels and mixtures of metabolites can see their effects added and potentiated (Nielsen et al., 2003). The biological effects (especially the effects of inhalation) of many fungal secondary metabolites are poorly documented (Nielsen et al., 2003).

As in other settings, although recognizably important, it is still not clear the effect biological agents have on the human health and heritage conservation in archives. In these particular institutions there is still a long way to go as two different populations must be protected. The interactions established between fungi and written heritage are still confusing and, as happens with humans, probably very complex. Despite the existence of health problems and complaints, data on human exposure and effects on staff and visitors of archives/libraries is very scarce.

The fact that there is an international recognition of the importance of indoor fungal communities, however, led to major developments in the area and the scientific community is closer to establish a dose-response exposure level and to design robust standards. Studies involving air and surface samples are important to achieve these goals.

2.6 Conclusions and Further Studies:

The presented review shed some light on the subject of fungal contamination in archives. Many authors have contributed to the study of the fungal communities in the air of archives. Some of the data are outdated but recent studies have also been performed. The most common genera found in the air of archives resemble the outdoor fungal flora as well as common contaminants of other unrelated settings. However, fungi like *Fusarium* spp., *Chaetomium* spp. and *Geotrichum* spp. display higher than usual frequencies.

In terms of fungal load, the studies performed in archives have returned high levels of air contamination by fungi. When displayed against the existing guidelines and guideline proposals, almost all of them cross even the highest level of 1000 CFU/m^3.

Human health was also considered in this analysis. The data obtained from the studies presented in Table 2.3 are also higher than recommended by the existing guidelines on indoor air quality. Only in one of the considered studies were all the results lower the than the Portuguese legislated limit of 500 CFU/m^3.

Also mentioned in this Chapter were the surface samples and how these can bring new insights on the presence of fungal contaminants in archival surfaces and in altered cellulose based samples.

Many fungi were cited in air and surface samples but a more thorough analysis makes one question which ones are really important. Despite a general lack of information on the subject, an attempt was made to highlight the fungi already associated with paper damage and the enzymes and metabolites produced by these fungi.

As happens in science, the conclusions one can take from this Chapter lead to new and important questions:

- The most common fungal genera present in archives/librairies are very similar to other environments but have a higher prevalence of *Fusarium* sp., *Chaetomium* sp. and *Geotrichum* sp., all associated with paper deterioration. Does the Portuguese scenario confirm this added presence?

- Most of the studied archives show contamination levels higher than desirable for both conservation and health safekeeping. What is the fungal contamination level in Portuguese archives and does it comply with national guidelines for indoor air quality and existing proposed levels for conservation?

- In many other settings, the surface samples present a more diverse array of fungal genera/species than air samples. This is usually related with ventilation and cleaning habits. In the two analysed studies this was not the case. What's the Portuguese scenario? Can surface samples be advantageous in fungal contamination assessment?

- Several methods of assessing fungal contamination have been discussed. A lack of comparability between some of these studies hampers global discussion on the subject. What are the best methodologies for determining the fungal communities present in archives considering all the possible locations where these may be relevant?

3. Methodologies for the evaluation of fungal communities in archives: study design

Published (with changes) as a journal article:

Pinheiro, A. C., Macedo, M. F., Jurado, V., Sáiz-Jiménez, C., Viegas, C., Brandão, J., & Rosado, L. (2011b). Mould and yeast identification in archival settings: Preliminary results on the use of traditional methods and molecular biology options in Portuguese archives. International Biodeterioration and Biodegradation, 65(4), 619-627

3.1 Introduction

Cultural Heritage – especially books and documents – have been under scope for fungal contamination. Fungi have been consistently isolated in our written heritage using the traditional culture methods (Zmyska, 1997; Corte et al., 2003). The main purpose of this study was to assess the fungal flora found on archives – air, surfaces and documents. Several methods were exploited to better achieve this goal. A combination of both traditional culturing methods and molecular biology protocols is now considered gold standard and efforts were made to include both methodologies in the present study.

Within the scope of the present research, a very recent analytical method is introduced – denaturing high performance liquid chromatography or DHPLC – which, once optimized, may allow the retrieval and separation of fungal DNA from a mixed sample in just 20 minutes per run. The basics on which it relies are known and applied in DGGE. In both, similar size yet sequence-different DNA fragments can be separated according to the decreased electrophoretic mobility of a partially melted double-stranded DNA molecule. DGGE uses polyacrylamide gels containing a linear gradient of DNA denaturants (Muyzer and Smalla, 1998) and DHPLC uses a ion-pair reverse phase HPLC column and an organic solvent for the DNA elution. DHPLC, being recent, is now starting to be applied in the field of human health genetics and we herein describe its primordial application to resolve fungal mixtures found in archival settings. Another protocol also applied in this work makes use of the automated fluorescence sequencer, here used to ascertain the identity of yeast colonies present in a mixture through the use of its characteristic ITS2 length.

The study design is presented next.

3.2 - Selected archives and Sampling Locations

The architectural and microclimatic characteristics of the buildings chosen to protect our written heritage are pivotal for their preservation. However, despite their common concern with the safekeeping of their documents, these spaces can be very different and the differences can arise even before any document was ever placed there. One can have buildings specifically built for the purpose of housing an archive (ad-hoc) – and these may or may not be furnished with the latest (or even the simplest) of equipment/conditions or one can have buildings or rooms adapted to the purpose of serving as a repository. Within each building type (with its benefits and problems) there can be very different purposes for each of the rooms since an archive usually houses reading room(s), conservation room(s), offices, etc…(Gallo et al., 2003). Regarding book conservation, also to be considered within a building is the position of these rooms (attic, basement, underground, etc…) whether or not they are exposed to sunlight, presence of windows, material's condition and storage equipment.

Four public access archives were selected for this study: Arquivo Distrital de Évora (ADE), Arquivo Histórico Ultramarino (AHU), Instituto de Habitação e Reabilitação Urbana – Forte de Sacavém (IHRU) and Torre do Tombo (TT).

The documentation kept by these archives is quite distinct in origin (dating from the IX to the XXI century and the conditions provided for their safekeeping are also quite diverse. Table 3.1 presents a summary of the main characteristics presented by the selected archives.

In Évora, the studied archive is one of the oldest in the country (formally created in 1916) and its creation is mostly due to the need to aggregate all the Diocese and City's documentation which had been temporarily kept by the city's Public Library. It was only recently separated from this institution and, at that time (1997) major efforts were made to improve the conditions of the building, equipment and access to the books. ADE is now almost reaching its full capacity and holds a vast and rich collection dating back to the XVI century *(http://adevr.dglab.gov.pt/).*

The building where the ADE is functioning was adapted to serve as an archive. It belongs to the Evora's University and dates back to the XVI century (Table 3.1).

The Arquivo Histórico Ultramarino (AHU) was legally created in 1931 in order to gather and provide the best conditions to safe keep all the scattered documentation related to the Portuguese Colonies. Its collection is now over 16 linear km and is almost all of it from the archives of the Portuguese Ultramarine Administration which functioned between mid-XVII century and 1974-1975. The AHU is another example of an adapted building since it is set on a Palace (Palácio do Pátio do Saldanha or Palácio da Ega) of recognized artistic and historical value. Its core dates back to the XVI century but almost every century has seen alterations made to the original construction. During the course of history, and before its present use as an archive, it served as a residence, as a hospital and as headquarters for the British and Portuguese troops. Since 1931, major improvements were performed to adapt this building to its present functions *(http://www2.iict.pt/?idc=100&idl=1*) (Table 3.1).

The IHRU collection and Library is housed in a former Fort (Forte de Sacavém) built in the XIX century during the Portuguese Civil War to protect Lisbon. Although also an adapted building, most of the areas where the documentation is kept were built for this purpose. It holds documentation from the XIX onwards mostly regarding historical buildings or constructions in Portugal and abroad *(http://www.portaldahabitacao.pt/pt/ihru/index.jsp)* (Table 3.1).

Torre do Tombo is the name given to the Royal archives of the Portuguese Kingdom and is considered, since the middle Ages, as the Central Archive of the Portuguese State. Its collections go back to the IX century and are kept in a variety of supports. The collection is now held in a specially-built location (built in 1990) where the conditions for its safekeeping, preservation and display have been considered *(http://antt.dglab.gov.pt)* (Table 3.1).

Table 3.1 Summary of some of the characteristics presented by the selected archives

	ADE	AHU	IHRU	TT
Location	urban	Urban (central city)	urban	Urban (central city)
Built ad-hoc	no	no	yes (partially)	yes
Adapted	yes	yes	yes (partially)	no
Mechanical ventilation system in the office areas	yes	yes	yes	yes
Mechanical ventilation system in the storage areas	no	no	yes	yes
Low oxygen storage rooms	no	no	yes (partially)	no
Windows	Public/staff areas: yes Storage areas: no	Public/staff areas: yes Storage areas: no/yes	Public/staff areas: yes Storage areas: no	Public/staff areas: no Storage areas: no

ADE's storage rooms are located both in the ground and first floor and none possesses any functioning window. There is, as mentioned in Table 3.1, no heating, ventilation and air conditioning system (HVAC) installed but the building characteristics (thick walls) are specially designed to avoid the intense heat felt in the summer and contribute to relatively stable temperature levels.

AHU's storage rooms are located in the ground floor (storage room 2) and basement (storage room 1), the restoration room is located at the ground level and the reading room is on the first floor. One of the efforts to transform this palace into an archive was the construction of underground storage areas (storage room 1, Table 3.2) and the installation of an HVAC system that would maintain the best conditions possible for documents preservation. However, and mainly due to budget restraints, the HVAC system is in need of repair and has not been able to maintain such conditions. The fact

that the storage areas are built underground also adds to the problems. The other storage area (storage room 2) has windows and no mechanical ventilation system. In terms of storage equipment, both storage areas are well equipped.

The building that now houses the IHRU collection suffered intensive renovations before becoming an archive and relies on mechanical systems for ventilation and maintaining proper temperature and relative humidity conditions. The storage area analysed has also a decreased level of atmospheric oxygen (14% instead of the usual 16%) and is equipped with an air filtering system.

The TT building, built to be the National Archive, presents mechanical ventilation systems in the entire building as the windows are only used for daylight illumination. The visited storage and office areas are located in the upper storeys while the restoration and higienization room are located in the basement.

In each archive, specific areas were selected for study and these always included at least one storage and one reading or office room: Table 3.2 presents the specific locations where the samples were taken for each of the studied rooms.

The storage rooms refer to the location where documents are kept when they are not being consulted by readers or being tended to by the Conservation/Restoration Laboratory. These locations are referred to as the restoration room in Table 3.2. Working rooms refer to the location where staff performs their activities (other than restoration). In the TT archive a study was also performed in the higienization room, where documents are cleaned before entering the collection.

The reading room is where the public is allowed to consult the documents kept at each of these archives.

Table 3.2 - Archives selected and locations where samples were taken.

		ADE	AHU	IHRU	TT
Sampling sites	Air samples	exterior	exterior	Exterior	exterior
		storage room 1	storage room 1	storage room	storage room
		storage room 2	storage room 2	restoration room	restoration room
		restoration room	restoration room	office room	higienization room
		reading room	reading room	reading room	office room
	Surface samples	storage room 1	storage room 1	storage room	storage room
		- floor	- floor	- floor	- floor
		- table	- ceiling	- shelves	- archival cases
		-archival cases	- archival cases	- archival cases	- documents
		- documents	- documents	- documents	
		storage room 2	storage room 2	restoration room	restoration room
		- floor	- floor	- floor	- floor
		- archival cases	- shelf	- shelves	- table
			- archival cases		- archival cases
		restoration room	restoration room	office room	higienization room
		- floor	- floor	- table	- floor
		- table	- table	- floor	- trays
					- archival cases
		reading room	reading room	reading room	office room
		- floor	- floor	- floor	- floor
		- table	- table	- table	

The air samples taken inside were always coupled with an outside evaluation for comparison. Generally, and given the areas of the rooms studied, only one air sample was taken for each location. The exception is the storage room 1 in the ADE archive where two air samples were taken given its large area.

Document's archival cases (DACs) is the name given to the samples taken from the document's outer protection and can relate to a cardboard case, a binding or a hardcover. In the floor, tables, shelves and DACs the sampled area allows for fungal quantification, since this area was predetermined (see 3.3.2). Document samples refer to chromatically or otherwise altered small surfaces of selected documents (see 3.3.3).

Two sampling seasons (summer and winter) were performed in order to account for climatic influences. A third sampling season was performed in the ADE archive since renovation work was taking place on the entrance to the upper floor (relatively close to storage room 1 and the office and reading rooms) and there was a possibility of analysing the impact this could have on the fungal communities present.

Samples were analysed using classical culturing methods and molecular biology protocols whenever needed (*mycelia sterilia* present in archival cases or otherwise unidentified fungi).

3.3 Sampling Protocol

3.3.1 Air Samples

Because fungi are known allergens air samples were retrieved during the sampling campaigns. A M Air Tester, Milipore, Massachussets, USA (Figure 3.1) was used for this effect and 250 litres of air removed for each location and season. Verhoeff et al. (1990) (as cited by Bueno et al., 2003) compared different methods for the identification and quantification of fungi and his review showed Slit and the N6-Andersen sampler (using DG18 and MEA as nutrient media) as the best methods in terms of precision and highest number of CFU/m^3.

Figure 3.1 - Indoor air quality studies include fungal assessment using air samplers such as this M Air Tester.

3.3.2 Surface samples (Floor, Table, Shelves, Trays and DACs)

Although not enforced by the NT-SCE-02, to establish a trustworthy assessment of the environmental health conditions of the chosen archival rooms, the dust settled in the shelves/working tables and floor was also sampled. Some fungal spores, due to their characteristics, fall much faster and are not caught on the air sampler (Buttner et al., 1993; Maggi et al., 2000; Duchaine and Mérieux, 2001).

In the present study, sterile cotton swabs (one for floor, tables and shelves and two coupled together and used at the same time for DAC´s) were used to sample a 100 cm2 square area with a 10x10 cm metallic square (Figure 3.2).

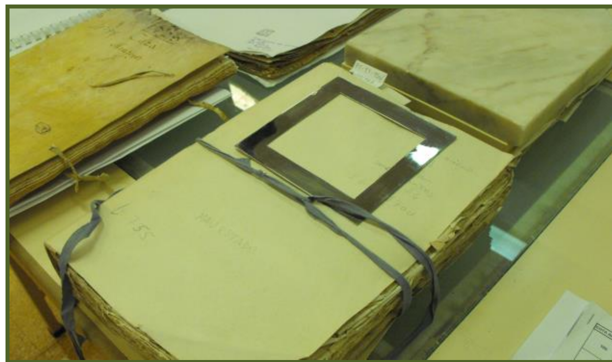

Figure 3.2 - The 10x10 cm metallic square used for area delimitation was disinfected between samples. The surface inside the area was streaked horizontally, vertically and in the diagonal.

The procedure used is described in ISO 18593 (2002) and was done in duplicate for each of the samples. Whenever considered relevant, other surfaces were also analysed.

3.3.3 Document samples

Because of dealing with valuable artwork the protocol related to the documents sampling was developed around a non-invasive swab sample. Visibly biodeteriorated documents were selected to perform this analysis and sterile cotton swabs (3) were used to remove any fungal mycelia present in the selected areas (normally chromatically altered areas) (figure 3.3). Two of the swabs were inoculated in growth media (MEA and DG18, one plate only) while the third swab was used for molecular biology protocols.

Figure 3.3 – Special areas were sampled for fungal presence analysis. A sterile cotton swab was used for the effect.

A control sample was also taken from the neighbouring and visibly undamaged area.

3.4 Sample Treatment and Analysis

3.4.1. Conventional Culturing Methods

When a given surface is swabbed and the swab streaked on a culture plate and incubated, the result will be a plate where spore germination or hyphae renewed growth has occurred. Culturing methods cannot and should not be excluded from any

fungal analysis – be it in an environmental sample or in an ancient piece of paper - because they give you the viable fraction of the fungal flora present in a given setting. This is especially relevant when trying to assess the impact a fungal population can have on human health which is also one of the objectives of this study.

The next lines will provide a brief description on the methodologies used to perform the study on fungal communities.

3.4.1.1 Air samples

After sample collection the plates were sealed and taken to the laboratory where they were left to incubate at 25-27°C for a week. The identification was based on the microscopic visualization (400x) of morphological characteristics using lactophenol cotton blue dye and illustrated manuals (Larone, 2002; Samson et al., 2004).

3.4.1.2 Surface samples (including documents)

The swabs (one from each of the sampled surfaces) were sealed, taken to the laboratory and streaked in the following culturing media (Samson et al., 2004): Malt Extract Agar (MEA) + chloramphenicol, dichloran-glycerol (DG18) (for xerophilic fungi). The inoculated media (original and duplicate) were placed at 27°C and remained there for a week (MEA) or 15-20 days (DG18). The identification was based on the microscopic visualization (400x) of morphological characteristics using lactophenol cotton blue dye and illustrated manuals (Larone, 2002; Samson et al., 2004, 2010). A negative swab was also streaked on each media used.

3.4.2 Molecular Biology Protocols

The presence of a large number of CFU's does not necessarily reflect the severity of the infestation. What one gets after performing these steps is the viable fraction i.e., the part of the fungal flora capable of growing in that specific media and environmental conditions. And even this is not entirely correct since the faster growth rate of one species can hamper the growth of other viable species (Stetzenbach et al., 2004). Some of these problems were minimized by advances in the methodology. The development

of dichloran-glycerol-18 agar (DG-18) – one of the media used in this study - is an example of such an advance since it has the ability of delaying the growth of fast growing fungi while allowing the growing of xerophilic species (low water content requirements), otherwise masked by the most common indoor contaminants (Cabral et al., 2010).

Other problems remained. The time needed to allow for fungi to growth and display their features, essential for fungal identification, can amount to a month. Also, when fungi infested surfaces are sampled these will be contaminated by many airborne fungal species and the ones growing on the culture media may not be the causative species and/or the causative species may no longer be viable.

Some fungal species simply cannot be cultured. According to Muyzer and Smalla (1998) 99% of all microorganisms in nature cannot be isolated and, therefore, identified under a microscope or show a very slow growth rate and/or very special media requirements. Many fungi from the Ascomycete group and the majority of Basidiomycetes do not grow on standard laboratory media (Salvaggio and Aukrust, 1981) and therefore the identification of these fungi can only be achieved through molecular biology methods. Moreover, when compared to the traditional culturing methods, not only do these allow the identification of non-culturable microorganisms which may have caused deterioration in the past, but they also enable the identification of cell debris which can be responsible for allergic reactions in those in contact with these documents (Wlazlo et al., 2008). Although molecular biology methods reduce drastically the time needed to perform a laboratory identification of fungi colonizing an ancient document, when the task is to analyse a mixed fungal sample (population) these modern techniques are still quite laborious - a problem that derives solely from the presence of more than one fungus.

There are many molecular biology methods currently in use for the genetic identification of fungi. Polymerase chain reaction is in the basis of them all but, once the DNA is extracted, one can choose different ways to proceed. Sequencing the PCR product has been one of the most promising methods for the molecular identification

of fungal DNA. There are several possible regions rDNA for amplification (see Figure 3.4), each one with its advantages and disadvantages.

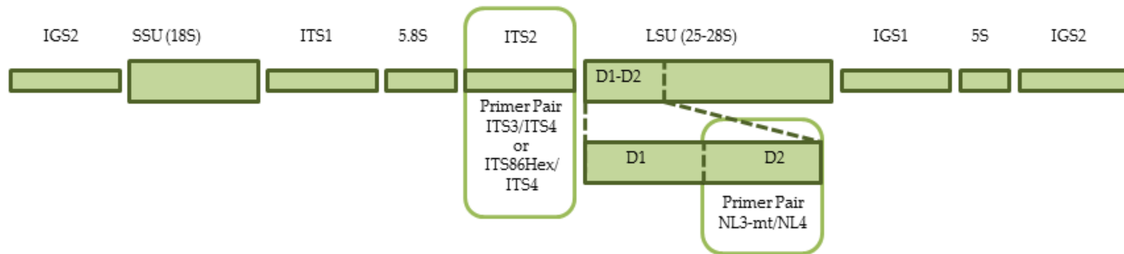

Figure 3.4 - Schematic representation of rDNA regions in the fungal genome

IGS= intergenic spacer, ITS= internal transcribed spacer, LSU=large subunit, SSU= small subunit.

The amplified region should, ideally, be sufficiently conserved amongst fungi in order to allow the amplification of several genera. However, it should also be variable enough to allow discrimination between closely related species. The 18S region evolves relatively slowly and is considered universal for fungi, i.e., it can be used to find consensus conserved regions within a group of fungi and to detect them but is not divergent enough to provide a distinction between them (Ninet et al., 2003). There is still no consensus on what region to amplify in order to achieve the best identification scores: ribosomal targets as the ITS1-5.8S-ITS2 or the LSU (25S-28S) are strong contenders in this challenge.

The D1-D2 region (in the LSU) has been exploited extensively (Abliz et al., 2004) with very good results in terms of GenBank identification scores, partially because there is a large contribution in this region and the database is, hence, very large. Because this is not the case with the ITS regions, the D1-D2 is sometimes used to confirm ITS1 or ITS2 results (Cano et al., 2004; Leaw et al., 2006). Nevertheless, and according to Hinrikson et al. (2005), the current identification of some *Aspergillus* species, especially the closely related, is best achieved using the ITS query sequences than the D1-D2 regions of the 25-28S region of the genome. Also, ITS has been recently established

as the bar-coding region of excellence (Rossman, 2007) as part of the European Consortium for the Barcode of Life initiative maintained by the Centraalbureau voor Schimmelcultures (CBS) and, therefore, an enrichment of the ITS region sequence database is to be expected. The comparison between these two regions in the identification of the fungi found in ancient documents is one of the major important aspects of this work and since they complement each other they will both be put to use.

3.4.2.1 DNA Extraction

Swab samples from documents

Regarding the swabbed sampled paper (or the small fragments of documents), one of the used swabs was collected and stored in a 300 μl TNE solution (1ml 1M Tris-HCl, pH8+ 2ml 5M NaCl + 0.2ml 0.5M EDTA, pH8 + 96.8 ml distilled H2O) at -70°C. For the DNA extraction the High Pure DNA Template kit from Roche (Mannheim, Germany) was used. To ease the DNA retrieval from all the fungi present in the gauze cotton swabs some minor alterations to the protocol were performed, including the use of 2mm and 4mm glass beads and/or a cell disrupter (FastPrep-24, MPBiomedicals, Solon, Ohio). The extraction protocol was also applied to a negative sample of TNE solution. DNA analysis was performed for all document samples.

Sterile Mycelia/Negative culture

DNA extraction was performed for all document samples and for archival cases which yielded negative cultures. In this case, the surface swab not streaked on malt and/or DG18 was submitted to the treatment explained in the last paragraph. Whenever possible, sterile positive cultures (*mycelia sterilia*) resulting from archival cases or documents were also analysed by molecular biology methods. A fragment of the colony was removed from the culture plate and submitted to DNA extraction using the High Pure DNA template kit (Roche). The High Pure DNA Template Kit (Roche Diag-

nostics Corp., Indianapolis, IN) was used to perform the DNA extraction of all yeast samples tested. No alterations were introduced to the recommended protocol.

3.4.2.2 PCR Protocols

Two DNA regions were mentioned earlier: 28S and ITS. Both regions are considered good starting points for sequencing and identification but, for one of the methods selected in this project (DHPLC), the fragment size obtained when amplifying ITS1 and ITS2 and the entire D1D2 region would result in a very large fragment - too large for the resolution needs of this study as shown earlier. The chosen fragments - ITS2 and D2 – are about 300bp in length. In order to proceed to fungal identification several regions of the fungal genome were exploited In Table 3.3 are the conditions and primers used for each sequence analysis.

Table 3.3 - PCR protocols used for fungal analysis.

Amplification	Primers	PCR Program		PCR mix
ITS1 – ITS2	ITS1 (forward) (White et al, 1990): 5'TCCGTAGGTGAACCTGCGG 3' ITS4 (reverse)(White et al, 1990): 5' TCCTCCGCTTATTGATATGC 3'	94ºC, 2 min 94ºC, 1 min 50ºC, 1 min 72ºC, 1 min 72ºC,5 min	35x	Buffer 10x: 5 ul MgCl2 25 mM: 6 ul DNTPs 100mM: 0.5 ul Taq polymerase 5u/ul: 0.25 ul Primers 100 uM:2x 0.25 ul DNA template: 20ng
ITS2	ITS3 (forward) (White *et* al, 1990): 5' GCATCGATGAAGAACGCAGC 3' ITS4 (reverse)(White et al , 1990): 5' TCCTCCGCTTATTGATATGC 3'	95ºC, 5 min 95ºC, 30 sec 54ºC, 1 min 72ºC, 1 min 72ºC, 6 min	35x	Buffer 10x: 5 ul MgCl2 25 mM: 6 ul DNTPs 100mM: 0.5 ul Taq polymerase 5u/ul: 0.25 ul Primers 100 uM:2x 0.25 ul DNA template: 20ng
ITS2 for AFCE	ITS86-hex (forward) (Turenne et al, 1999): 5' GTGAATCATCGAATCTTTGAAC' 3' ITS4 (reverse)(White et al, 1990): 5' TCCTCCGCTTATTGATATGC 3'	94ºC, 4 min 94ºC, 1 min 55ºC, 1 min 72ºC, 1 min 72ºC, 10 min	30x	Buffer 10x: 5 ul MgCl2 25 mM: 6 ul DNTPs 100mM: 0.5 ul Taq polymerase 5u/ul: 0.25 ul Primers 100 uM:2x 0.25 ul DNA template: 20ng
D1-D2	NL1-GC (forward) 5' GCATAT CAATAAGCGGAGGAAAAG 3' NL4 (reverse) 5' GGTCGGTGTTTCAAGACGG 3'	94ºC, 5 min 95ºC, 30 sec 51ºC, 1 min 72ºC, 1 min 72ºC, 7 min	30x	Buffer 10x: 5 ul MgCl2 25 mM: 6 ul DNTPs 100mM: 0.5 ul Taq polymerase 5u/ul: 0.25 ul Primers 100 uM:2x 0.25 ul DNA template: 20ng
D2	NL3 - mt (forward) (Pinheiro et al, 2011): 5' GAGACCGATAGCGCACAAGT 3' NL4 (reverse) 5' GGTCGGTGTTTCAAGACGG 3'	94ºC, 5 min 95ºC, 30 sec 51ºC, 1 min 72ºC, 1 min 72ºC, 7 min	30x	Buffer 10x: 5 ul MgCl2 25 mM: 6 ul DNTPs 100mM: 0.5 ul Taq polymerase 5u/ul: 0.25 ul Primers 100 uM:2x 0.25 ul DNA template: 20ng

Primer NL3-mt was created to substitute the MicroSeq commercial kit for the D2 region since its use implicated elevated costs. Yeasts, filamentous environmental and standard fungi isolates were used to assess the identification ability of the in-house method to amplify the D2 region by comparing the sequencing results obtained with this method with the commercial MicroSeq amplification and sequencing kits. To test the primer specificity for fungal DNA, the primer pair NL-3mt /NL4 was applied to human (serum from a pregnant woman) and bacterial DNA (*Staphylococcus aureus, Staphylococcus epidermitis, Klebsiella sp., Enterococci sp., Escherichia coli, Pseudomonas aeruginosa, Clostridium sp.* and *Salmonella sp.*). No amplification product was detected when using human or bacterial DNA template. The sensitivity was estimated using the dilution technique and a Nanodrop (ThermoScientific). The original yeast sample used for this test had 3.48 ng/ul, so amplification can be achieved with very low amounts of DNA.

All amplification protocols were performed in a MyCycler Personal Thermal Cycler (BioRad Inc.) and two negative controls (for amplification and extraction) were included in every amplification.

3.4.2.3 Amplified Product Purification and Sequencing

After PCR amplification, 5 ul aliquots were electrophoretically separated in a 2% agarose gel in 1x Tris-Borate-EDTA buffer stained with SybrSafe (Alfagene) or Gel Red (Biotium, California, USA). A 100-bp DNA molecular weight ladder (Biorad, California, USA) was included in each run. Gel pictures were obtained using the Gel Doc system (Biorad, California, USA).

Selected amplified products were submitted to purification and sequencing.

After positive amplification, 2 ul of Exo-Sap IT (USB Corporation) were used for every 5 ul of DNA sample and the mix was then submitted to 15' at 37°C followed by 15' at 80°C.

Sequencing protocols were designed for the amplified regions of interest. These consist of 25 cycles of 96°C (10sec), 51°C (5 sec) and 60°C (4 min).

The ABI Prism 310 Sequencer (Applied Biosystems, California, USA) was used for direct sequencing of all the protocols tested. Sequences were analysed using Chromas Lite and the FASTA sequences were aligned for a consensus sequence using Clustal W software. The result was, subsequently, compared against the BLAST database. A similarity of >98% to 28S rRNA sequences of type strains was used as the criterion for identification. The sequencing protocol was repeated whenever a definite identification was not obtained.

The resulting sequence was aligned with ClustalX and matched with GenBank NCBI database (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*).

3.4.2.4 DHPLC methodology

In clinical research, once the best sequence for a correct identification has been chosen and the protocol optimized one has merely to proceed to the sequencing part. Because, in the case of archives we are not dealing with clinical samples – where there is a low probability of *infection* by more than one fungus – we need to find a way to separate the amplified DNA from a mixture of origins order to be able to sequence each one. Nowadays this is usually done with DGGE and cloning. Although DGGE offers the advantage that individual bands can be excised, re-amplified, cloned and sequenced, many methodological difficulties, such as co-migration of bands with similar sequences, double or multiple bands from identical sequences, formation of chimeric and heteroduplex molecules and limited phylogenetic information from sequenced bands are usually encountered in the analyses (Janse et al., 2004; Gafan and Spratt, 2005; Nikolausz et al., 2005). In addition, one band may not necessarily represent one species and one bacterial/fungal species may also give rise to multiple bands because of multiple 16S rRNA genes with slightly different sequences (Gelsomino et al., 1999).

Denaturing high performance liquid chromatography - or DHPLC - a very recent technology, has emerged as an alternative and has already been applied to study populations (Domann et al., 2003; Barlaan et al., 2005; Pirotta and Garland, 2006) and mixed *Candida* sp. samples (Goldenberg et al., 2005). It can also be used to detect mutations in a specific gene (Keller et al., 2001; Jongbloed et al., 2005). As with bacterial samples, the microscopic approach to fungi identification lacks the discriminating capacity for the assessment of a community's diversity, especially in complex populations, and DHPLC attempts to overcome this problem. This technique allows the identification of each component of a mixture based on their genetic variation as submitting the amplified double-stranded DNA to an appropriate temperature leads the strands to partially denature. The right interaction between the ion pairing reagents and the cartridge matrix allows the different components of the mixture to be eluted at different times. In a highly complex mixture of microbial DNA this method can be used simply to study the population dynamics or the effects of human action over a popula-

tion – as the introduction of antifungal substances. What we will be attempting, however, goes beyond the community monitoring analysis as a whole as this system also allows the sample fraction collection which can, in many cases, be immediately re-amplified and sequenced circumventing the need for cloning. Regarding the technique setbacks, its resolution (as happens with DGGE) may not yet be sufficient, as DHPLC peaks may represent co-migrating amplicons from different species (Goldenberg et al., 2007).

DHPLC can be run in two basic methods depending on the temperature: non-denaturing or partially denaturing. At non-denaturing conditions (50°C), the amplicon is fully double-stranded and DNA separation is based solely on fragment size and is independent of sequence. Sizing results are similar to results obtained from an agarose gel and PCR products can be checked for purity.

The use of non-denaturing temperatures and a potential specific time of retention for each species could lead us to believe possible the creation of a size database and the use of these size differences as an identification marker. The already mentioned ITS2 region (+/- 300bp) is highly variable between different species (Turenne et al., 1999) and it is defended by some authors (Turenne et al., 1999; De Baere et al., 2002) that its length variation can alone serve as an identification marker. ITS2 size determination has already been used successfully in the identification of *Candida* species using automated fluorescent capillary electrophoresis (AFCE), a method also being applied in this study but to the identification of culturable yeast colonies present in a mixture (see Chapter 7, Clinical Studies. However, when applied to a complex mixture of filamentous fungi - and given the universe of species one can find in the environment – it will be relatively easy to find different *taxa* with the same amplicon length, a fact that renders this technique insufficient (Fujita et al., 2001; Hinrikson et al., 2005).

When using partially denaturing temperatures in dHPLC (>50°C), size and sequence play a role in defining the elution time. Figure 3.5 illustrates the usefulness of the technique as a mixture of three fungal species of known origin *Alternaria alternata* (Scientific Institute of Public Health, Brussels), *Aspergillus niger* (from the Quality in

Water Analysis Scheme quality test) and *Penicillium spinulosum* (from the RINGTEST quality test), all of them amplified for the D2 region, are submitted to a DHPLC program at 57°C, 59% buffer B (acetonitrile) at a 0.9 ml/min flow rate. These fungi were selected because *Alternaria* sp., *Penicillium* sp. and *Aspergillus* sp. are common paper contaminants (Zyska, 1997, see Chapter 2).

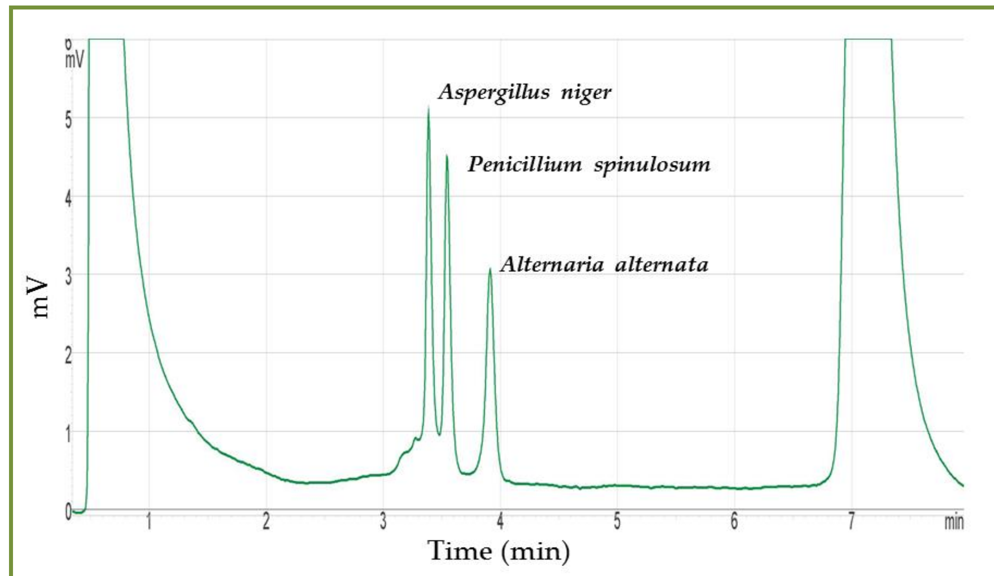

Figure 3.5 - DHPLC chromatogram of a mixture of amplified DNA from three different fungi (*Alternaria alternata*, *Aspergillus niger* and *Penicillium spinulosum*).

The amplified products are 298 basepairs in length and the mixture of the three provides a simple peak when run at 50°C, 40%B (data not shown).To achieve separation the following conditions were selected: Temperature: 57°C, Wave optimized Buffer B (acetonitrile): 59%, DNASep Cartridge 0.9ml/min. The targeted region was D2. (Pinheiro et al, 2008, 2010)

At partially denaturing temperatures the column/eluents will separate DNA according to sequence (GC content) and molecular conformation which is a very useful complement to the size difference mentioned above and follows the same idea of DGGE.

Given the choice of possible genomic regions one has still to consider a DHPLC limitation: the longer the fragment, the harder it is for the DHPLC system to identify the differences in the sequences analysed and so the method is most precise for sequence fragments of no more than 500 bp. This reason supports our choice of using two different fragments to accurately identify the fungal components of our mixtures:

ITS2 from the ITS region and the D2 part of the D1-D2 genomic region. This way, both sequences studied will have an average 300 bp length. Regarding the D2 region, when compared to the 600bp D1-D2 region, the size reduction is not expected to bring any deficit in the quality of the sequence in terms of identification scores since this smaller region is targeted by commercial kits as the MicroSeq D2 LSU 28S Fungal Identification and Sequencing kits from Applied Biosystems with very good discriminating power (Ninet et al., 2003).

DHPLC optimization

The elution of the samples depends on a series of factors such as column temperature and buffer B gradient rate and, so conditions have to be optimized. The example given in Figure 3.5 was a result of a series of tests on temperature, buffer gradient and rate of flow. The separation began to occur at 57°C and 59% B but other combinations (59°C, 55%B) also resulted in a good definition of the three peaks (data not shown). However, to optimize the protocol for future samples several fungal species were selected and their ITS2 and D2 sequences were analysed by the Wave (v.4) DHPLC sequence analyser program. A predicted temperature was automatically selected by the Wave program and it was around this suggestion that a range of analytical temperatures was chosen (between 59 and 63°C): since the collected samples will present an unknown composition just one temperature would not cover all the possibilities in terms of fungal presence. As with the D2 region (see Figure 3.5), it is possible to use the ITS2 region to separate the components of a mixture and the following example is an illustration of that (see Figure 3.6).

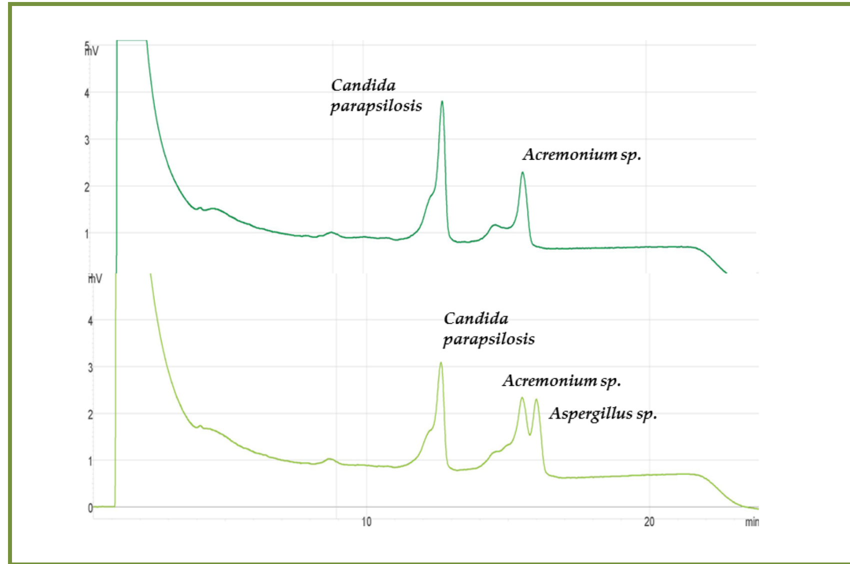

Figure3.3.6 - Top: DHPLC chromatogram of a mixture of amplified DNA from two different fungi (*Candida parapsilosis* and *Acremonium* sp.) using partially denaturing temperatures (59°C, 55%B) and a 0.5 ml/min flow rate. Bottom: DHPLC chromatogram of a mixture of amplified DNA from three different fungi (*Candida parapsilosis* and *Acremonium sp*. from above to which Aspergillus sp. was added) using the same conditions as above. In this example, the amplified region is the ITS2 (Pinheiro et al, 2010)

For both amplicons, the program used was the double stranded single fragment and the elution buffer acetonitrile. The column used was the DNASep®, made from alkylated nonporous polystyrene-divinylbenzene (PS-DVB) copolymer microspheres for high performance nucleic acid separations. The negative and positive amplification controls were included in every run.

For some of the samples currently under study a series of tests still have to be performed to assure the best resolution. The reason behind the choice of flow rate lies on the proximity of the peaks and on how difficult it is to separate them for collection. A higher flow rate produces a quicker run and sharper peaks which makes them more easily identifiable but, when too close, more difficult to collect. In this case it is best to choose a slower flow rate.

In the field work, once real mixed samples are collected, extracted and amplified together they are first run on the DHPLC at 50°C to check the quality of the ampli-

fied product and are then rerun through the column at the temperature range mentioned above. Peak collection allows the retrieval of the amplified DNA which can then be sequenced.

In the absence of an automated collector it is possible to collect the peaks manually for which a previous study was already performed. The sample represented in the chromatogram peak elutes a few seconds before the peak is visible on-screen. After collection it is not necessary to perform any subsequent steps before amplification and sequencing.

DHPLC performance tests

In order to establish the detection limit for this methodology, a yeast sample amplified DNA amount was determined using a Nanodrop 2000 (Thermo Scientific, Massachussets, USA). The initial *Candida parapsilosis* sample had 705 ng/ul and the 1:10 and 1:100 dilutions amounted to 70 and 6 ng/ul, respectively. All three samples were run on DHPLC sizing program (50°C, 40% B, flow rate 0.9 ml/min). The DHPLC is able to detect a PCR product with DNA amounts higher than 5 ng/ul (see Figure 3.7).

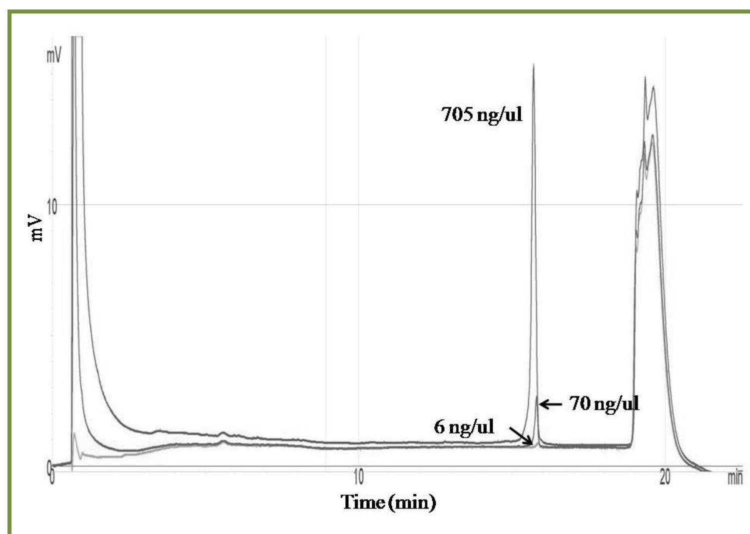

Figure 3.7 - Detection limits for the DHPLC DNASep Cartridge.

A pure Candida parapsilosis D2 region amplicon (300bp) was used to perform this test and the minimal amount of DNA detectable by the device were 5 ng/ul. The program used was non-denaturing at 50°C, 40%B, 0.9 ml/min.

The performance of a single species (in this case, *Candida parapsilosis*) at several temperatures was also tested. This was necessary to guarantee that when working with a single species one only obtains one peak at partially denaturing temperatures (Figure 3.8).

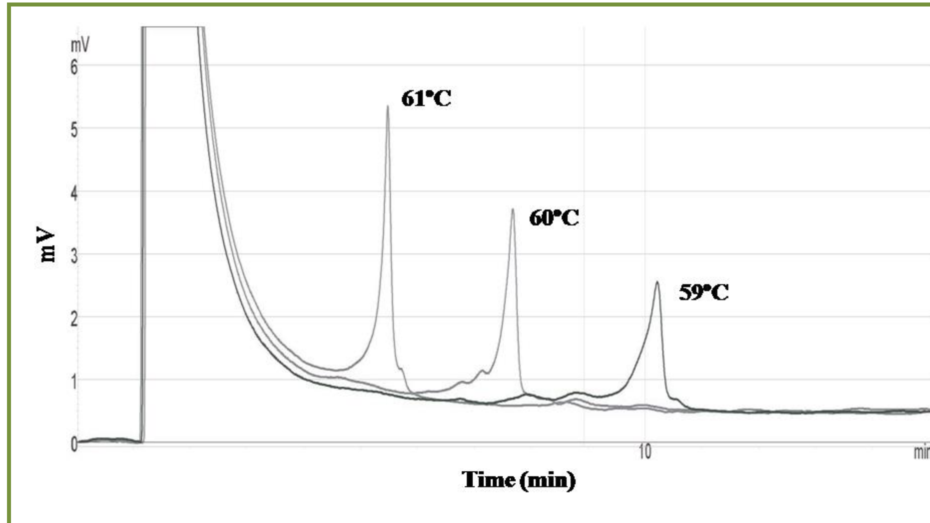

Figure 3.8 - Performance of a single species at partially denaturing temperatures. This test was performed with a culture collection *Candida parapsilosis* and the partially denaturing temperatures were 59°C (55% B), 60°C (51.2% B) and 61°C (54.2% B). The rate flow used was 0.5ml/min.

The use of ITS1, ITS2 or both these regions have several supporters (de Baere et al., 2002; Hinrikson et al., 2005). Opposing the choice of these regions is the still handicapped sequence database available (Hinrikson et al., 2005) (Sujita et al., 2003) and the difficulties the ITS region can present when applied to a fungal community (Ihrmark et al, 2012). Also noted was the greater difficulty in amplifying the ITS region, a difficulty felt by other authors (Michaelsen et al, 2006), which was not such an evident problem when using the D2 region from the 28S large subunit. Until full optimization of the ITS amplification, the D2 was considered the best choice and the DHPLC method here presented was developed and optimized for use with this region.

3.4.2.5 AFCE methodology

Alternative methods for the identification of clinical or environmental yeasts are based on DNA extraction, amplification and analysis (Borman et al., 2008). Automated fluorescent capillary electrophoresis (AFCE) is commonly used with the ITS2 region of the rRNA (Turenne et al., 1999; de Baere et al., 2002). In this method, the size differences in the ITS2 region are detected as one of the primers used for amplification is marked with a fluorescent label and the amplification product is detected by a sequencer using fragment size program analysis. In routine practice, the AFCE method is library dependent, improved by the constant growth of a robust ITS2 size fragment database. Amplified DNA samples were diluted with deionized sterile water before individual analysis. A negative and a positive control were used in each run. One μl of the PCR product was added to the electrophoresis mixture (0.5 μl of the ROX-500 marker and 10 μl of deionized formamide) The samples went through a denaturation step for 3 min at 95°C and the sample injection was carried out at 5 kV for 15 sec, followed by an electrophoresis at 60°C and 15 kV during 30 min.

To create the database all runs were done in triplicate. The ABI Prism 3130XL Genetic Analyser from Applied Biosystems (California, USA) was used for analysis.

3.5 Indoor Air Quality Studies

Fungi are the main focus of the performed and now presented analysis but they are also a vital part of any indoor air quality study. And since the scope of this thesis encompasses both conservation and health needs it is appropriate to present the data from the indoor air quality studies performed in the selected archives. The methodology is as follows.

For the chemical/physical and comfort parameters active sampling was used and the equipment is listed in Table 3.4. The procedures mentioned in NT-SCE-02 were followed during measurements.

Table 3.4 – Parameters analysed and equipment used to perform the indoor air quality analysis.

Parameter	Equipment
Relative Humidity and Temperature	Handheld 3016 IAQ Lighthouse
Particulate Matter (PM2.5 and PM10)	Handheld 3016 IAQ Lighthouse
Carbon Dioxide(CO_2)	Babuc LSI Sistems
Ozone (O_3)	Babuc LSI Sistems
Volatile Organic Compounds (VOCs)	Multi Rae RAE
Carbon Monoxide(CO)	Multi Rae RAE
Formaldehyde	First-Check Ion Science

A five minute measurement, as mentioned in the technical note NT-SCE-02 was used to perform these evaluations and an exterior determination was used for comparison purposes. Relative humidity (RH), temperature (T) and particulate matter (PM) were assessed during a second visit to the premises. Volatile Organic Compounds were also checked twice in the AHU in a follow-up procedure.

Chapter 2 left us with important questions to address. To answer them and pursuit the objectives set in Chapter 1 a methodology needed to be defined. The one presented in this Chapter 3 gathered information from various studies and the results obtained from its use are presented next, in Chapter 4.

4. Aerobiology of Fungi in archives

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4.1 Introduction

It is known that microorganisms such as fungi can damage cultural heritage, namely the one of organic origin. Biodeterioration of paper by fungi in archives and libraries is a well-documented phenomenon (see Chapter 2). Fungal metabolic and mechanical action can cause severe biodeterioration and their presence is particularly feared when the thermo hygrometric conditions favour their development. This is, therefore, one of the reasons why museums and archives, amongst other cultural sites, monitor and attempt to control the relative humidity and temperature by use of costly air conditioning and ventilation systems. However, when there is already a contamination, it´s important to know how dangerous (or not) is the fungus in question since different methodologies and procedures may have to be adopted depending on the type of fungus.

According to Chapter 2 there are several fungi positively associated with biodeterioration. This association between action and effect has, however, been hard to achieve for most of the cases. According to Caneva et al. (2003), effects such as erosion, spots, pigmentation and changes in the mechanical characteristics of paper have been the result of the action of the following genera: *Alternaria, Aspergillus, Chaetomium, Mucor, Myrothecium, Penicillium, Rhizopus, Stachybotrys, Trichoderma, Trichothecium* and *Ulocladium* (see Chapter 2).

Fungi can also have an impact on the health of the people working or attending archives, libraries or museums. When it comes to fungal contamination there is still a long way to come in terms of defining what is dangerous or not as far as fungal load and present species but efforts have been made to produce standards of air quality and in Portugal these were first set by the Decree Law 78/2006, 79/2006 (April 4th), NT-SCE-02 and more recently by the Ordinance n. 353-A/2013, December 4th, for some specific buildings. For all building types, as defined by the Decree-Law n. 78/2006 and 79/2006, April 4th, up until 2013, the total fungal CFU/m^3 could not exceed 500, there could be no visible fungal growth and the inside concentration had to be lower than

the one measured outside (I/O ratio lower than one). If this last condition was not met then further studies were needed in order to know the fungal flora. The requested conditions for air quality certification are presented in Table 4.1.

Table 4.1 – Requirements for air quality certification. These were binding up until 2013 for all building types and were established by the Decree-Law 78/2006, 79/2006 and the NT-SCE-02.

Low quality environment defined by:	
Fungal Load ≥ 500 CFU/m³ or	
Visible fungal growth or	
Indoor concentration higher than outside. In this case, the following conditions had to be ascertained:	
Presence of relatively uncommon species as long as:	Mixture of relatively uncommon species≥ 150 UFC/m3
	One uncommon species ≥ 50 UFC/m³
Confirmed presence of:	*Aspergillus fumigatus, Stachybotrys sp.* or other toxinogenic or pathogenic fungi (**)

*Common fungi: *Cladosporium sp., Alternaria sp., Penicillium sp.*

(**) Toxinogenic or pathogenic fungi: *Stachybotrys chartarum (S. atra), Fusarium sp., Aspergillus versicolor, A.flavus, A. fumigatus* and *A. niger, Histoplasma capsulatum* and *Cryptococcus neoformans.*

The 2013 legislation, applied so far to offices and services buildings, somewhat alters these premises as only when the inside CFU/m³ is higher than the outside measurement is the location considered to be unhealthy. If this condition exists then further studies are needed. There must not be visible fungal growth and the conditions shown in Table 4.2 must prevail:

Table 4.2 -Requirements for air quality certification for office and services buildings. These were recently established by the Ordinance 353-A/2013, December 4th.

Species		Conformity requirement
Common	*Cladosporium sp.*	Mixed species ≤ 500 CFU/m3
	Penicillium sp.	
	Aspergillus sp.	
	Alternaria sp.	
	Eurotium sp.	
	Wallemia sp.	
Uncommon	*Acremonium sp.*	Each species ≤ 50 CFU/m3
	Chrysonilia sp.	
	Trichothecium sp.	Mixed species ≤ 150 CFU/m3
	Curvularia sp.	
	Nigrospora sp.	
Pathogenic	*Cryptococcus neoformans*	Presence not admissible
	Histoplasma capsulatum	
	Blastomyces dermatidis	
	Coccidioides immitis	
Toxinogenic	*Stachybotrys chartarum*	Each species: less than 12 CFU/m3
	Aspergillus versicolor	
	Aspergillus flavus	
	Aspergillus ochraceus	
	Aspergillus terreus	
	Aspergillus fumigatus	
	Fusarium moniliforme	
	Fusarium culmorum	
	Trichoderma viride	

Since the study of the microflora of archives is still very incipient in Portugal, the present research was designed to determine the fungal load and species of fungi found in four Portuguese archives, in both storage rooms and reading/working rooms and considering air and surface samples (floor, shelves and archival cases). The results were then analysed from a paper conservation and human health point of view. As happened with the Ordinance 353-A, designed for office and services buildings, it is recommended to create specific norms for specific buildings. None have been created specifically for archives, with their dual purpose. In the attempt to aid in the definition

of guidelines for these particular settings the obtained results were analysed according to both legislated values and conditions.

4.2 Results and Discussion

4.2.1 Air samples

The air samples can be characterized in terms of fungal load (quantitative approach) and/or in terms of their composition (qualitative analysis).

4.2.1.1 Fungal Load

The total viable fungal count collected in the air samples is presented in Figures 4.1-4.4 for all four archives and considering all sampling seasons.

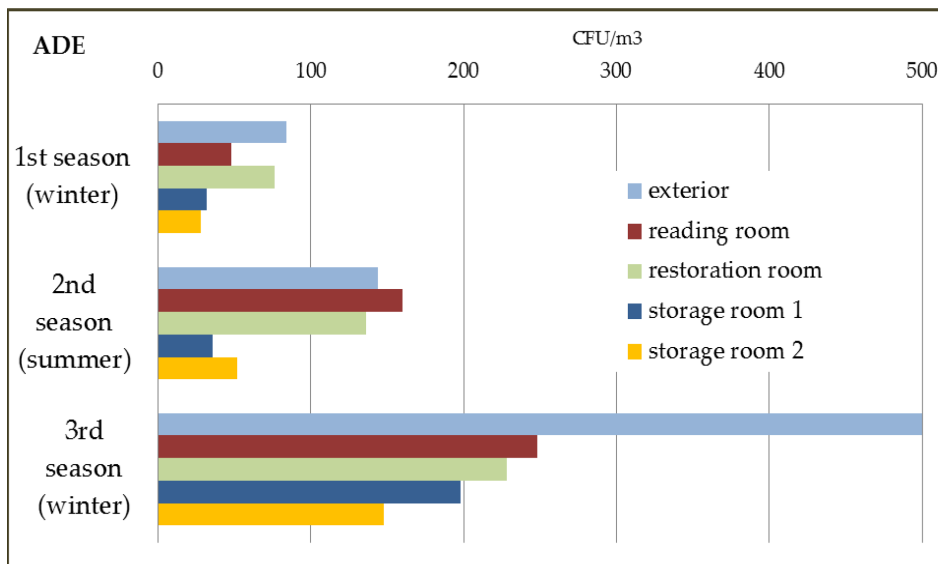


Figure 4.1 - Total viable CFU/m³ in the air samples taken from the selected locations in the ADE archive

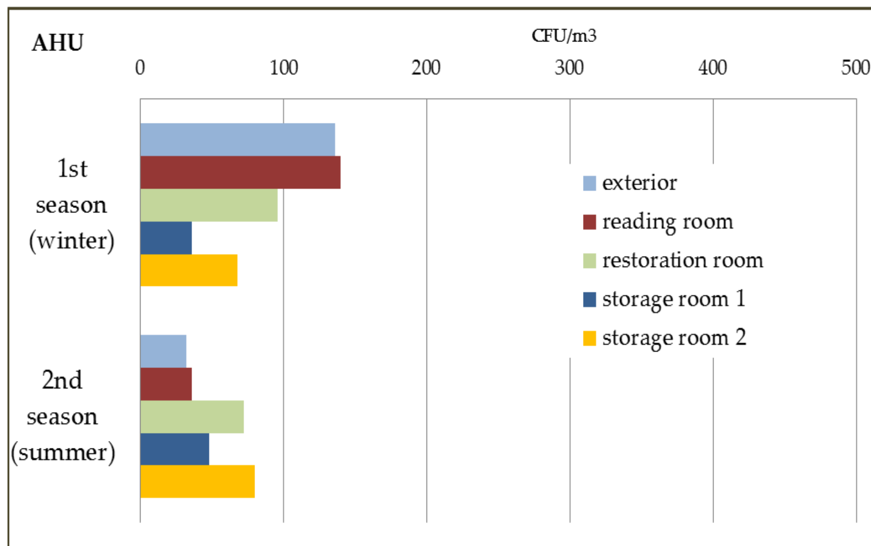


Figure 4.2 - Total viable CFU/m³ in the air samples taken from the selected locations in the AHU archive.

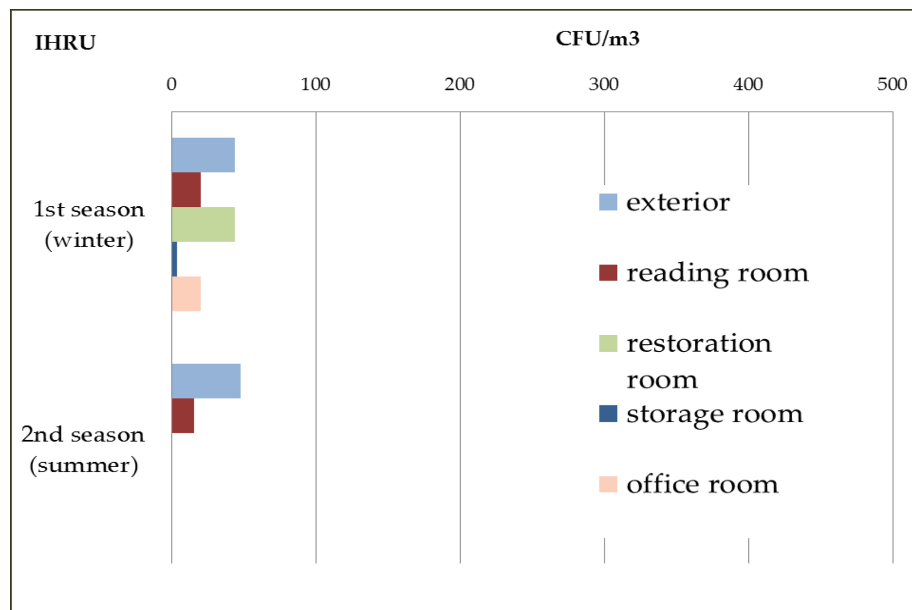


Figure 4.3 – Total viable CFU/m³ in the air samples taken from the selected locations in the IHRU archive.

A problem with the media plates used for air assessment made it impossible to perform the analysis in the restoration and storage room in the summer season. The office room sample returned zero colonies in the summer.

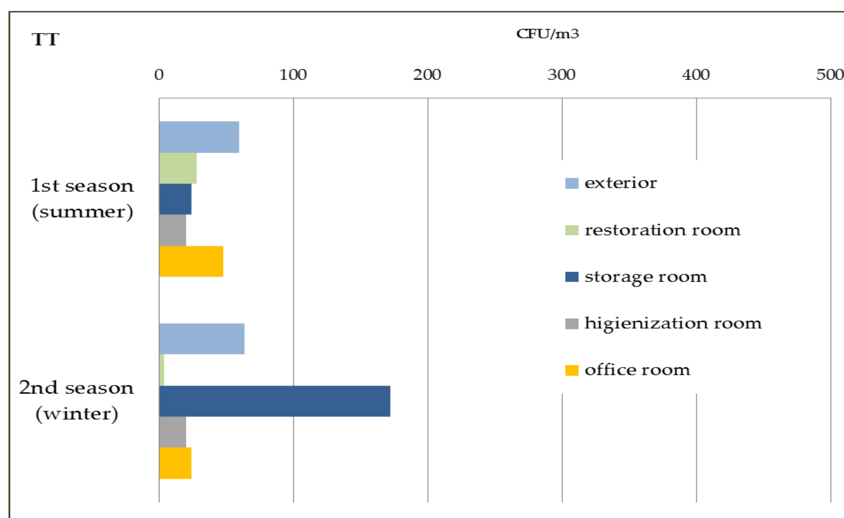


Figure 4.4 - Total viable CFU/m³ in the air samples taken from the selected locations in the TT archive.

The two seasons chosen for analysis are normally diverse in what concerns fungal contamination. In the spring and summer the levels of fungal load tend to be higher and the diversity of encountered species is also higher. On the contrary, winter and autumn, colder and rainier seasons, show a decreased level of fungal load and less diversity in its contents. ADE was analysed a third time due to construction and the interest in assessing its impact on the fungal flora.

In terms of fungal load, and contrary to several studies, there was no increase in the summer *vs* the winter samples as can happen in libraries (Bueno et al.,2003) and in other settings (Ali-Shtayedh et al.,2002, Panagopoulou et al., 2002; Medrela-Kuder, 2003; Martins-Diniz et al.,2005, and Ramachandran et al.,2005.) Only ADE (comparing the first two seasons) show a higher fungal load in the summer season. In the other three archives, the tendency is either reversed or unnoticed.

Fungal Load and Human Health

The indoor limit of 500 CFU/m³ (both according to the 2006 and 2013 Directives) was not reached in any of the rooms, for any of the seasons. If ADE's 3rd season is not considered (since the conditions when sampling was performed are considered abnormal), the highest value was found in the TT storage room with 148 CFU/m³ and the

lowest was experienced in TT's restoration room and IHRU's storage room, both with 4 CFU/m³.

The indoor/outdoor ratio (I/O) - which compares the fungal load determined inside with the one determined outside – should always be lower than one since fungal contamination is mainly from external sources (Nevalainen, 2007). In the selected archives, the I/O was lower than one in most of the analysed rooms.

Fungal load and paper conservation

In terms of fungal load the limits proposed by several authors and presented earlier (Table 1.1) are compared with the results obtained in the four archives studied and for the storage rooms, where documents are kept most of the time.

The comparison is presented in Figure 4.5:

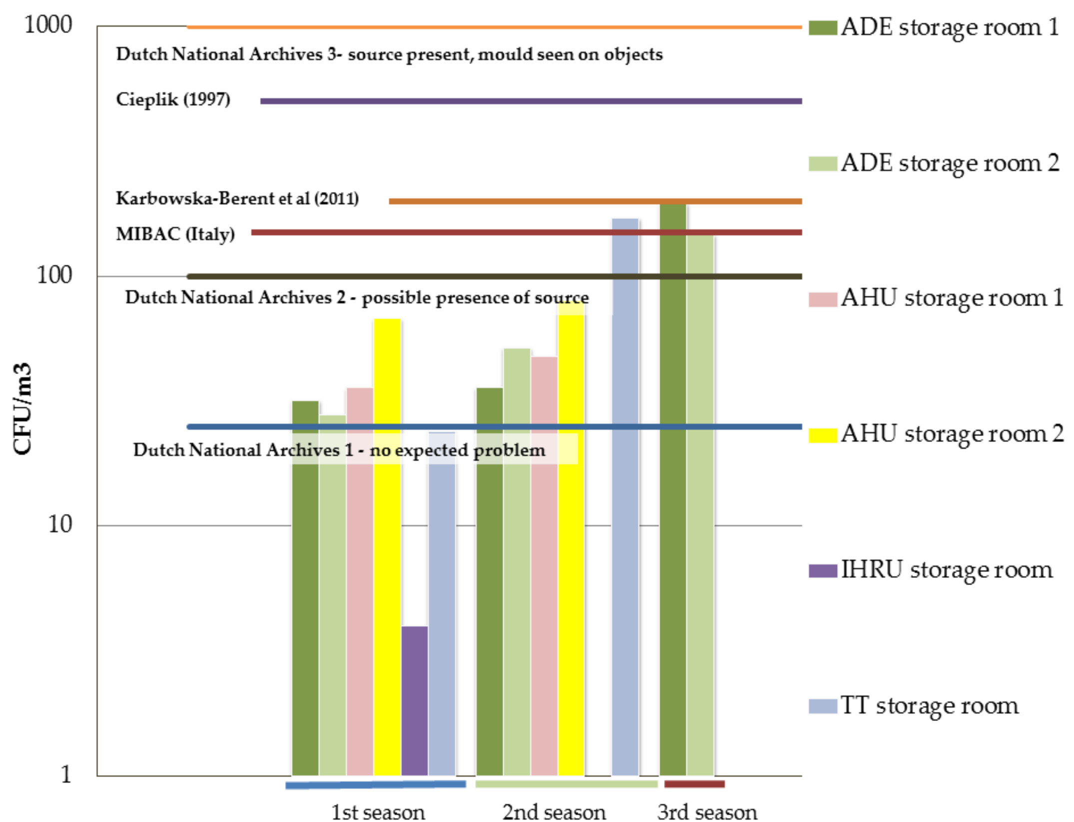


Figure 4.5 – Air quality levels compared with some of the established limits for conservation (see Table 1.1).

Please mind the logarithmic scale.

Most of the studied Portuguese archives show levels lower than the recommended by the Italian Ministry of Culture which is the only governmental standard depicted. The TTs storage room (2nd season) and the ADE's storage rooms (3rd season) are the only ones crossing the Italian *barrier*.

ADE's third sampling season coincided with renovation work taking place in the archive and this may have been responsible for the increased contamination levels while in the TT archive the cleaning activities with water might have been responsible for the increase in the *Penicillium* population in this storage area in the second season.

IHRU was only tested once but no problem is to be expected in the sampled deposit room. This room was designed to provide only 14% O_2 rate which may aid in the low level of contamination.

AHU levels are considered good in both seasons and for both storage areas.

If the Dutch National archives are to be followed, then only the IHRU storage room presents optimal results since no problem is to be expected from the air readings results. It was not possible to confirm this high quality in the second season.

The Dutch National Guidelines also predict the existence of a source when levels cross the 25CFU/m^3. This prediction is correct for the TT archive, assuming water cleaning activities to be responsible for the higher results in the second season. ADE's and AHU's levels were always above this limit which may render more useful the transformation of this proposed guideline into an increase between seasons. Instead of defining the 25 CFU/m^3: no expected problem and above 100 CFU/m^3 (4x higher): source present, it could be more reliable to assume an increase in fungal count as a predictor of a fungal source.

The other existing study performed in Portugal (Nunes et al, 2013) found lower levels of fungal contamination in the air samples performed (between 1 and 16 CFU/m3 in the several storage rooms assessed).

To evaluate the selected environments in terms of paper conservation one could go no further since the proposed guidelines are based on limit values alone without

considering species or genera definition. This is not true, however, for human health guidelines.

4.2.1.2 Fungal Communities and Human Health

Unlike the existing guidelines for paper conservation, indoor air quality levels for human health demand the characterization of the fungal flora as explained in Tables 4.1 and 4.2. As such, the identification of the elements composing the fungal communities encountered in the air samples was performed to the species levels whenever possible and/or recommended.

As mentioned, no indoor location presented higher than 500 CFU/m^3. Up until 2013, two other conditions had to be met for the environment to be considered healthy: no visible fungal growth (condition met in all locations except for the AHU, storage room, both seasons) and an I/O ratio higher than one: ADE, 2nd season, reading room; AHU, 1st season, reading room and second season for all the rooms studied; IHRU, 1st season, restoration room and TT, 2nd season, storage room. With the most recent legislation only the locations with an I/O ratio higher than one demanded further studies (even if inside those with a regular I/O there is active fungal growth as happens with the AHU storage room!)…

Arquivo Distrital de Évora (ADE)

Three sampling seasons were considered at the ADE archive. In Figure 4.5 are presented the results from the first season, performed in the winter.

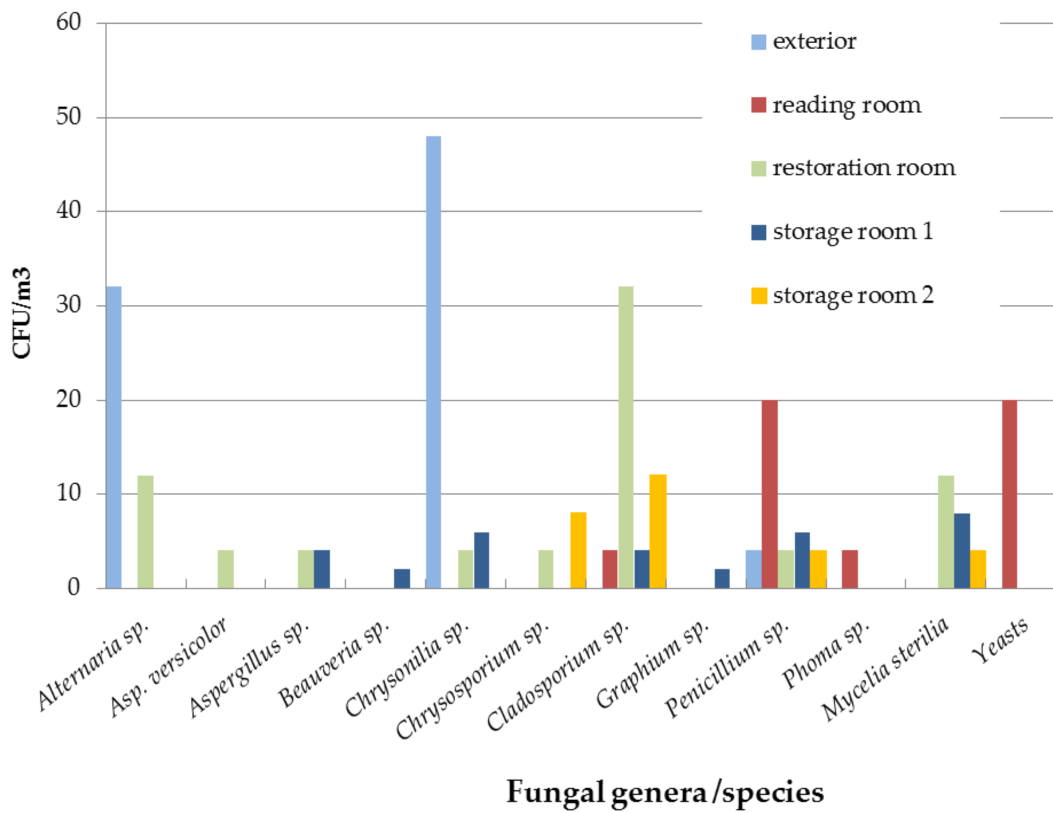


Figure 4.6 – Fungal load and diversity on ADE's air sample, 1st season, winter.

As mentioned, the exterior sample presents higher fungal loads but inside measurements reveal the presence of *Aspergillus versicolor* in the restoration room. This is the only potentially toxinogenic fungi encountered. Indoor contamination is also noted, since the outdoor air presents the commonly found *Alternaria* sp. and *Penicillium* sp but inside air is more diverse in its fungal elements. The high prevalence of *Chrysonilia* sp. outside is probably affected by the remarkable growth rate this fungus presents when in culture which may not mirror its real frequency outdoors.

The 2nd sampling season, performed in the summer, gave the following results (see Figure 4.7):

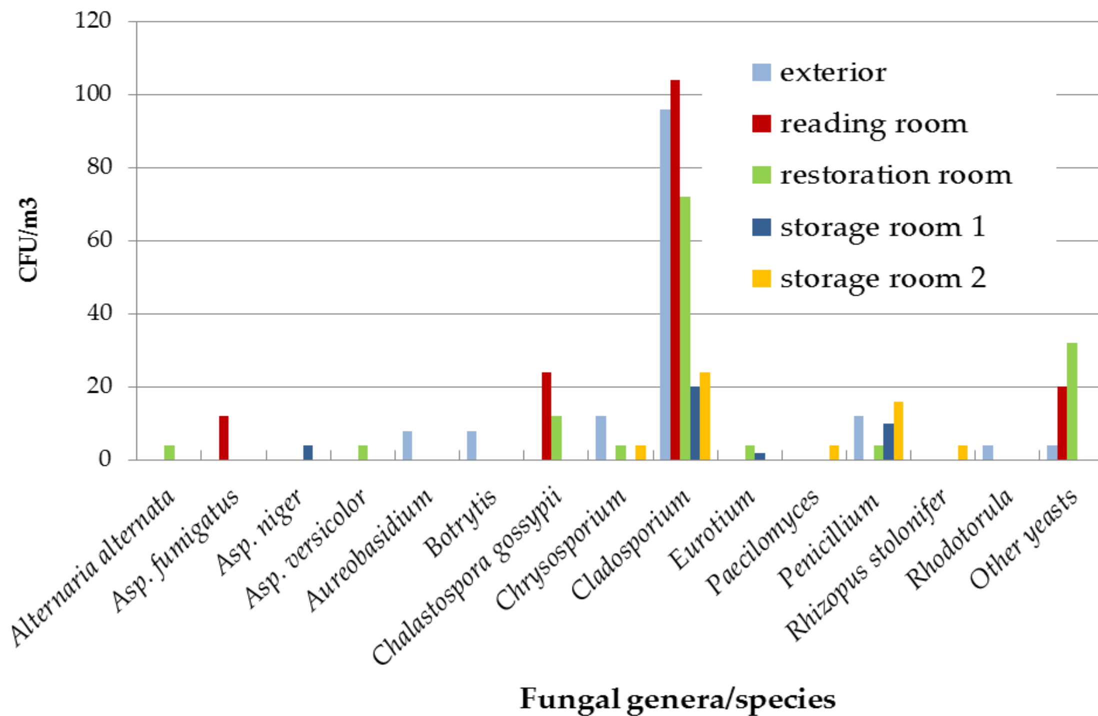


Figure 4.7 – Fungal load and diversity on ADE's air sample, 2nd season, summer.
Note: Chalastospora gossypii was firstly identified as mycelia sterilia but in this season a similar fungus appeared in an archival case sample and the DNA was extracted and amplified for analysis. This method allowed for the identification of this fungi in the air samples (see surface samples for details).

The variety of fungi found during summertime is wider and the fungal load is also higher (see figure 4.1), which comes in agreement with several studies where this trend is noted (Ali-Shtayedh et al.,2002, Panagopoulou et al., 2002; Medrela-Kuder, 2003; Martins-Diniz et al.,2005, and Ramachandran et al.,2005.)

Cladosporium sp. displays a strong presence inside which is probably related to its also prominent presence outdoors. This influence is more noticeable, as expected, in the two rooms with windows (reading and restoration room).

In the only room with an I/O ratio above one (reading room), the air samples returned the presence of *Aspergillus fumigatus* (12CFU/m^3), a toxinogenic fungus considered relevant by both the NT-SCE-02 and the most recent Ordinance. Other potentially toxinogenic fungi are *Asp. niger* (only for NT-SCE-02) and *Asp. versicolor*, both absent from the control sample.

Although none of the rooms analysed during the third season in ADE theoretically show an I/O ratio superior to one, this may be due to the type of fungi covering the entire exterior air sample taken at this season. Figure 4.7, corresponding to the ADE 3rd air sample, in November, is presented next.

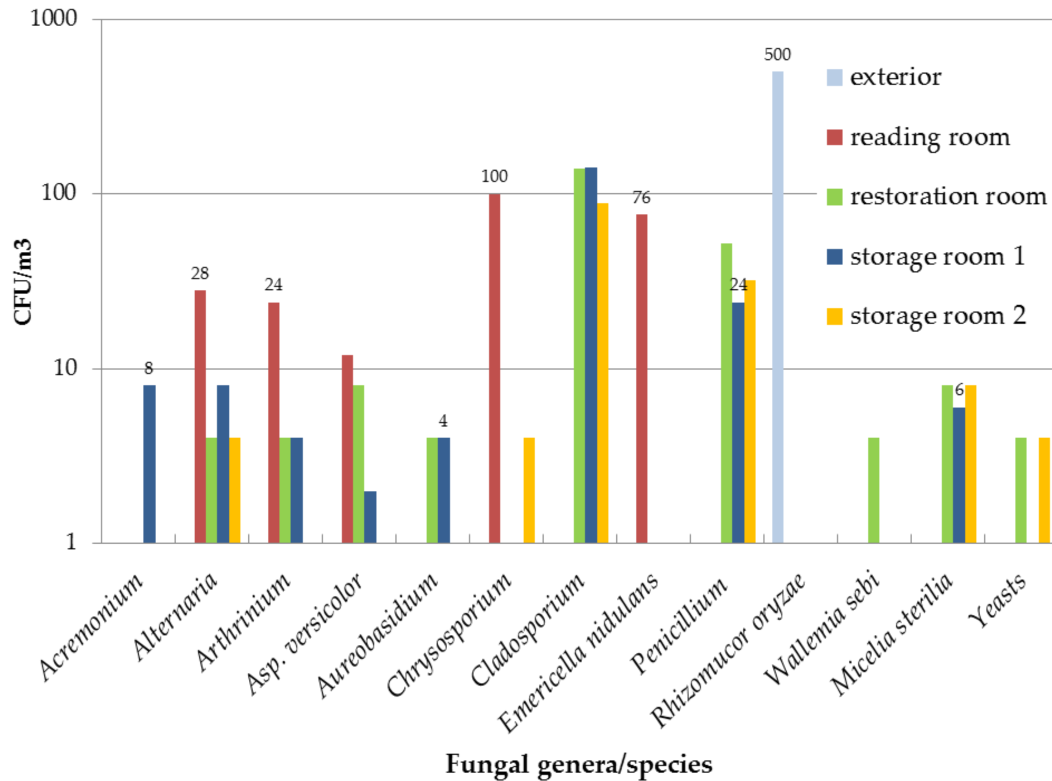


Figure 4.8 – Fungal load and diversity on ADE's air samples, 3rd season, winter. Some of the values are presented to ease comprehension.
Please mind the logarithmic scale.

In this case, the 500 CFU/m^3 corresponds to an uncountable level but while this can be true for *Penicillium* or *Aspergillus*, *Rhizomucor oryzae* spreads incredibly quickly over the plate leaving it almost impossible to count the colonies. For this reason, 10 colonies can create the same scenario as 100 but these different interpretations can translate into a total disregard for all the rooms considered since their study depends on I/O ratio.

One of the striking differences when comparing this season with the previous two is the higher fungal count since all of the rooms now cross the 100 CFU/m3. Toxinogenic fungi such as *Asp. fumigatus* and *versicolor* are present and the last one in levels ≥ 12 CFU/m^3.

Arquivo Histórico Ultramarino (AHU)

In AHU, the first season showed an I/O ratio higher than one in the reading room. In Figure 4.9 are the results for all the locations sampled in winter.

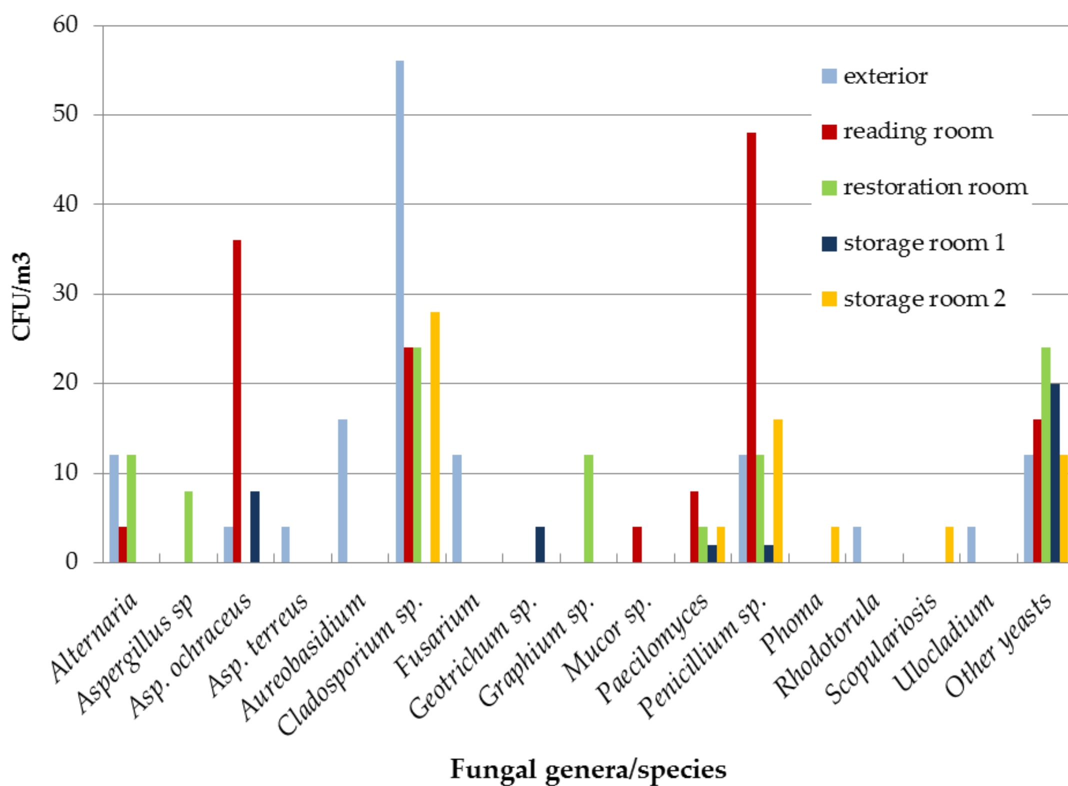


Figure 4.9 – Fungal load and diversity on AHU's air sample, 1st season, winter.

Apart from the I/O ratio, the pivotal reason to conduct further studies according to recent legislation, there is also a higher than 12CFU/m^3 of *Aspergillus ochraceus*, a potentially toxinogenic fungi as considered by the most recent Ordinance. It must also be pointed out that there was active fungal growth in the storage room 1 and this room presents an unsuspecting ratio.

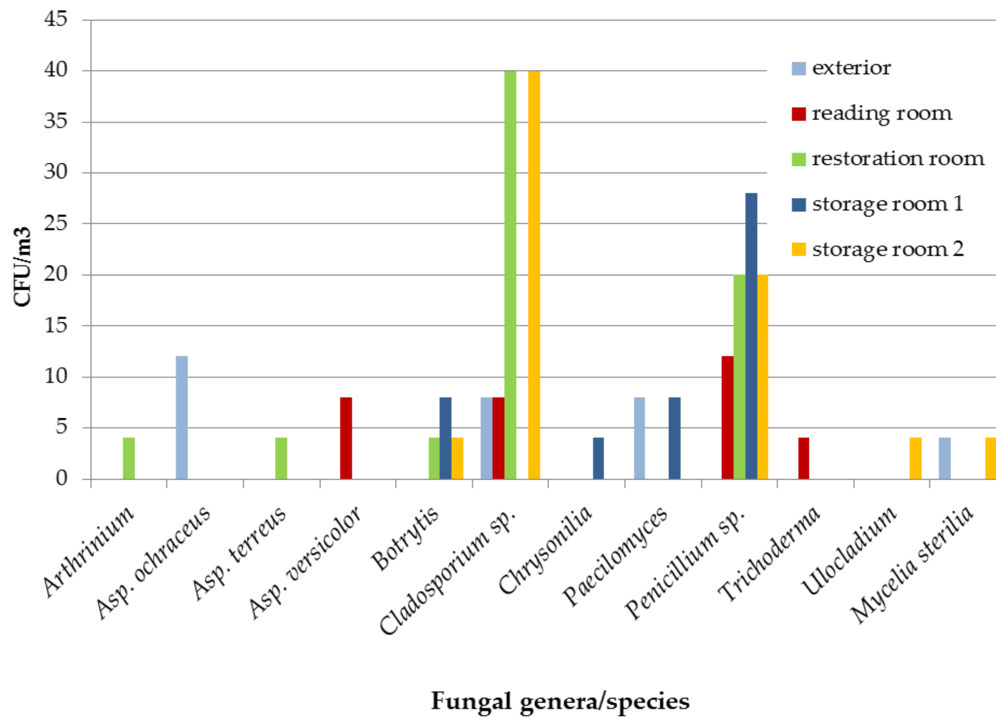


Figure 4.10– Fungal load and diversity on AHU's air samples, 2nd season, summer.

In this second season, all rooms present higher than one I/O ratios and therefore all should be further inspected in light of the new legislation. The reading room, reincident in what ratios are concerned, still presents *Asp. versicolor*, but this time in lower concentrations.

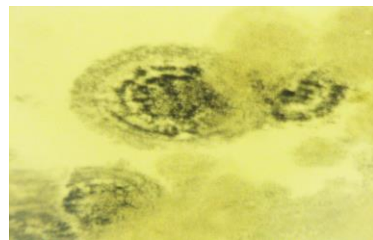
The restoration room demands no further studies but in storage room 1, the fungal growth is much more visible now and curiously the levels of *Penicillium* are greatly increased (1400%). According to Samson et al. (2004, 2010) *Penicillium* species can be indicators of humidity problems.

Figures 4.11 to 4.14 illustrate the fungal growth progression between the two sampling campaigns. A leaking pipe was the reason behind this problem.

Winter 10

Figures 4.11 and 4.12 – Ceiling showing the first signs of fungal growth after a pipe has started leaking.

Summer 11



Figures 4.13 and 4.14 – One year after, the ceiling shows profuse fungal growth of *Penicillium sp.* and *Stachybotrys chartarum*.

By the time one and a half years had passed since the onset of the problem, the staff was already fearful of entering the room since what was once a black spot had spread, despite (non-professional) cleaning of the area. A mask was advised for whoever entered the room and the institution was alerted to the need to fix the water problem as the only means of effectively eliminate the fungal contamination. Only some of the documents were moved to other storage areas.

Instituto de Habitação e Reabilitação Urbana (IHRU)

Air samples taken in the IHRU archive, 1st and 2nd season, revealed no potentially toxinogenic fungi. In the first season, the restoration room presented levels of contamination equal to the ones measured outside and the entire fungal flora was constituted of *Penicillium sp.*, considered by the legislation as a common contaminant (Figure 4.15).

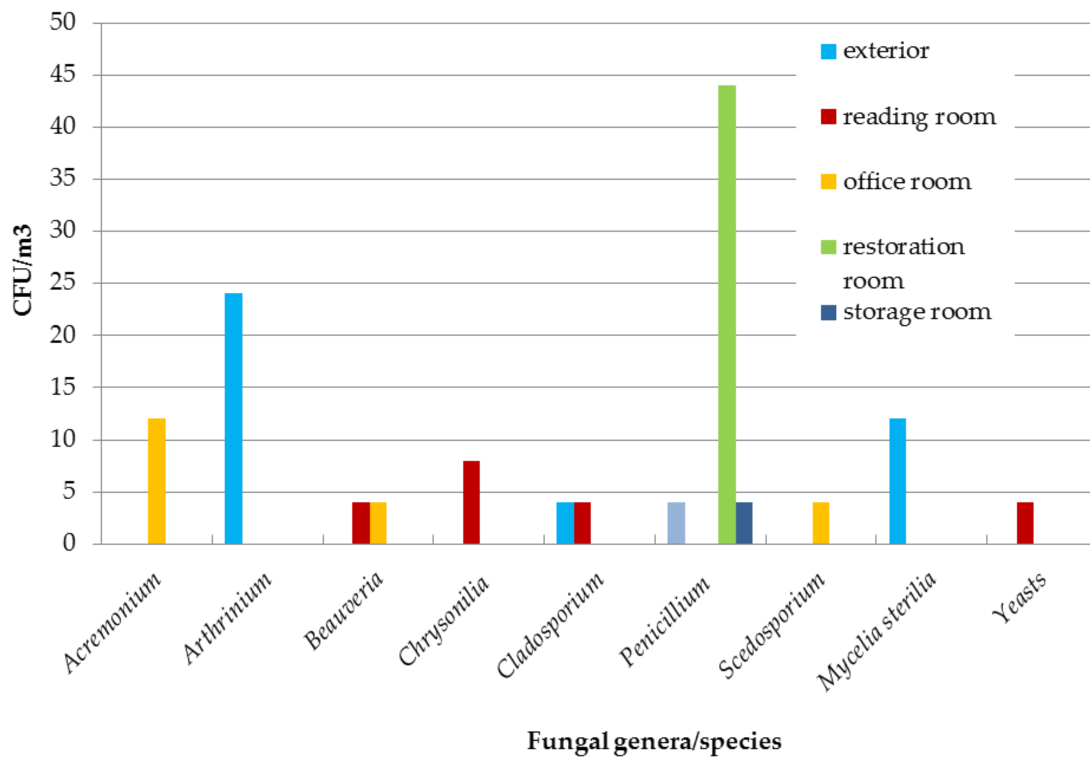


Figure 4.15 – Fungal load and diversity on IHRU's air sample, 1st season, February

As mentioned, *Penicillium* spp. tends to be more common in locations where the humidity level is higher. The restoration room is a *clean* room where water related activities take place and the water presence might be partially responsible for the fungal flora. Unfortunately, in the second season it was not possible to retrieve any data from most of sampled rooms in the first season due to technical problems with the media used for culturing (Figure 4.16).

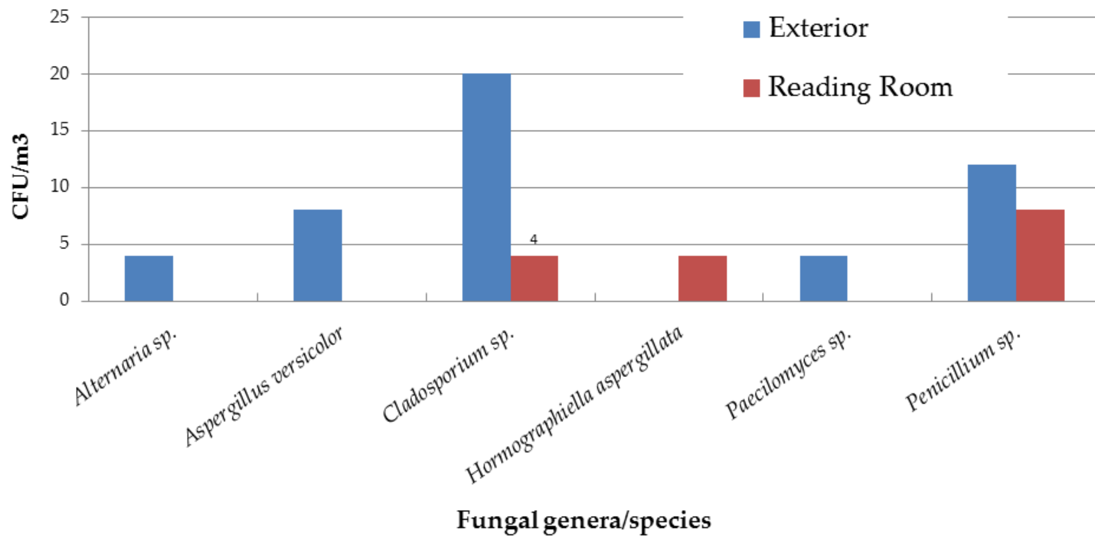


Figure 4.16 – Fungal load and diversity on IHRU air sample, 2nd season, April. The office room returned zero colonies.

Inside, only the reading room returned a readable result and the levels and fungal flora present were very similar between the two seasons. Despite having considered as viable the culture plate used to assess the air contamination in the office area, the authors recognize the improbability of a zero CFU/m^3.

Torre do Tombo (TT)

The results in the first season are all within desirable limits: all below 500 CFU/m^3, no I/O ratio above one, no potentially toxinogenic or pathogenic fungi and no visible fungal growth. As before, indoor contamination does occur (see Figure 4.17).

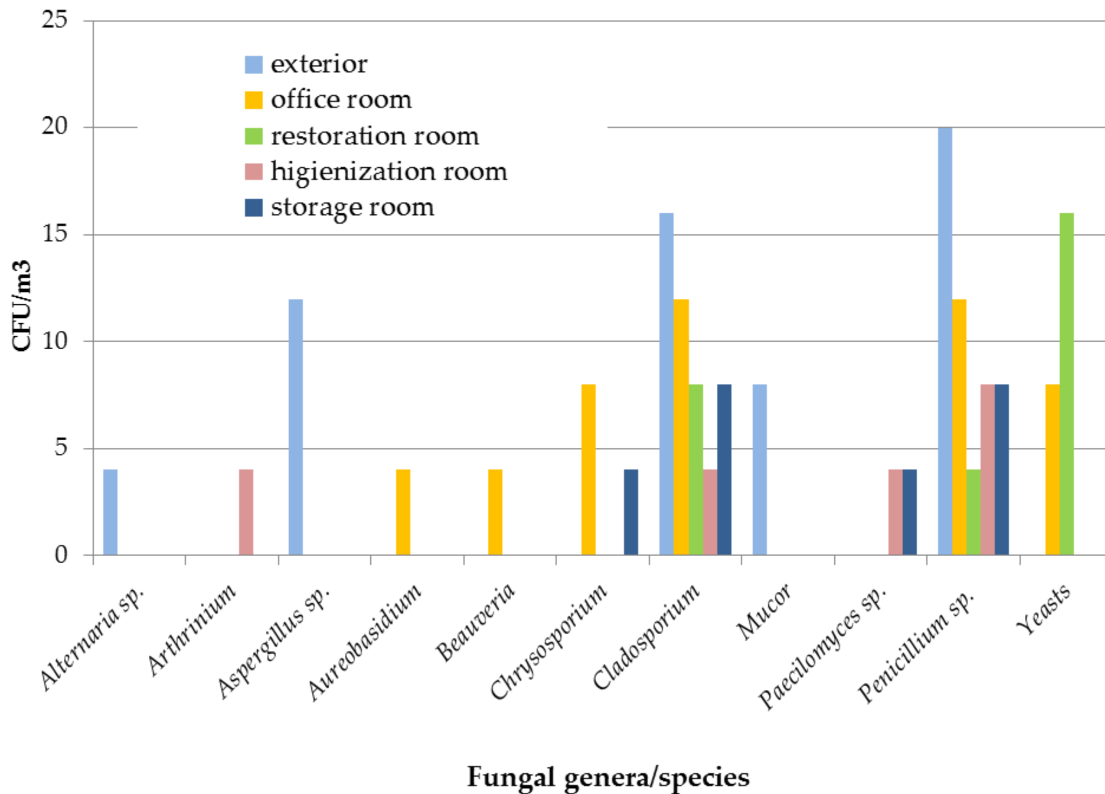


Figure 4.17 – Fungal load and diversity in TT's air sample, 1st season, April.

In the second season (see Figure 4.18) the storage room presents high levels of contamination by *Penicillium* species. In the first season the contamination level by this genus is much lower and does not reach the 10 CFU/m^3 in this room. *Asp. versicolor* - in levels considered *acceptable* by the recent legislation– are found in the office room for this second season. According to the 2006 legislation this room and the higienization room also present the potentially toxinogenic fungi *Fusarium* sp.

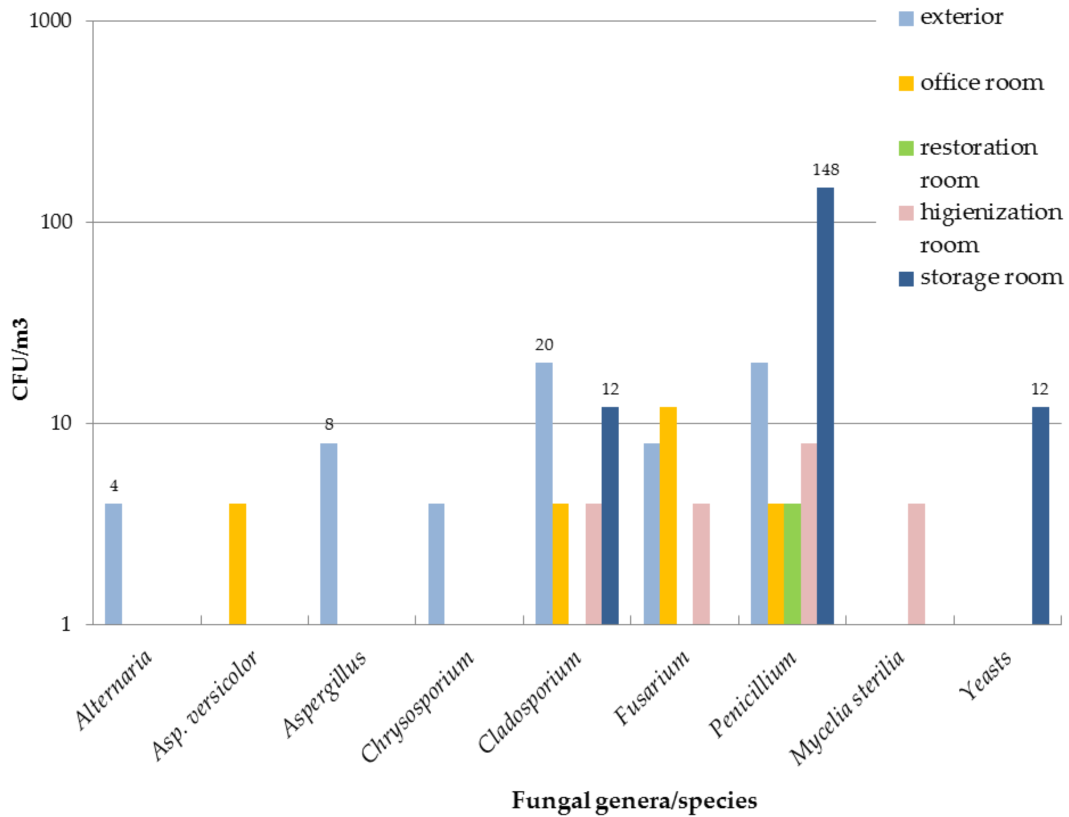


Figure 4.18 – TT air sample, 2nd season, winter. Please mind the logarithmic scale.

In terms of relative humidity - one of the factors to be considered for an higher fungal load and for the presence of certain fungi – the storage room shows very high levels (higher than 70%) and this is the room with the higher fungal load and with high levels of contamination by *Penicillium* spp. and yeasts - two fungi that thrive in moist environments.

4.2.1.3 Global Analysis

The results from the archive have been discussed separately but Figure 4.19 brings us the total frequency and the maximum concentration for each fungal genera and/or species identified in the indoor air of the selected locations. Considerations on the presence of a given species are taken from both the human health and the paper conservation perspective.

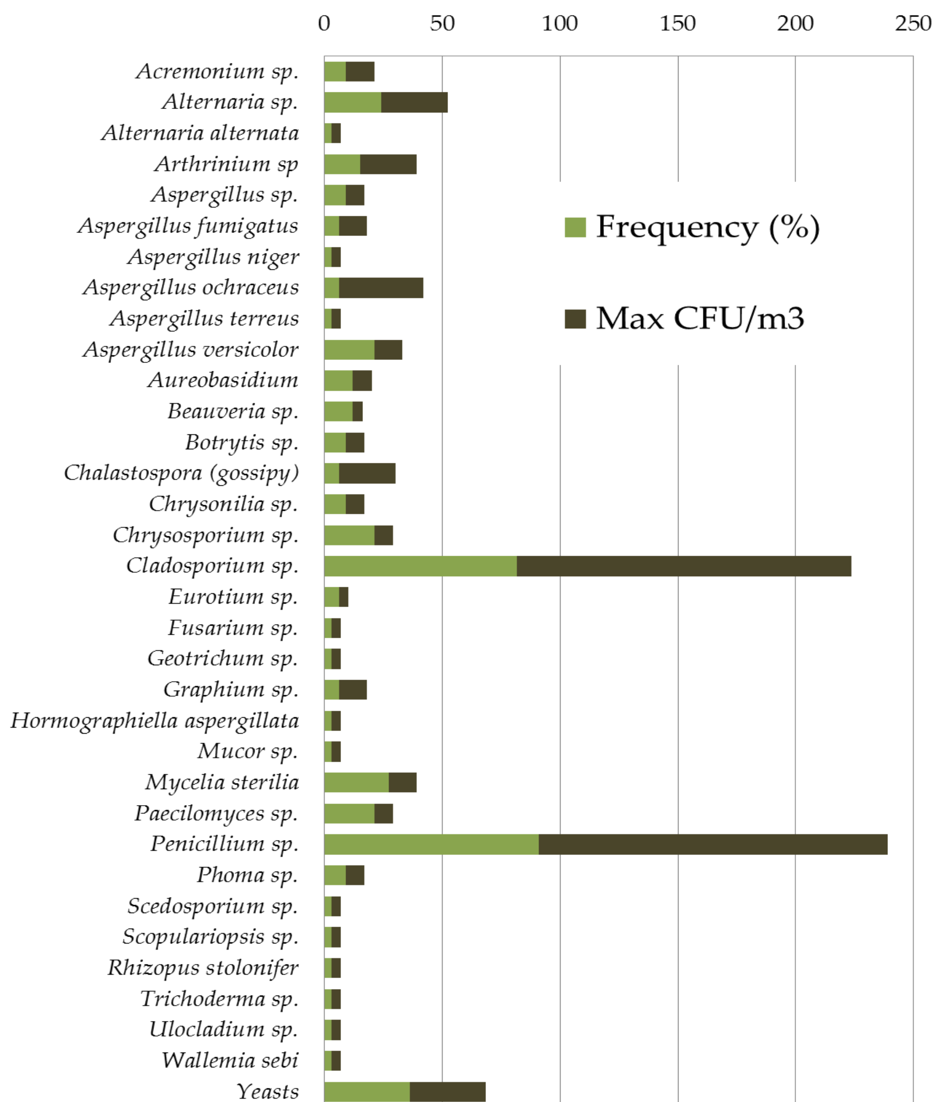


Figure 4.19 - Air samples performed in four Portuguese archives and analysed in terms of frequency (presence/total number of samples) and maximum number of CFU/m^3.

Comparing with the global results presented in Table 2.1, the yeasts stand out as one of the most commonly present in air samples retrieved from these archives. The other most represented fungi follow the trend depicted in Table 2.1 with *Penicillium* sp. and *Cladosporium* sp. taking the lead. From the *Aspergillus* group, *Aspergillus versicolor* is the most represented and this fungus is known for being able to produce mycotoxins under indoor environment (Salkinoja-Salonen et al., 2003).

The also high presence of *mycelia sterilia* is a pointer to the difficulties encountered when using just culture methods and it is a reminder of the large proportion of fungi that are probably not receiving the best conditions to grow and sporulate.

The other existing study performed in Portugal (Nunes et al, 2013), also found *Penicillium, Aspergillus* and *Fusarium*, to be the well represented in the air samples. However, *Cladosporium* sp and yeasts were not found in Nunes et al. (2013) study.

Table 4.3 summarizes the results obtained in the sampling campaigns according to relevant requirements established by both legislations (2006 and 2013).

Table 4.3 - Summary of the results obtained in the sampled air according to relevant requirements established by both legislations (2006 and 2013).
The plus sign means that at least one of the rooms presents the condition mentioned in the left column.

Assessed Conditions	Archive								
	ADE			AHU		IHRU		TT	
	W	S	W	W	S	W	S	S	W
I/O > 1	-	+	-	+	+	-	-/nd*	-	+
Visible fungal growth	-	-	-	+	+	-	-	-	-
Fungal Load ≥ 500 CFU/m3	-	-	-	-	-	-	-/nd*	-	-
Mixture of uncommon fungi ≥ 150 CFU/m3**	-	-	-	-	-	-	-	-	-
One uncommon fungi ≥ 50 CFU/m3	-	-	-	-	-	-	-	-	-
Presence of potentially pathogenic fungi	-	-	-	-	-	-	-	-	-
Presence of potentially toxinogenic fungi ≥ 12CFU/m3	-	+	+	-	-	-	-	-	-
Presence of potentially toxinogenic fungi	+	+	+	+	+	-	-/nd*	-	+

Assessed Conditions	Archive								
	ADE			AHU		IHRU		TT	
	W	S	W	W	S	W	S	S	W
Indoor contamination	+	+	+	+	+	+	+	+	+

W= winter; S= Summer * the culturing media made it impossible to perform the evaluation in all but three location in the second season; ** according to the 2006 legislation supported by the NT-SCE-O2, all but *Penicillium*, *Cladosporium* and *Alternaria* are considered uncommon.

Indoor/Outdoor ratio was above one in 21% of the samples. Applying the most recent legislation to the archives would mean a disregard for:

- the visible fungal growth observed in the AHU storage room which would only be considered relevant in the second sampling season, when the I/O ratio is over one.
- the presence of potentially toxinogenic fungi in 75% of the rooms where they existed in AHU, both seasons considered.
- the presence of potentially toxinogenic fungi in 83% of contamination cases in ADE, all three seasons considered.
- the presence of potentially toxinogenic fungi with concentrations higher than 12CFU/m^3 in the ADE's reading room, third season. Only after considering that the exterior third air sample taken in the ADE archive can, in fact, represent a much lower CFU count, would there be reason to discuss the presence of toxinogenic fungi in levels higher than accepted.
- the presence of toxinogenic fungi in the TT's working room, second season, while the reading room (the only one with an I/O ratio above one) requires no further comments in terms of air quality.

We have seen how quantitative guidelines can be followed as it is possible to establish a ratio between inside and outside levels of fungal load (Rao et al. 1996). Be-

tween indoor and outdoor environment it is also possible to establish a qualitative relation. According to Nevalainen (2007), the exterior air is one of the main sources of indoor fungi and, as such, some coincidental data are to be expected when comparing outside with inside results. When significantly different from the outside profile, indoor air quality may also mean a leakage issue or potential health problems (Kemp et al., 2002). In all places studied there were different inside genera/species which suggests indoor contamination (Kemp et al., 2002). Indoor contamination has been proven very common and is expected in locations where human activities take place.

The distribution of the fungal flora was analysed according to the definitions proposed by the Portuguese Air Quality standards (33 data entries were considered from Figure 4.19, *mycelia sterilia* was excluded) in 2006 and 2013.

Figure 4.20 refers to the 2006 Decree-Law:

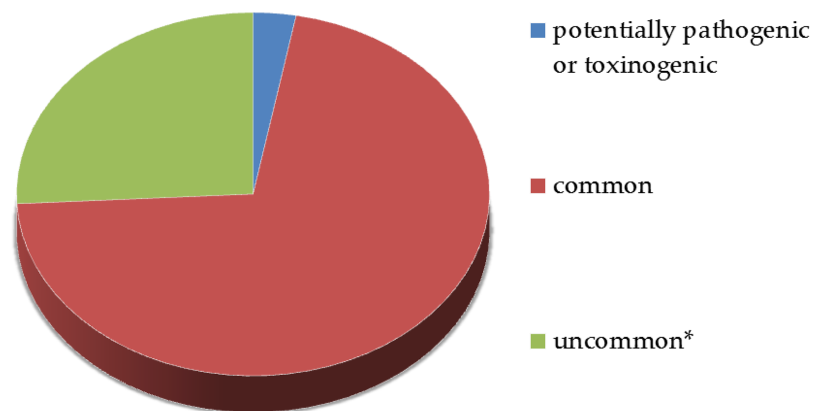


Figure 4.20 - Air sample fungal flora classified according to the class definitions proposed by the Decree-Law 78/2006 and 79/2006 supported by the NT-SCE-02.
*The uncommon include: all but Penicillium, Cladosporium Alternaria sp. plus considered potentially toxinogenic or pathogenic.

According to the 2006 legislation:

- 3% of the fungi found are considered potentially toxinogenic. No pathogenic fungi were found.

- The depicted uncommon species (all but *Penicillium, Alternaria and Cladosporium* + the potential toxinogenic) make up for 26% of the fungal diversity. In one of the locations, yeasts are present in higher than 50 CFU/m³.
- All of the considered common can be considered detrimental to paper heritage (see subchapter 4.2.2 for details).

The 2013 legislation separates the potentially toxinogenic from the potentially pathogenic, delimitates the uncommon fungi and alters the group of potentially toxinogenic fungi (Figure 4.21).

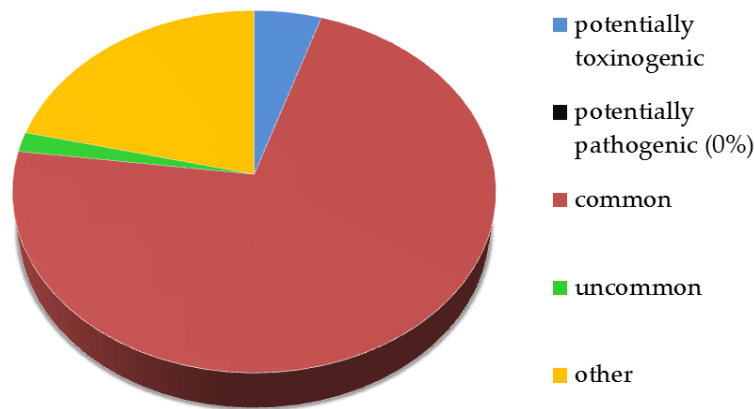


Figure 4.21 – Air sample fungal flora classified according to the class definitions proposed by the Ordinance 353-A/2013, December 4th.

According to the 2013 legislation (Figure 4.21):

- 5% are potentially toxinogenic (the *Fusarium* found in the TT's higienization room was not defined to the species levels and, as such, it was not considered as potentially toxinogenic in this case). *Trichoderma* sp. identified in the AHU archive (2nd season, April) was also not considered since this particular genus was not included by the NT-SCE-02 as potentially toxinogenic when the original study was performed and the identification was not pursued to the species level. Due to the widening of the toxino-

genic spectra (mainly in the *Aspergillus* group), this percentage is higher than the one considered using the 2006 values.

- 72% are considered common. In these, most are considered detrimental to paper conservation (*Penicillium sp., Aspergillus sp., Cladosporium sp., Alternaria sp.* and *Eurotium sp.)* (see sub-Chapter 4.2.2 for more details).
- About 2% are considered uncommon. None of these crosses the 50 CFU/m^3 mark.
- The remaining 21% are not considered relevant in terms of indoor air quality studies. One third of these is considered detrimental to paper heritage conservation and should be considered relevant if air quality standards are to be defined for such locations (see sub-Chapter 4.2.2 on surface samples for more detail).

As mentioned, and as far as conservation is concerned, no further remarks can be taken from the characterization of air samples since the existing guidelines are only quantitative. However, this characterization is extremely relevant since from the initial compilation performed by Zyska (1997), 19% of the total fungi identified could be a source of health problems (Mesquita et al., 2009). It is not clear in Zyska's review if the locations sampled include only storage rooms or rooms where people can normally be found but it is not strange to find people working in the storage areas and these are not usually considered relevant in indoor air quality studies. Also some of the fungi identified in the present study and recognizably involved in the deterioration of paper can produce mycotoxins and these may pose yet another health problem to the staff and visitors of library, galleries and museums (Mesquita et al., 2009).

In the consulted literature the most common genera found in air samples retrieved from archives were *Alternaria, Aspergillus, Chaetomium, Cladosporium, Fusarium, Geotrichum* and *Penicillium*. Almost all of these were also found in the air of the selected archives. Surprisingly, yeasts stood out in the present study as a frequent contaminant and more studies are needed to ascertain their true potential for biodeterioration. As-

sessing the results merely by the numbers has the disadvantage of neglecting the type of fungi present in each of these locations since some of these have a greater importance than others as far as biodeterioration is concerned. It is the case of *Trichoderma*, for instance, present in AHU's storage room 2 in the second season.

We have seen in Chapter 1 how the some of these fungi can deteriorate cellulose based materials when kept in contact under the right conditions. The fungi found in the air establish a circuit with the surrounding surfaces where they are deposited and from which they can, again, return to the aerosol. Surface samples are a great tool in understanding this interaction. Surface studies are also extremely relevant in complementing air samples since some of the fungi present – and this example occurred in one of the sampled archives – are only detected when they are deposited in the surfaces since they are very difficultly airborne.

4.2.2. Surface samples

As will be shown in the following lines, the spectrum of fungi found on the surfaces is (almost) always much wider than the one obtained with the air samples, which corroborates the need to perform both assessments concomitantly and also contradicts Harkawy (2011) and Zielińska-Jankiewicz et al. (2008) studies since their results point to a higher diversity in air samples (Chapter 2). It is important to mention that no dermatophytes (fungi that cause skin diseases in animals and humans) were found. This is true for all the surfaces sampled in all of the archives studied. Since there are no established limits for surface samples the results will be discussed per archive and considering both indoor air quality for human health and paper collection's conservation.

As highlighted in the methodology, Chapter 3, the use of traditional culturing methods gives important information. However, some of the surface samples tested did not presented any growth using culture media or this was unidentifiable because the developed mycelia was sterile and did not exhibited the morphological features on which traditional identification methods are based. The use of modern molecular biol-

ogy techniques based on PCR and presented in Chapter 3 made it possible, in some of the samples tested, to achieve identification. In others, DNA amplification was not obtained.

4.2.2.1 Surface samples from Arquivo Distrital de Évora

Figures 4.22 and 4.23 present the data from surface samples (tables, floor and archival document cases) in the ADE archive for the first of the three seasons. Presented (x-axis) in the charts (for all the archives) are the fungi identified in these surfaces and known to have caused damage to paper based documents/books as mentioned in the introduction section (*Alternaria* sp., *Aspergillus* sp., *Chaetomium* sp. , *Mucor* sp., *Myrothecium* sp., *Penicillium* sp., *Rhizopus* sp., *Stachybotrys* sp., *Trichoderma* sp., *Trichothecium* sp. and *Ulocladium* sp. (Caneva et al., 2003), *Cladosporium* sp., *Fusarium* sp. and *Paecilomyces* sp. (Pasquariello and Maggi, 2003) and *Eurotium* sp. (Michaelsen et al., 2006) and yeasts (Corte et al., 2003, Michaelsen et al., 2010a, Chapter III). The remaining fungi are grouped under "Others".

To further ease data comprehension, the archival document cases are presented separately for all of the archives. This way, and because these cases are the primary line of defence against accumulation of spores from the environment onto the documents, it is easier to identify potential damage inflictors.

Figure 4.22 presents the data from the first season, performed in the winter.

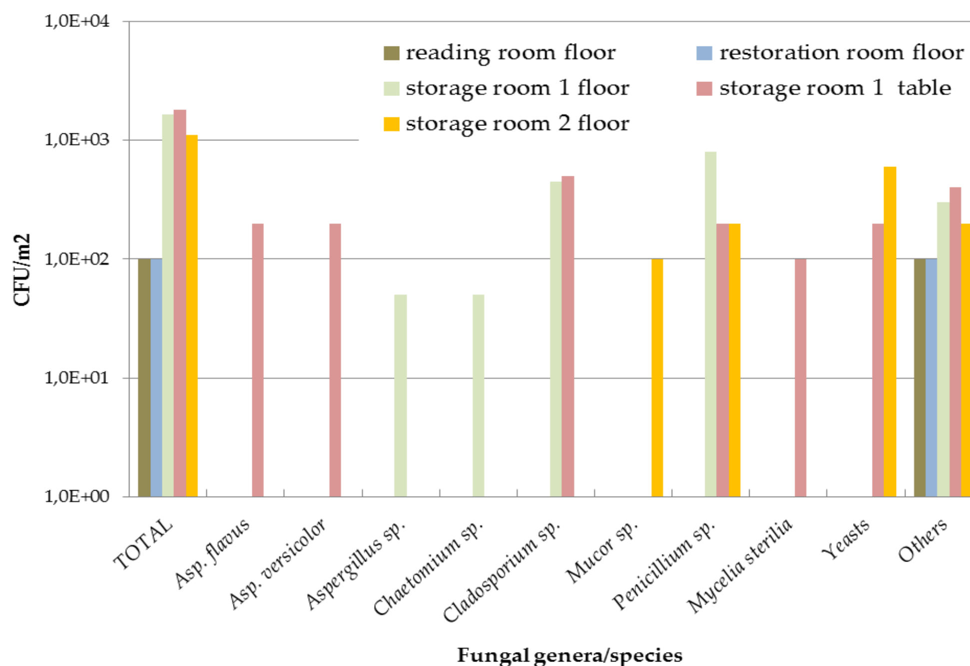


Figure 4.22 – Fungal load and diversity on surface samples in ADE 1st season, winter. No growth: reading room table, restoration room table. In the x-axis are the fungi associated with paper biodeterioration. Others include: *Beauveria* sp., *Chrysonilia* sp., *Chrysosporium* sp., *Graphium* sp., *Phaeoacremonium* sp., *Phialophora* sp., *Phoma* sp., *Scopulariosis* sp., *Scedosporium* sp. and *Scytalidium* sp. Please mind the logarithmic scale.

Total fungal load varied between 100 and 1800 CFU/m2 and the highest values were determined in the storage rooms (storage room 1: table and floor and storage room 2: floor). These three surfaces presented fungal counts higher than 1000 CFU/m^2. When comparison is made between total air and surface samples, the surface samples retrieved twice more species/genera and some of these of relevance: the already mentioned *A. niger* (in the document archival cases), *A. flavus* and *Chaetomium* sp., for instance. All of the fungi identified in the air samples were also present in the surface samples but, as seen, the reverse is not true.

Archival document cases were sampled from both storage rooms but only one (in storage room 2) returned a positive result. The fungal load obtained in this sample reached the 5500 CFU/m^2, a much higher value than the one obtained in the other surfaces samples from the same room. In ADE's particular case, and in the first (winter)

season, potentially toxinogenic fungi like *Aspergillus versicolor, flavus* and *niger* (Figure 4.22) were present.

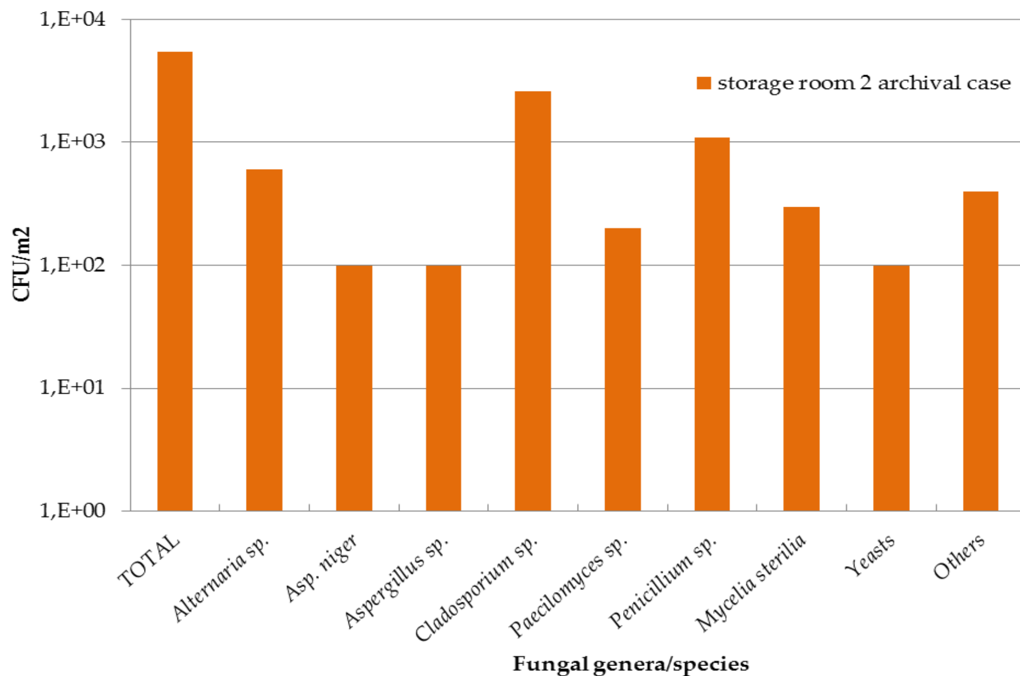


Figure 4.23 – Fungal load and diversity on the archival cases in ADE, 1st season, winter. No growth: storage room 1 archival case. In the x-axis are the fungi associated with paper bio-deterioration. Others include: *Arthrinium sp., Chrysosporium sp., Phoma sp. and Scytalidium sp.* Please mind the logarithmic scale.

The variety of fungi obtained in the document archival case was also much wider, with three fungal genera identified in the floor of the storage room vs 11 identified in the archival case.

Assuming that fungi identified in the pavement and other surfaces may also be present on documents (in case these were exposed), it´s necessary to alert to their bio-deterioration activity. Considering just the fungi present on the archival case and as presented in Table 2.7, amylase, cellulase, protease and pigment production are associated with *A. niger* and *Alternaria* sp. *Cladosporium* sp. can be associated with cellulase production and *Paecilomyces* sp. associated with lipase production. The *Penicillium* gen-

era is a very wide group (with 21 entries in Table 2.7) but, according to the consulted literature, only some of the species have been associated with identifiable metabolites.

One of the archival cases sampled in the first season returned a culture negative result but by using the DNA analysis it was possible to ascertain the presence of *Pithomyces sp.* Berk. & Broome. According to Menezes (2009), this is a cellulase producing fungus only identified once before in shelves by Maggi (2000).

The second document archival case sampled (this time in storage room 2) showed a positive culture but one of the colonies present was unidentifiable through classical methods (classified as *mycelia sterilia*). Using DNA analysis, it was possible to determine the presence of *Corynascus sepedonium* (C.W. Emmons) Arx (synonyms: *Thielavia sepedonium* and *Chaetomidium sepedonium*). *Thielavia* sp. was considered common by Zyska in the 1997 review but since then it has only been isolated once in a document sample (di Bonaventura et al., 2003).

The yeast colonies also present in this sample were attributed to *Cryptococcus albidus* (fragment length analysis: 350bp), a yeast not yet identified in document samples.

In summer, 2nd season, the variety widens as had happened with the air samples between the first and the second season.

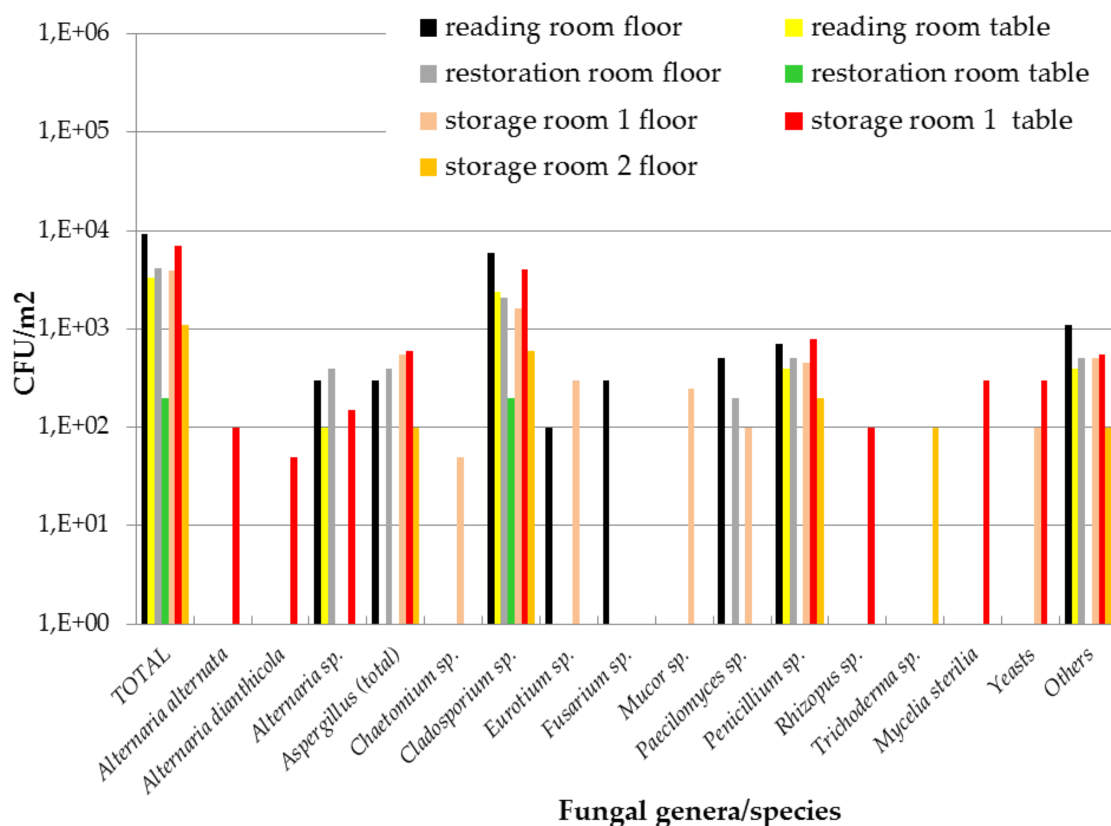


Figure 4.24 - Fungal load and diversity on ADE surface samples, 2nd season, summer. In the x-axis are the fungi associated with paper biodeterioration. All sampled areas showed positive growth. Others include: *Acremonium* sp., *Arthrinium* sp., *Aureobasidium*, *Beauveria*, *Botrytis*, *Chrysosporium* sp., *Emericella nidulans*, *Scopulariopsis* (fusca) and *Scytalidium*. The *Aspergillus* species identified are presented separately (next figure) to ease comprehension. Please mind the logarithmic scale.

The fungal load in the sampled surfaces does not reach the 10000 CFU/m^2 but it is higher than the obtained in the 1st season. The more affected rooms are the reading and storage room 1. As expected, the presence of *Cladosporium* sp. is noticeable as this was also the most frequently found fungi in the air samples. Storage room table presents again one of the highest values.

Fusarium spp. is considered potentially toxinogenic in the 2006 legislation and is present in the reading room. This particular fungus was not identified in the air samples. According to the 2013 Ordinance, *Trichoderma* sp. is not considered toxinogenic

unless it is *Trichoderma* viride and further studies were not taken with this sample since the most recent legislation had not been issued yet.

The *Aspergillus* species identified in the surface samples are presented in Figure 4.25.

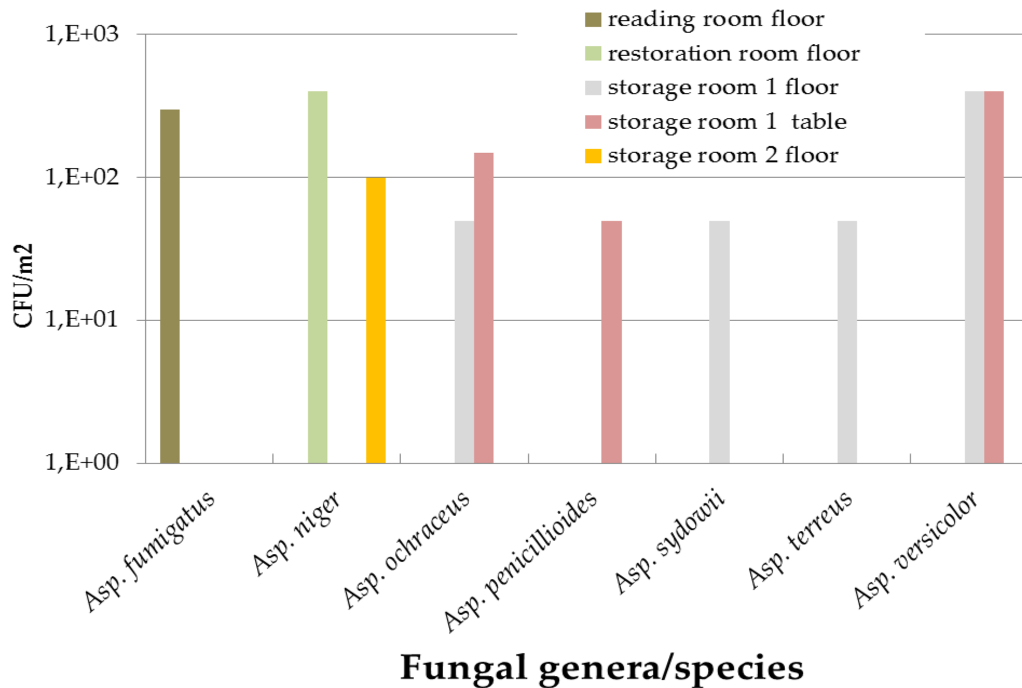


Figure 4.25 – Fungal load and diversity of *Aspergillus* species identified in the surfaces from ADE (2nd season, summer).

In the x-axis are the fungi associated with paper biodeterioration. Reading room table and restoration room table presented no *Aspergillus*. Please mind the logarithmic scale.

Considered potentially toxinogenic species of *Aspergillus* are present: *fumigatus, niger, versicolor, ochraceus* and *terreus*. These last two were not present in the air samples in this season nor in the surface samples in the previous season (winter). The storage room 1 presents the highest levels of contamination by *Aspergillus* species with 550 and 600 CFU/m^2 in the floor and table, respectively.

As before, the archival cases are presented separately (see Figure 4.26).

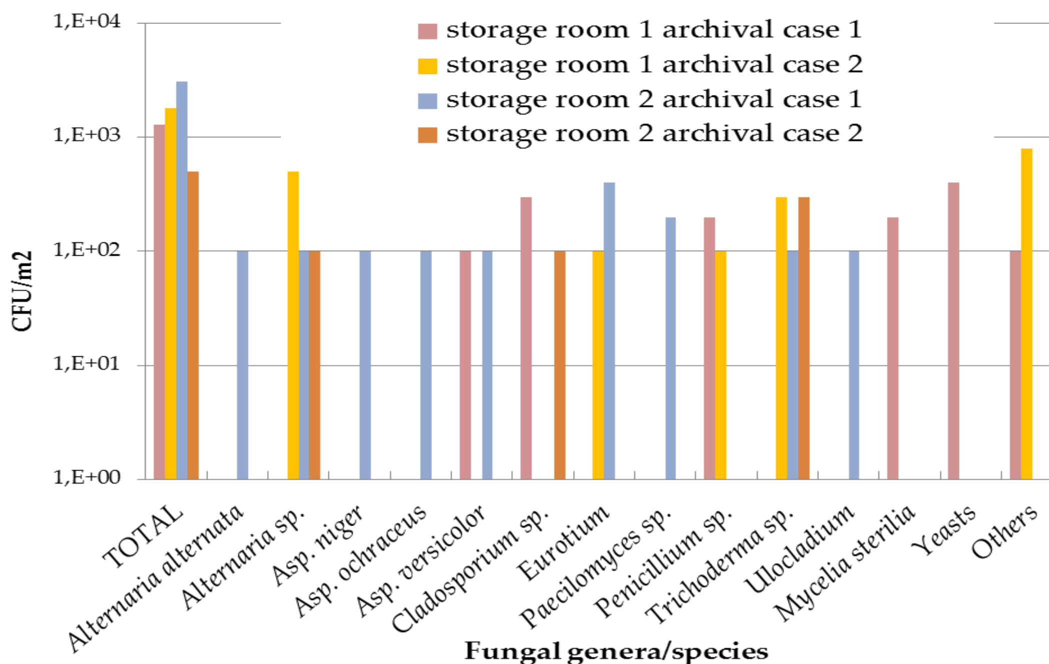


Figure 4.26 - Fungal load and diversity on Archival cases from ADE, second season, summer.

In the x-axis are the fungi associated with paper biodeterioration. All sampled areas showed positive growth. **Others include:** ***Acremonium, Botrytis, Chrysosporium*** **sp.** ***and Scopulariopsis (fusca).*** Please mind the logarithmic scale.

The document archival cases present a lower fungal load than the remaining storage room areas (Figure 4.24 and 4.25). *Trichoderma* sp., which had been present only the storage room 2 is now identifiable in both storage room's archival cases.

In archival cases and consulting again the present species/genera in Table 2.7 it is possible to conclude that the majority of these fungi produce at least protease, amylase or cellulase and some of these can excrete pigmented metabolites (*Alternaria* sp. - depending on the species - and *Asp. versicolor*).

Only 29% of the fungi encountered in the surface samples were also present in the air samples. The following fungi were found on both inside air and surface samples: *Alternaria alternata, A. fumigatus, A. niger, A. versicolor, Chrysosporium* sp., *Cladosporium* sp., *Eurotium* sp., *Paecilomyces* sp. and *Penicillium* sp.In the second season four archival cases were sampled. Two of them required no molecular biology protocol

since the cultures were positive, there was no *mycelia sterilia* and there were no yeasts in the sample.

Mycelia sterilia was only found on the 1st archival cases of both rooms. In storage room 1, this unknown contaminant was identified using DNA analysis and returned the presence of *Humicola grisea*. This fungus has already been identified in the air of archives and librairies (Bueno et al.,2003; Ruga et al.,2008) is mentioned as common in altered document samples in the review by Valentin et al. (2003) but has not been identified since then in paper samples.

The *mycelia sterilia* present in the other document archival case corresponded to *Chalastospora gossypii* (synonym: *Cladosporium porophorum, Alternaria malorum*). Since the genomic sequence returned more than one species, the identification was based also in the morphological features of the culture. This is the first occurrence of this fungus in document samples.

Yeasts were found on one of the archival cases (storage room 1, document cover 1). Identification through D2 sequencing was performed but the score obtained was low and the fragment length analysis performed returned just one peak which was not yet in our database. Further studies were pursued with D2 sequencing and DHPLC was used in an attempt to separate a possible mixture. DHPLC did, in fact, returned the presence of two elements but, despite all efforts, it was not possible to achieve a species level. The yeasts sequenced after DHPLC collection (Figure 4.27) returned the presence of *Exophiala sp.* and *Cryptococcus sp.*

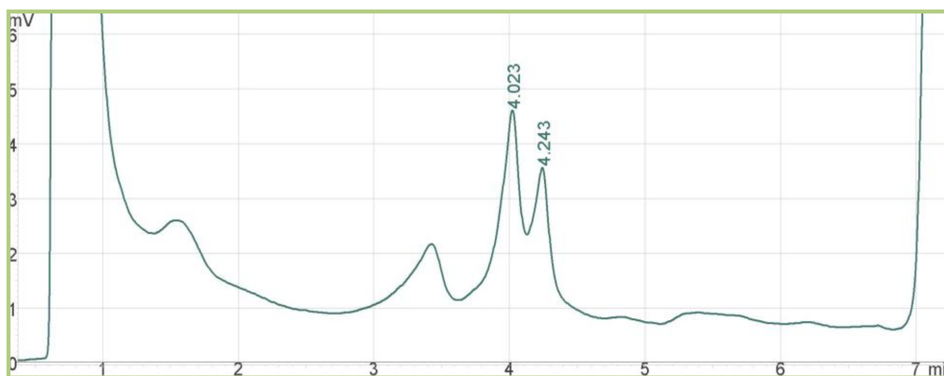


Figure 4.27 - DHPLC chromatogram of a mixture of two yeasts. The run was performed at 61°C/55%B at a 0.9ml/min rate flow. The first peak, collected at minute 4 at 63°C corresponds to *Cryptococcus sp.* and the second peak, collected at minute 4.2 corresponds to *Exophiala sp.*

In the third season (winter) the variety of fungi is even greater. Since the biological contaminants of particulate matter include fungi (Leese et al, 1997), the construction work taking place during this last sampling season may have played an important role on these results (Figure 4.27).

Thirty eight different fungal elements are now identifiable in this archive. The lowest contamination level (1500 CFU/m^2) is verified in reading room and the highest in storage room 1 (18800 CFU/m^2). The control sample air taken at this archive returned the sole presence of *Rhizomucor oryzae*, fast growing fungi that can hamper the normal development of other contaminant fungi. Therefore, contamination by other species probably exists but is not as easily assessed.

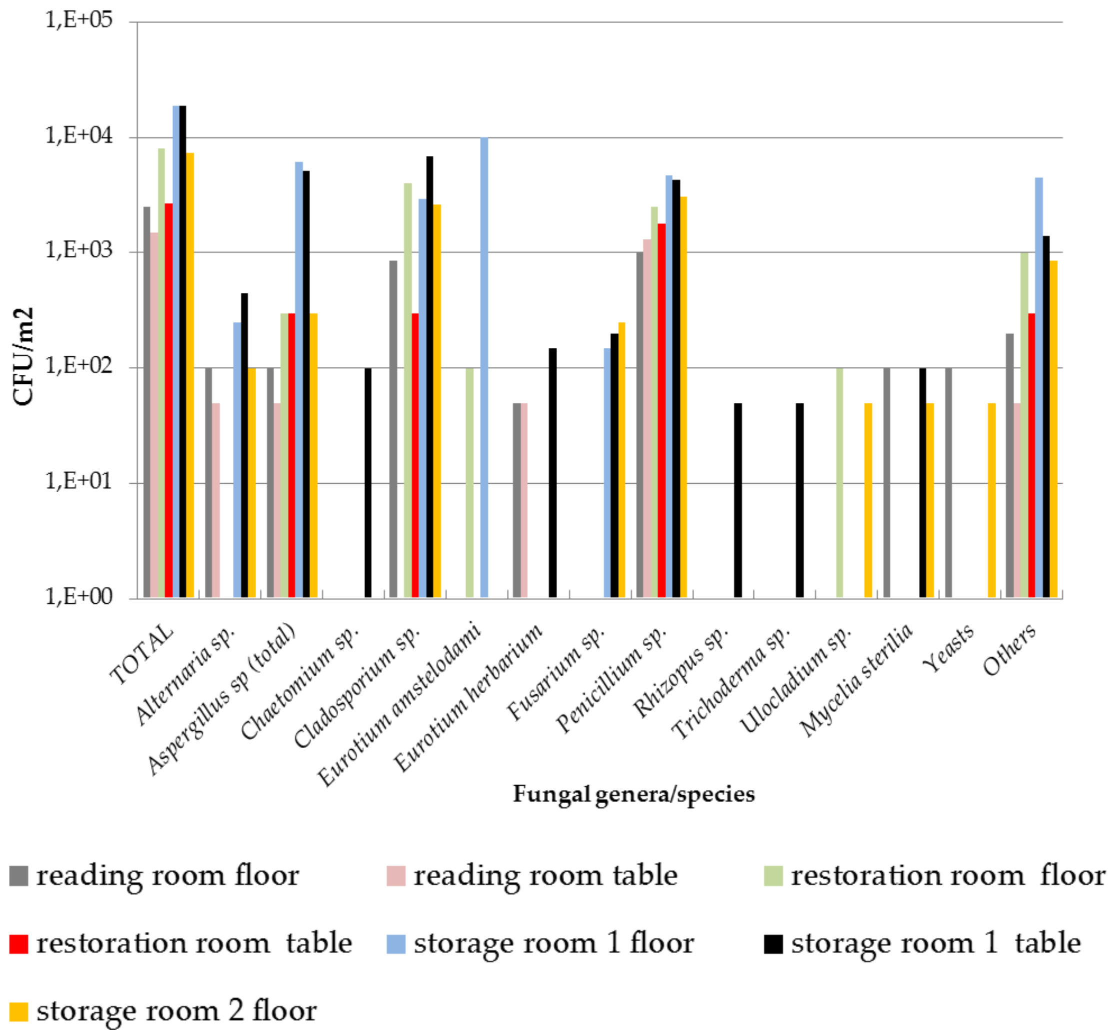


Figure 4.28 – Fungal load and diversity on ADE's surface samples, 3rd season, winter. In the x-axis are the fungi associated with paper biodeterioration. Sampling was performed during construction work. All sampled areas showed positive growth. Others include: *Aureobasidium sp., Beauveria sp., Botrytis sp., Chrysonilia sp., Chrysosporium sp., Emericella nidulans, Emericella sp., Epicoccum purpurascens, Exophialia sp., Geomyces pannorum, Gliocladium sp., Hormographiella, Phoma glomerulata, Rhizomucor sp., Scedosporium sp., Scopulariosis fusca and Scopulariosis sp*. The *Aspergillus* species identified are presented separately to ease comprehension. Please mind the logarithmic scale.

Indoor air samples (see Figure 4.8) taken at this same season show only a spectrum of 10 identifiable fungi. In the potentially toxinogenic fungi, the presence of *Aspergillus flavus, niger, ochraceus* and *terreus* is worth noting since the indoor air samples

taken during this season have only returned the presence of *Asp. versicolor*. *Fusarium* sp., present in the storage rooms, exhibits the same pattern.

The *Aspergillus* species identified in ADE's surface samples, 3rd season, winter, are presented separately to ease comprehension (Fig. 4.29).

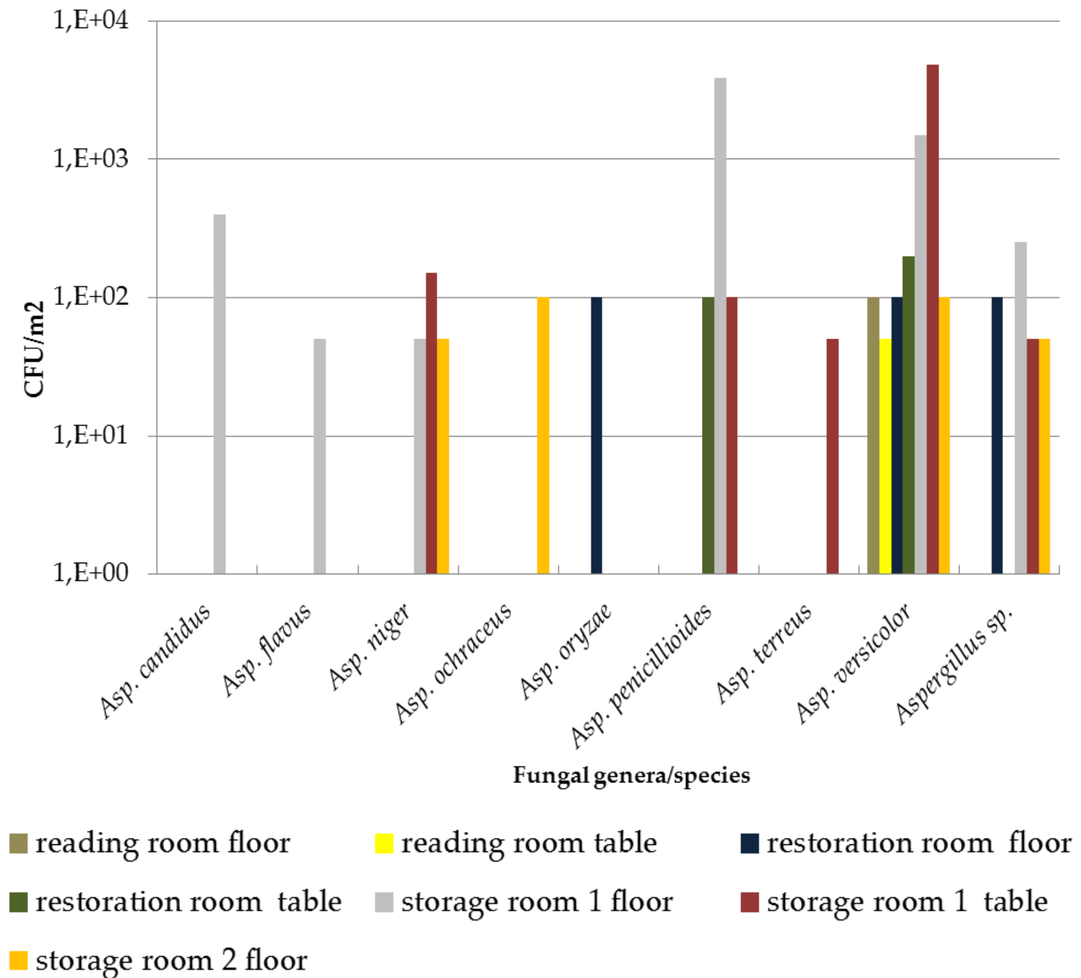


Figure 4.29 - Fungal load and diversity on the *Aspergillus* species identified in ADE's surface samples, 3rd season, winter. Sampling was performed during construction work. Please mind the logarithmic scale.

Again, *Aspergillus* species are specially represented in the storage room 1. On archival cases we can now find 33 different genera/species of fungi. In previous seasons the maximum number was 15. Again, Table 2.7 can tell us to expect potential biodeterioration activity from the presence of most of these fungi.

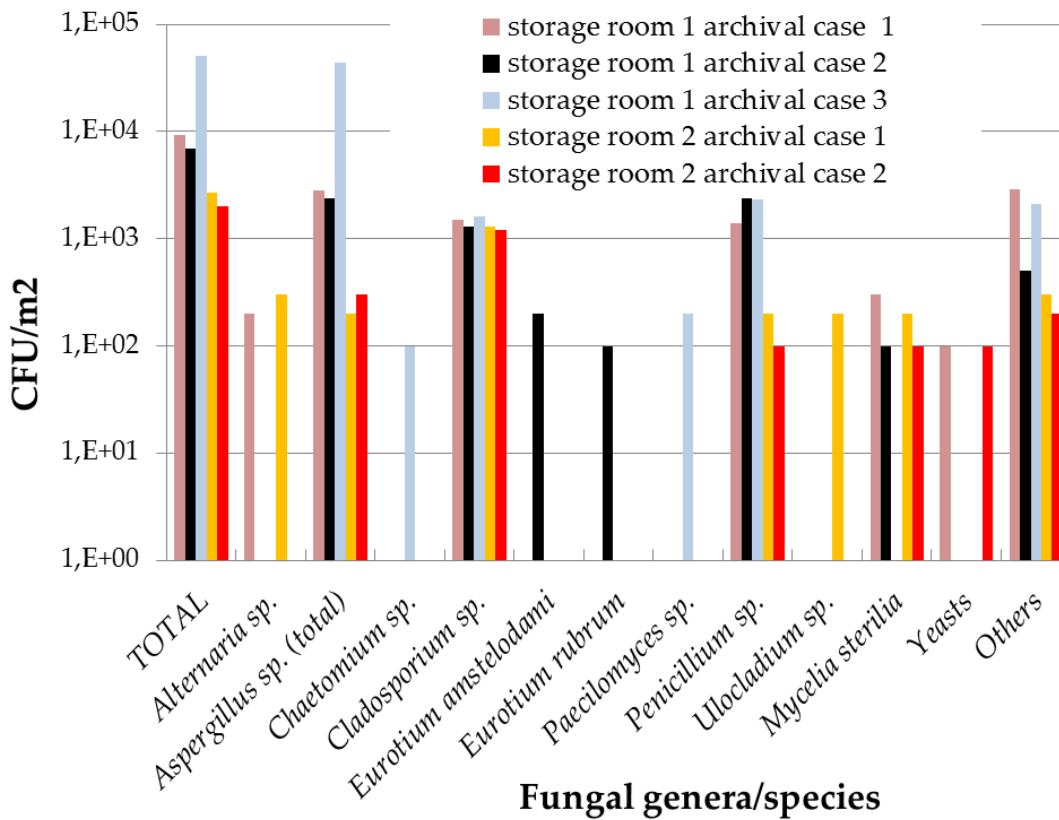


Figure 4.30 – Fungal load and diversity on ADE's Archival cases, 3rd season, winter. In the x-axis are the fungi associated with paper biodeterioration. All sampled areas showed positive growth. Please mind the logarithmic scale. Others include: *Beauveria, Chrysonilia, Emericella* sp., *Epicoccum purpurascens, Exophialia, Geomyces pannorum, Hormographiella, Scopulariosis* (fusca). Please mind the logarithmic scale.

Storage room 1 document archival cases presented the highest level of contamination, reaching the 50600 CFU/m^2. As mentioned, the other surfaces in the same storage room presented half this concentration while in other seasons the maximum obtained was 1800 CFU/m^2.

The *Aspergillus* found in the archival cases are also more varied than in the previous seasons, as shown in Figure 4.31.

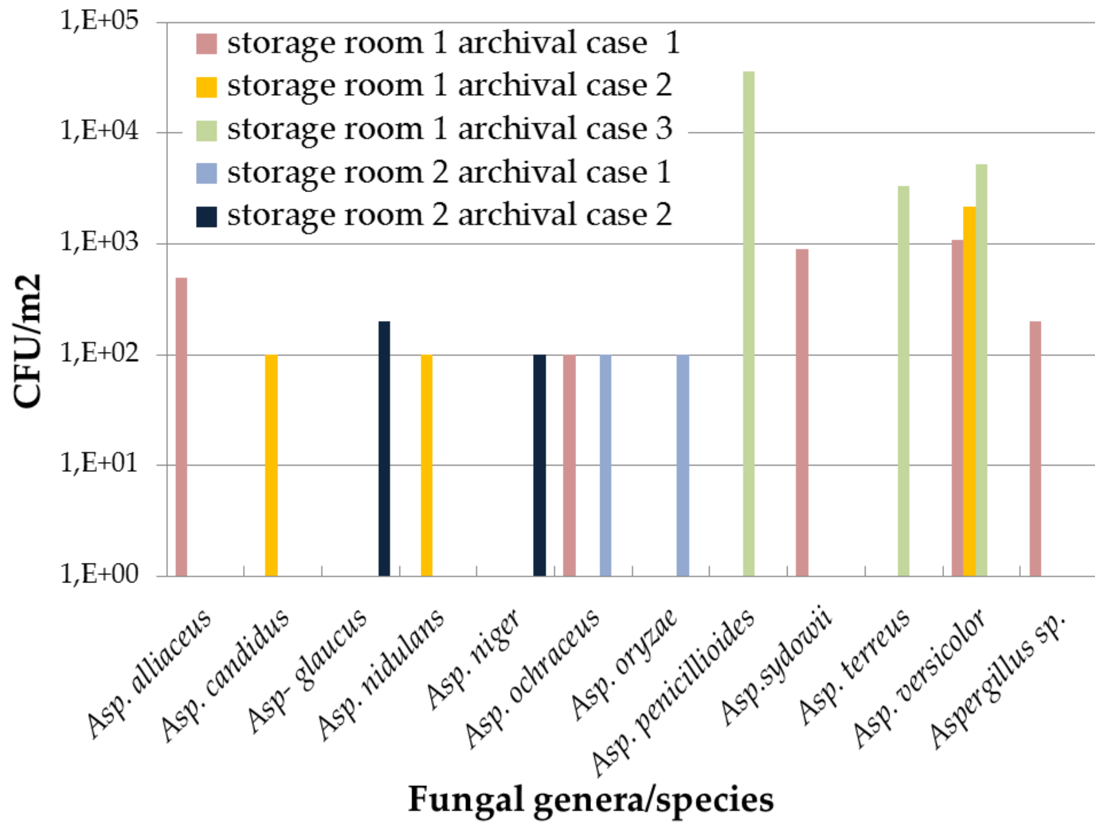


Figure 4.31 - Fungal load and diversity of the *Aspergillus* species identified in ADE's archival cases in the 3rd season, winter. In the x-axis are the fungi associated with paper biodeterioration. Sampling was performed during construction work. Please mind the logarithmic scale.

Aspergillus niger, ochraceus, terreus and *versicolor* are considered potentially toxinogenic. *Aspergillus penicillioides* crosses the 1000 CFU/m2 in the only archival case where it was identified and this archival case also presents the highest level of contamination. This fungus was also found in high concentrations in the floor from this same storage room but its levels were much lower in the previous season and inexistent in the first season.

Between the first (winter) and the second season (summer), storage room 1 contamination levels doubled but between the first and the last season they increased 10x in fungal load. Storage room 2 has maintained its surface fungal load from season 1 to season 2 but the fungal CFU/m^2 increased six times between the last and the previous

seasons. The fact that this room is located further from the place where construction work was taking place explains this difference.

Valentin (2007) compared cellulose objects with other surfaces in archives, suggesting a different behaviour in terms of contamination and time needed to adjust to new storage conditions. In this study, the relationship between the surfaces in storage and non-storage areas was evaluated in terms of fungal contamination by potential biodeterioration fungi. The results for the ADE archive, all seasons considered, are shown in Figure 4.32.

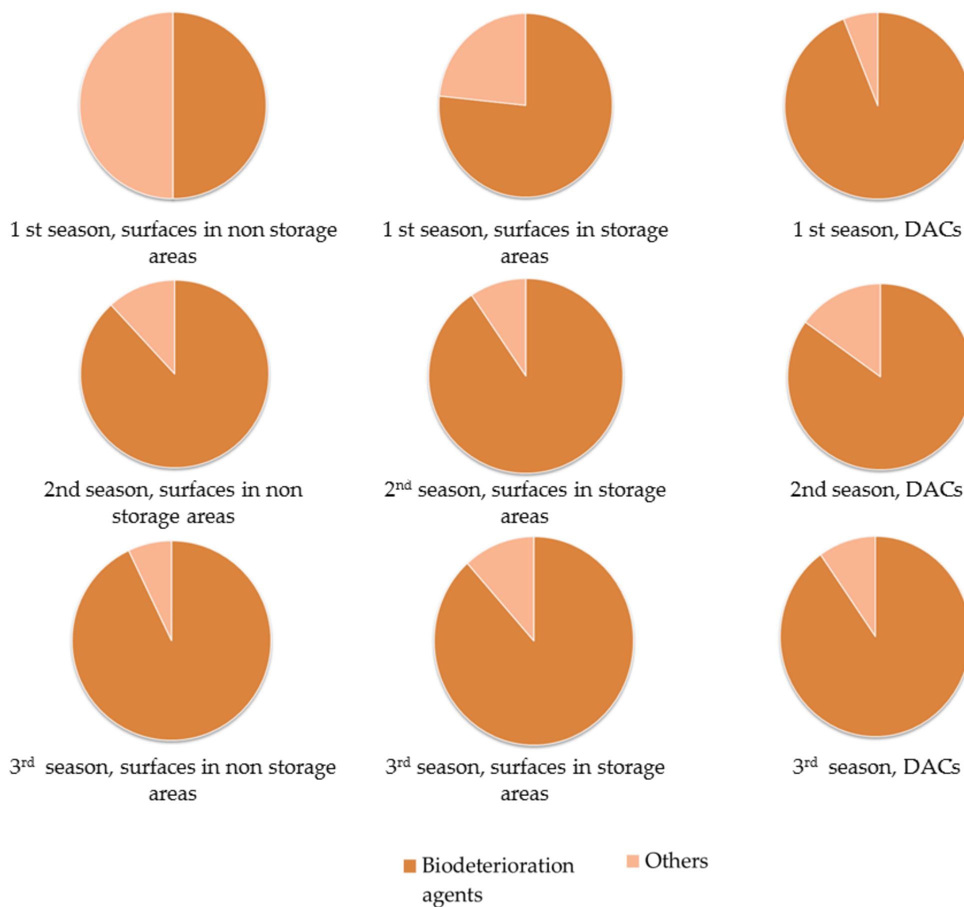


Figure 4.32 - Percentage of the fungal population associated with paper degradation in the ADE archives for all three seasons.

Fungi that are considered biodeterioration agents comprise most of the fungal communities present at the surfaces of the ADE archive. In the first season the storage areas seem to be more contaminated by potential biodeterioration agents but the fol-

lowing two seasons don't show the same trend. In the last season the values might have been influenced by the construction work since the fungal flora showed great variation and all the rooms were influenced both in terms of fungal load and introduced fungal flora.

In the last season, five archival cases were sampled and four of them were submitted to DNA analysis. Two of them showed the presence of both *mycelia sterilia* and yeasts (storage room 1 DAC 1 and storage room 2 DAC 2) but the *mycelia sterilia* was not possible to reisolate and identify in any of these mixed samples. Only the yeasts were submitted to further testing in one of the samples and these were identified as *Ustilago sp.* Despite attempts made with the DHPLC (to *clean* the sample) it was not possible to confidently achieve a species level. According to the consulted bibliography, there is no previous record of *Ustilago* genera (or species) in document samples. The other yeast sample was sequenced and returned the presence of *Aureobasidium pullulans*.

*Gymnascell*a sp. and *Nectria* were the fungus identified by molecular biology protocols in the remaining two *mycelia sterilia* samples. In both cases, visual identification and comparison with the BLAST results was vital to ascertain the genera of the fungal contamination.

4.2.2.2 Arquivo Histórico Ultramarino Surface Samples

In the AHU two sampling seasons were performed. Figure 4.33 shows the surface samples for the first season:

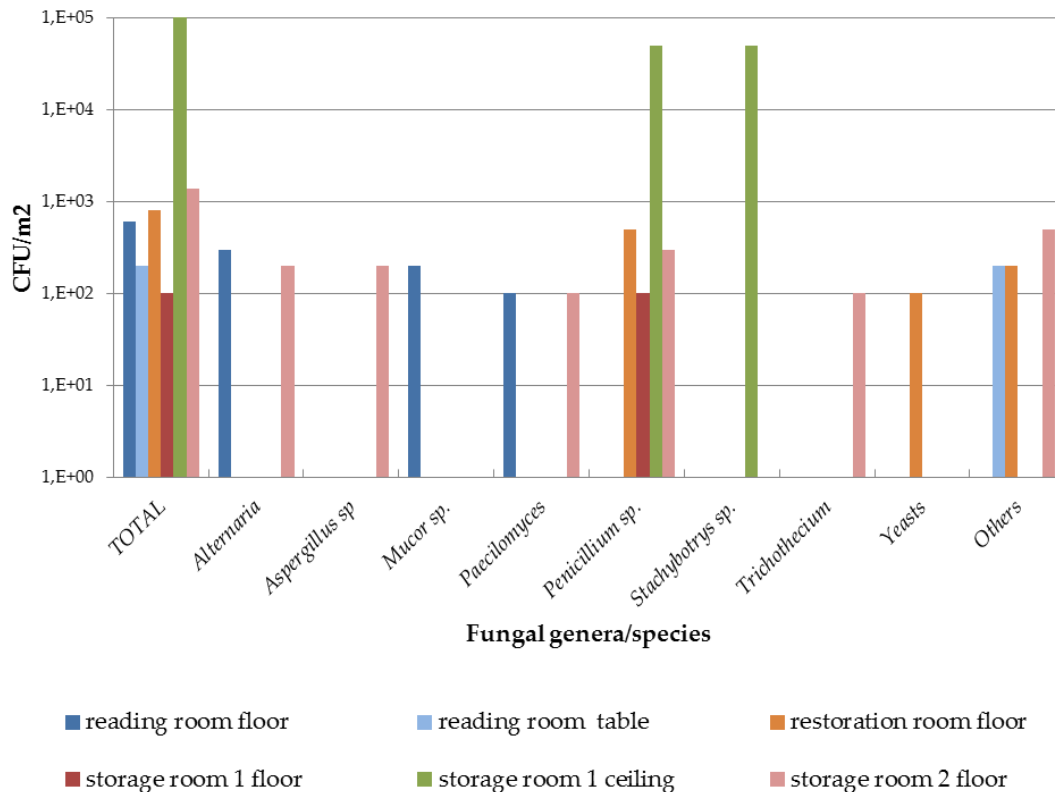


Figure 4.33 – Fungal load and diversity on AHU Surfaces, 1st season, winter.
In the x-axis are the fungi associated with paper biodeterioration. Others include: *Aureobasidium, Cladophialophora, Curvularia, Graphium sp., Nigrospora, Rhodotorula and Scopulariopsis.* Please mind the logarithmic scale.

Total fungal load varied between 100 CFU/m2 (storage room floor) and 100000 CFU/m2 in the storage room ceiling. Both *Stachybotrys* sp. and *Penicillium* sp. are indicators of bad indoor air quality. Stachybotrys sp., a known mycotoxin producer and inducer of health problems, was not present in the air sample taken from the same location which reinforces the introduction of surface samples in air quality studies.

Encountered both on surface and air samples: *Alternaria, Aspergillus sp., Mucor, Paecilomyces, Penicillium, Rhodotorula,* and *Scopulariopsis* (44%of the number of fungal genera/species encountered in the air samples). Contrarily to recent results, there was a wider diversity in air samples. No archival cases were analysed at this first season.

In the second season, the variety is again higher in the surface samples. The archival cases analysed in the storage rooms are presented in Figures 4.35 and 4.36.

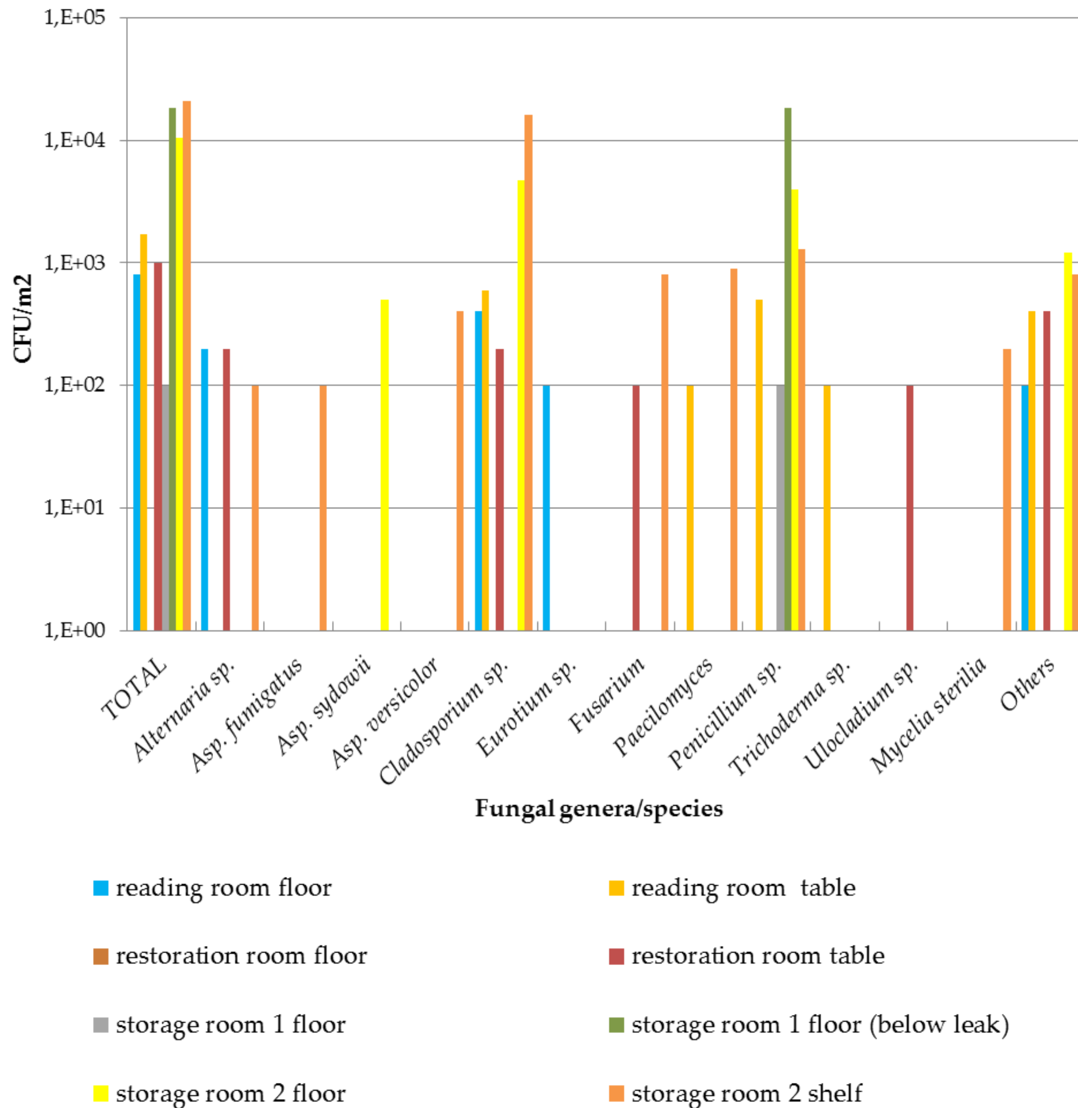


Figure 4.34 – Fungal load and diversity on AHU's surface samples, 2nd season, summer.

In the x-axis are the fungi associated with paper biodeterioration. No growth: restoration room floor. Others include: *Acremonium, Aureobasidium, Beauveria, Botrytis, Phialemonium, Ramichloridium, Scedosporium, Scopulariopsis* and *Scytalidium.* Please mind the logarithmic scale.

Storage room 2 shows no *Aspergillus* sp. in the air sample taken at this time but the surface samples show the presence of potentially toxinogenic *Asp. fumigatus* and *A. versicolor.*

The fungal contamination on the document archival cases sampled in storage room 2 is presented in Figure 4.35.

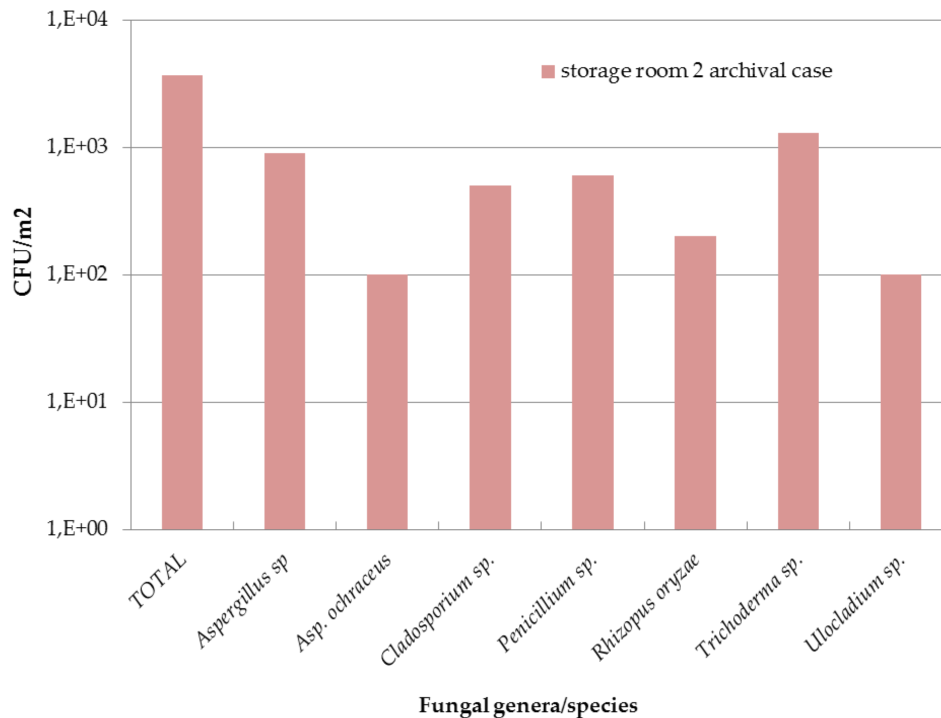


Figure 4.35 – Fungal load and diversity on AHU's archival case from storage room 2. Please mind the logarithmic scale.

Especially relevant in terms of conservation, *Trichoderma* sp., *Ulocladium* sp. and *Rhizopus arrhizus* were not identified in other surfaces. *Rhizopus arrhizus* and *Trichoderma* sp. were also not present in the air sample taken from this location.

Storage room 1 floor fungal flora is entirely comprised of *Penicillium* sp., a fact that could go unnoticed with just the air samples since the air sample taken from this location reveals a normal and varied fungal flora. If the visible fungal growth had not been so easily spotted this fact could be an indicator of a potential problem in this room. The presence of this fungus is also noteworthy in the archival cases in this room (Figure 4.36).

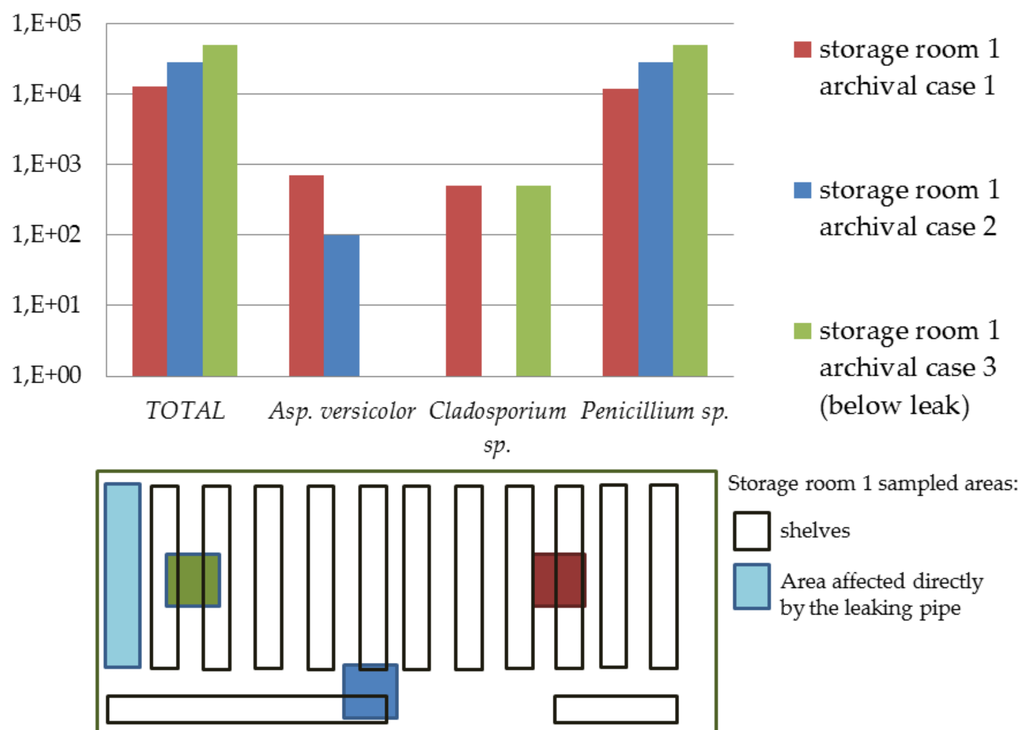


Figure 4.36 – Fungal load and diversity on the archival cases sampled from storage 1. Below is a depiction of the location where they were kept in relation to the water affected area. Please mind the logarithmic scale.

The main indicator of potential problems – *Penicillium* sp - suffers a gradual increase as the sampled area approaches the area affected by the leaking pipe. *Cladosporium sp*. population remains virtually the same but Asp. *versicolor* seems to give way to the *Penicillium* proliferation near the affected area.

The fungi identified on archival cases from both storage rooms (*Asp. ochraceus, Asp. versicolor, Asp. sp., Cladosporium, Penicillium, Rhizopus, Trichoderma and Ulocladium*) are all part of the list considered to encompass fungi already proven to cause paper biodeterioration.

In terms of the fungal elements found in all of the sampled surfaces the presence of potential biodeterioration agents was found to be:

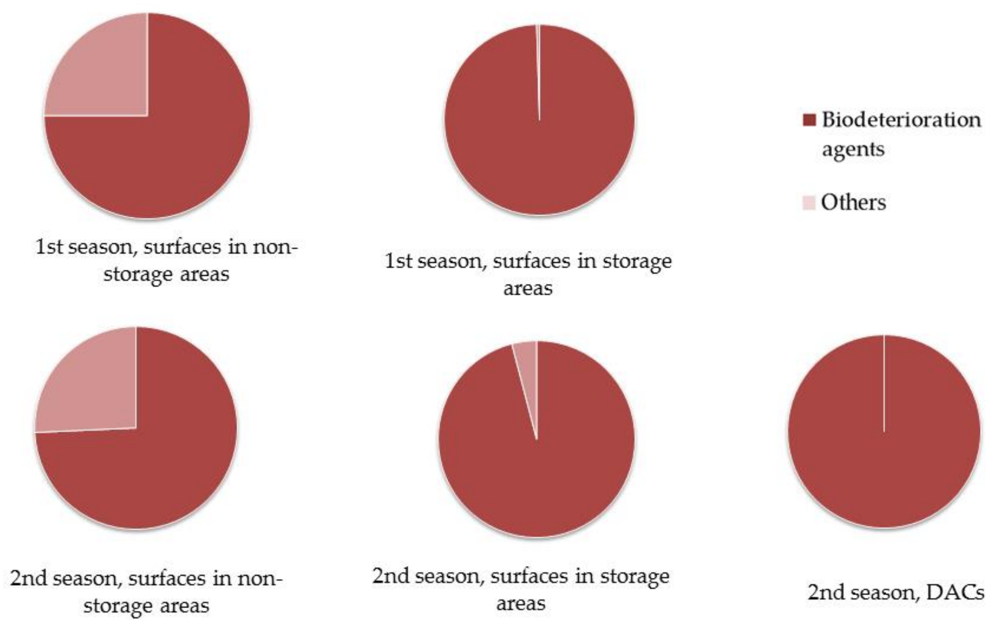


Figure 4.37 - Fungal population distribution in all surfaces tested in AHU for both considered seasons.

A tendency is again noticed for the accumulation of certain genera/species of fungi on archival cases and storage inorganic areas. Possible bias would be the leaking pipe in storage room 1 which, by inducing the proliferation of fungi such as *Penicillium*, a potential biodeterioration agent, could be impairing the results. When analysing just the storage room 2, with no leaking pipe problem, the results are as presented next:

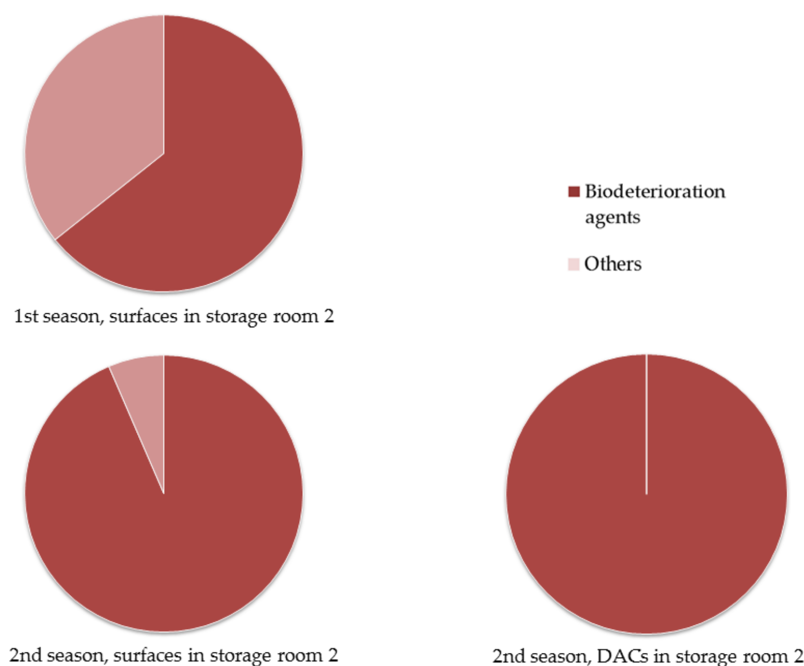


Figure 4.38 – Storage room 2 distribution of fungal types according to their biodeterioration potential.

The document archival cases in storage room 2, unaffected by bias, are 100% contaminated by potential biodeterioration agents. The remaining surfaces do show a higher contamination by this group of fungi in season 2 but not in season 1.

Storage room 1 suffered from a high humidity level caused by the leaking pipe over some of the storage areas (see Figure 4.36) and *Penicillium sp.* was the only fungus found on surface areas other than archival cases. On these two other potentially damaging fungi were identified.

The archival cases analysed in the second season did not return any *mycelia sterilia* or demanded any species identification. Therefore, no molecular biology protocols were applied to these samples.

4.2.2.3 Instituto de Habitação and Reabilitação Urbana (IHRU) Surface Samples

Surface samples were executed for both seasons in all rooms where air samples were also performed. Figure 4.39 presents the results for the first season (winter):

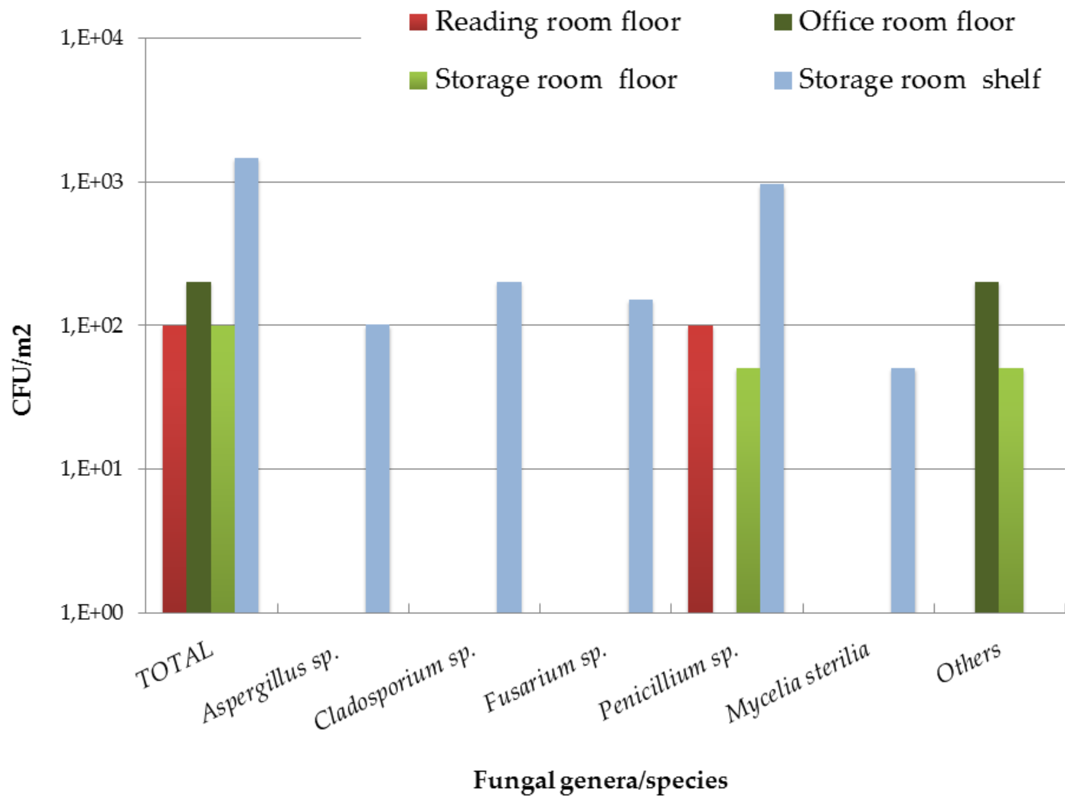


Figure 4.39 - Fungal load and diversity on IHRU's surface samples, 1st season, winter. In the x-axis are the fungi associated with paper biodeterioration. Negative growth: Working room table, Storage room document cover 2 and Restoration room floor and shelf. Others include: *Arthrinium* sp., *Chrysonilia* sp. and *Phoma* sp. Please mind the logarithmic scale.

Levels of fungal load varied between 100 and 1450 CFU/m2 in the storage room shelf. This higher fungal load coincides with a higher variety of species, of which *Fusarium* sp. is especially noteworthy. Only *Penicillium* is present both in the air and sample surfaces taken in this room.

In the restoration room, the *Penicillium* sp. found as frequent in the air samples did not mirror its presence in the surface samples taken since both returned a negative culture.

Figure 4.40 - Fungal load and diversity on IHRU's document archival cases, 1st season, winter.

In the x-axis are the fungi associated with paper biodeterioration. Negative growth: Storage room archival case 2. **Others include: *Phialemonium* sp. Please mind the logarithmic scale.**

Two document archival cases were sampled in the storage room but only one of them returned a positive culture (Figure 4.40). The *Aspergillus* species found, *Asp. flavus*, is considered potentially toxinogenic and was not identified in other surface samples or air samples from the same room.

In term of fungal load, the level of contamination is quite similar between the document case and the other sampled surfaces.

The fungal flora present in one of the two archival cases analysed in season 1 was fully identified with traditional culturing methods but the second one did not return any colony and the DNA analysis also revealed a negative result

The second season (summer) showed a wider variety of fungi as depicted in Figure 4.41. *Aspergillus* species are detailed in figure 4.42 to ease data comprehension.

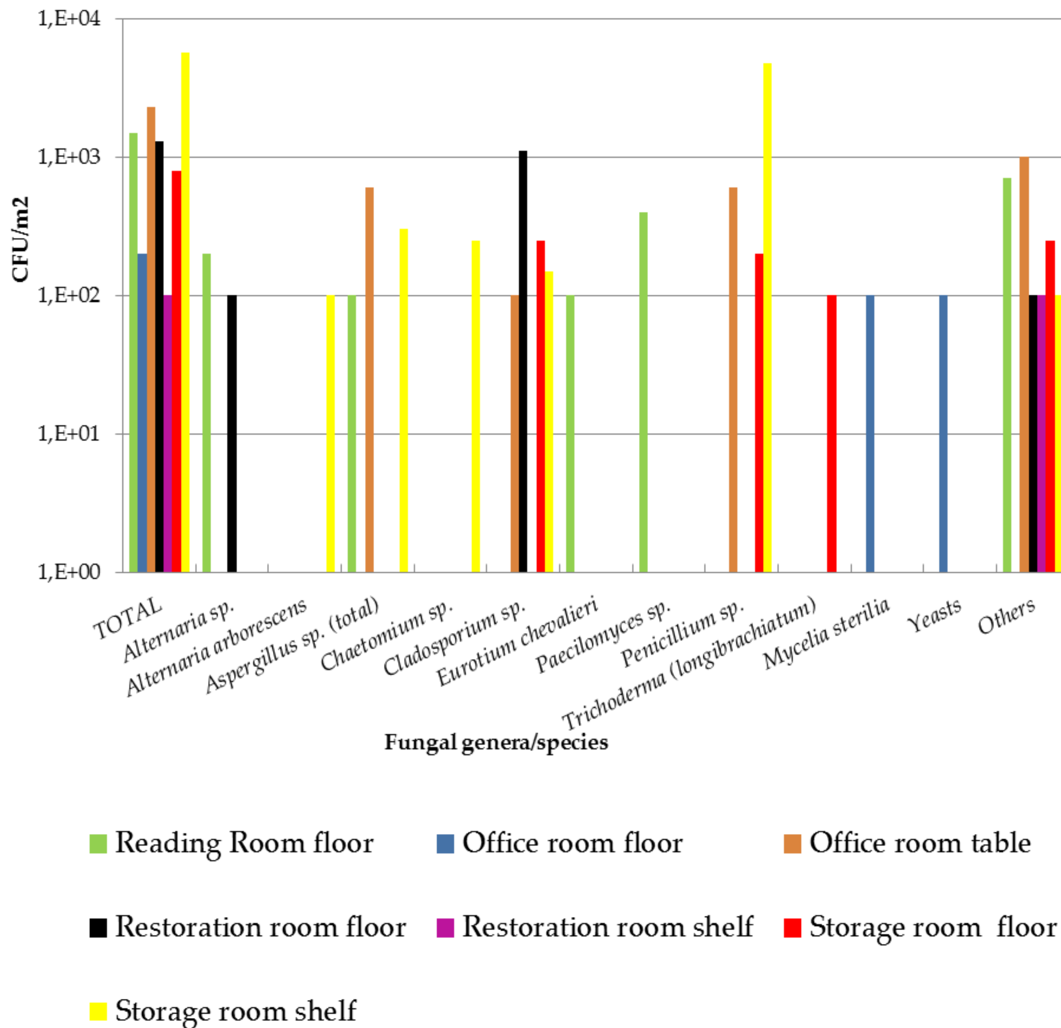


Figure 4.41 - Fungal load and diversity on IHRU's surface samples, 2nd season, summer.

All cultures positive. In the x-axis are the fungi associated with paper biodeterioration. Others include: *Arthrinium* sp., *Beauveria* sp., *Chrysonilia (sitophila)*, *Chrysosporium* sp., *Exophialia* sp., *Phoma* sp., *Scedosporium* sp., *Scytalidium* sp. and *Stemphylium* sp. Please mind the logarithmic scale.

The fungal load was now between 100 and 5750 CFU/m2. The air sample retrieved from the reading room showed the presence of *Cladosporium* sp., *Hormographiel-*

la aspergillata and *Penicillium* sp., a total dissonant display when compared to the surface sample taken from the same location. Again, this case clearly illustrates the benefits of performing both analyses.

From the *Aspergillus* sp. analysis, *A. versicolor* and A. *ochraceus* are considered potentially toxinogenic (Figure 4.42).

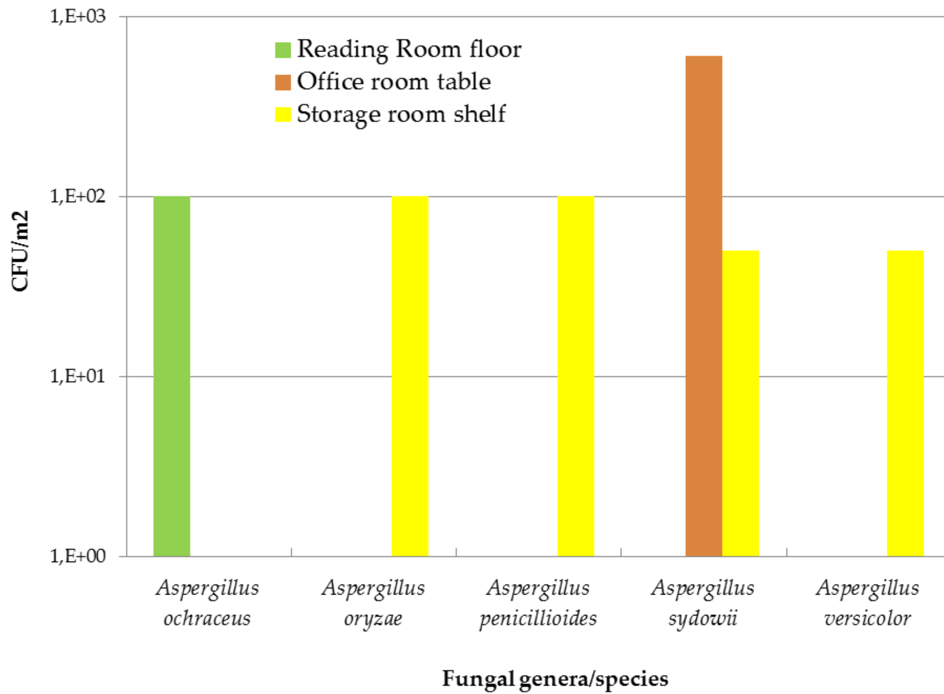


Figure 4.42 - Fungal load and diversity on IHRU's surface samples, 2nd season.
In the x-axis are the fungi associated with paper biodeterioration. No *Aspergillus* was present in the office room floor, restoration room floor, restoration room shelf and storage room floor. Please mind the logarithmic scale.

As had happened before, only one of the document archival cases returned a positive result in the second season (Figure 4.43).

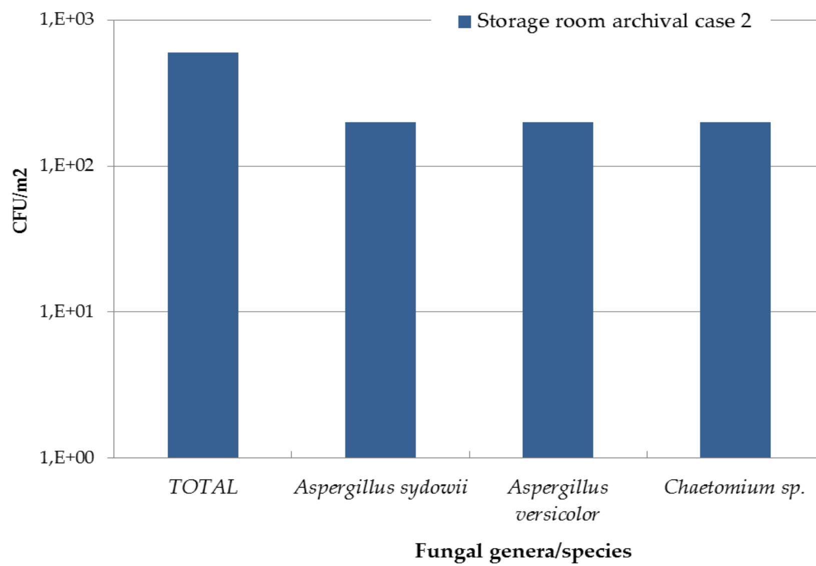


Figure 4.43 – Fungal load and diversity on IHRU's archival case, storage room 2, 2nd season, summer. Negative culture: archival document case 1. Please mind the logarithmic scale.

In terms of the species found in all of the sampled surfaces (both seasons considered), the presence of potential biodeterioration agents was found to be:

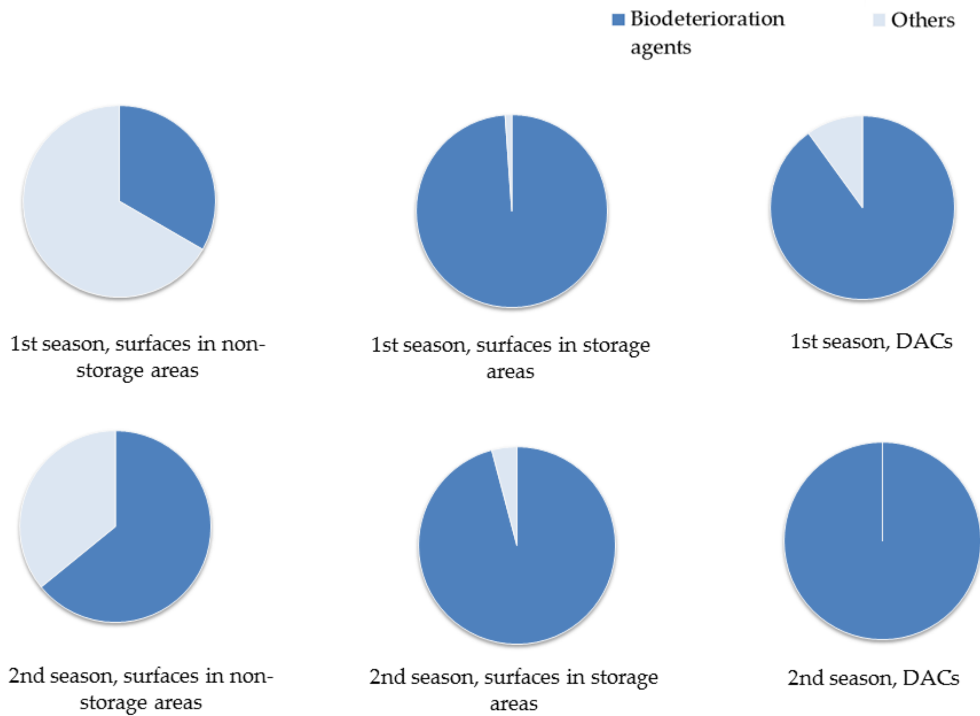


Figure 4.44 - Fungal population distribution in all surfaces tested in IHRU for both considered seasons.

As was seen in the AHU archive, the surfaces sampled in non-storage areas seem to present a lower level of contamination by potential biodeterioration agents than the surfaces from locations where cellulose based materials are kept or present. In the storage areas cellulose or inorganic materials present similar levels of contamination.

As has happened in the first season, classical culturing methods were sufficient to determine the fungal flora in one of the analysed archival cases and the negative culture obtained for the second case was not followed by a positive DNA analysis.

4.2.2.4 Torre do Tombo (TT) Surface Samples

Seven different genera of moulds were identified in the air samples for the first season (summer). Yeasts were also identified in the air samples. No room presented potentially toxinogenic fungi and all the counts were lower than the measurement performed outside.

In the TT archive and for the first season the following surface sample results were obtained (Figure 4.45).

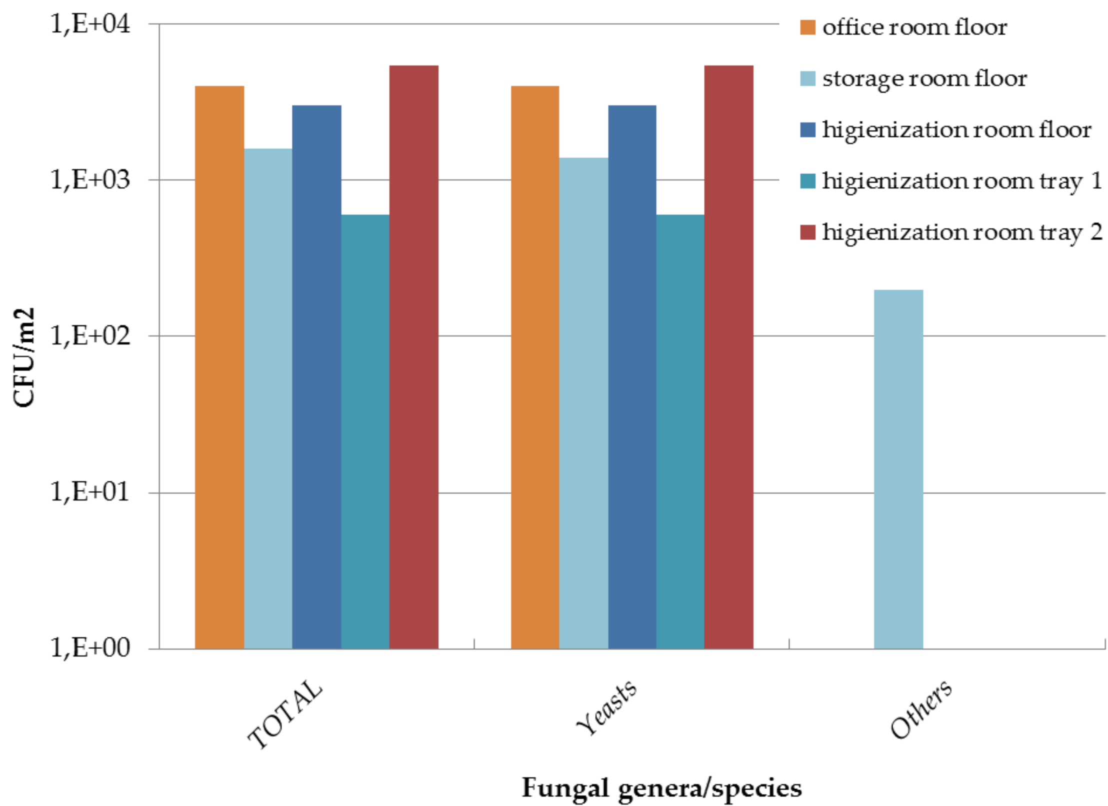


Figure 4.45 - Fungal load and diversity TT surface samples, 1st season, winter. Negative growth: restoration room floor and table. In the x-axis are the fungi associated with paper biodeterioration. Others include: *Chrysosporium* sp. and *Scopulariopsis* (fusca).Please mind the logarithmic scale.

Total fungal load varied between 600 and 5400 CFU/m2. All of identified genera were yeasts, exception made to the storage room floor where *Chrysosporum* sp. and *Scopulariopsis (fusca)* were identified also. Yeasts are generally present where water is readily available which may indicate recent cleaning activities. The air samples taken in February do not predict the encountered surface contamination scenario in these rooms.

Very different results were obtained when sampling the archival document cases in this first season (Figure 4.46).

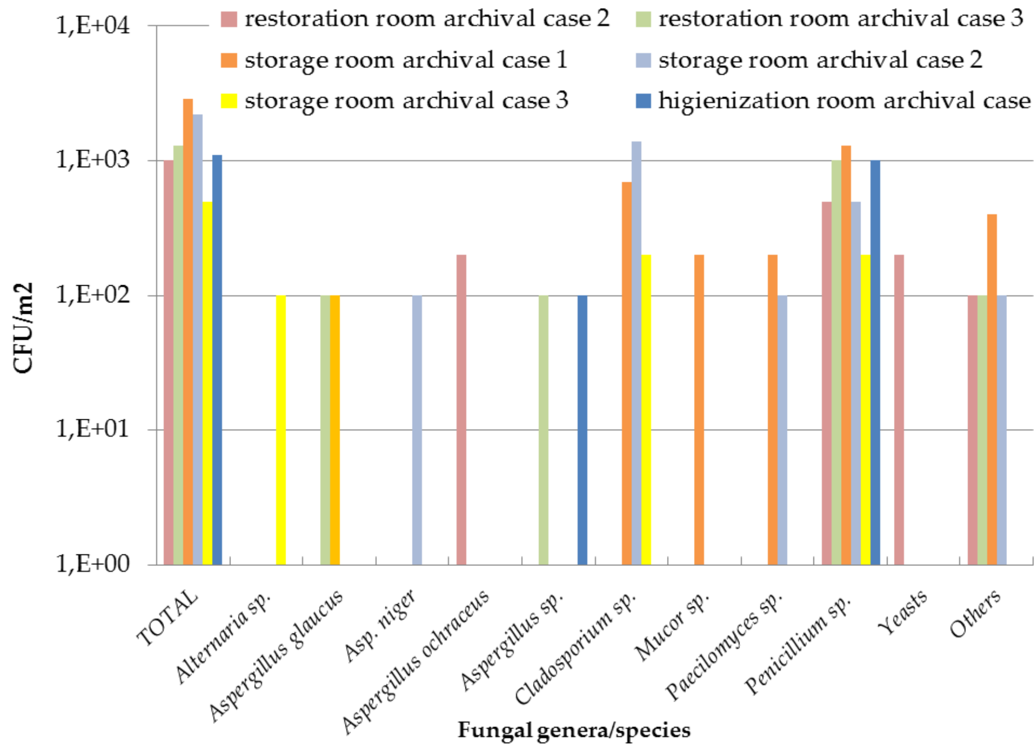


Figure 4.46 - Fungal load and diversity on TT's surface samples, archival cases, 1st season, summer.

Negative growth: restoration room archival case 1. In the x-axis are the fungi associated with paper biodeterioration. Others include: *Arthrinium* **sp.**, *Chrysosporium* **sp.** and *Scytalidium* **sp.**Please mind the logarithmic scale.

The fungal load for archival cases varied between 500 and 2900 CFU/m2. Nine genera were identified in the archival cases sampled for this season and in the *Aspergillus* genera two of the identified species are considered potentially toxinogenic (*Aspergillus niger* for the 2006 legislation and *A. ochraceus* for the 2013 ordinance). When compared to the results obtained in the other surface samples in the storage room, restoration room and higienization the differences are astounding which is yet another indication of the cleaning activities which may have altered the fungal flora in the floor and tray samples. In the air samples for these three locations the fungal flora is more easily related to the one found in the DACs but the variety is wider in these last ones.

Regarding the use of molecular biology protocols, these were needed and applied in a series of samples. From season 1 only one document archival case produced

a negative culture and no success was obtained with the DNA analysis protocols tested. The fungal communities present in the remaining archival cases samples were all identified through their morphological features. One of the samples contained pink yeast like fungus which was attributed to *Rhodotorula glutinis.* The trays sampled in the higienization room contained only yeasts which were sequenced and identified as *Debaryomyces hansenii* and *Cryptococcus laurentiis.*

The air samples in the second season presented some irregularities in terms of indoor air quality requirement (see Figure 4.18). For surface samples, the variety found in this second season, is wider, both comparing with the first season and with the air sample from season 2.

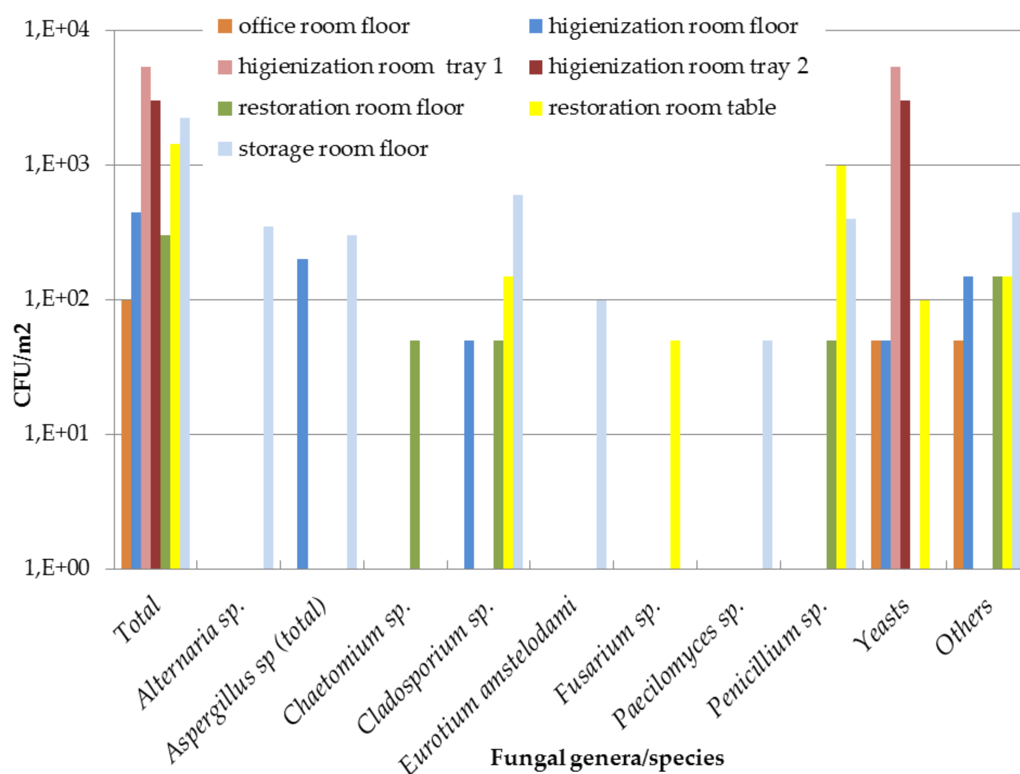


Figure 4.47 - Fungal load and diversity on TT surface samples, 2nd season, winter. All cultures positive.

In the x-axis are the fungi associated with paper biodeterioration. Others include: Arthrinium, Arthrographis, Aureobasidium, Chrysosporium sp., Geomyces sp., Hormographiella aspergillata, Scopulariopsis sp. and Scytalidium dimidiatum. Please mind the logarithmic scale.

The fungal load varied between 100 (office room floor) and 5400 CFU/m2 (higienization room tray 1). Again, yeasts comprise the entire fungal flora presented in both sampled trays. Sixteen mould genera were identified in the surfaces and only four were identified in the air.

The *Aspergillus* sp. (total) mentioned in the above figure can be further detailed in species (see Figure 4.48) and this reveals the presence of potentially toxinogenic *Asp. niger* and *Asp. versicolor.*

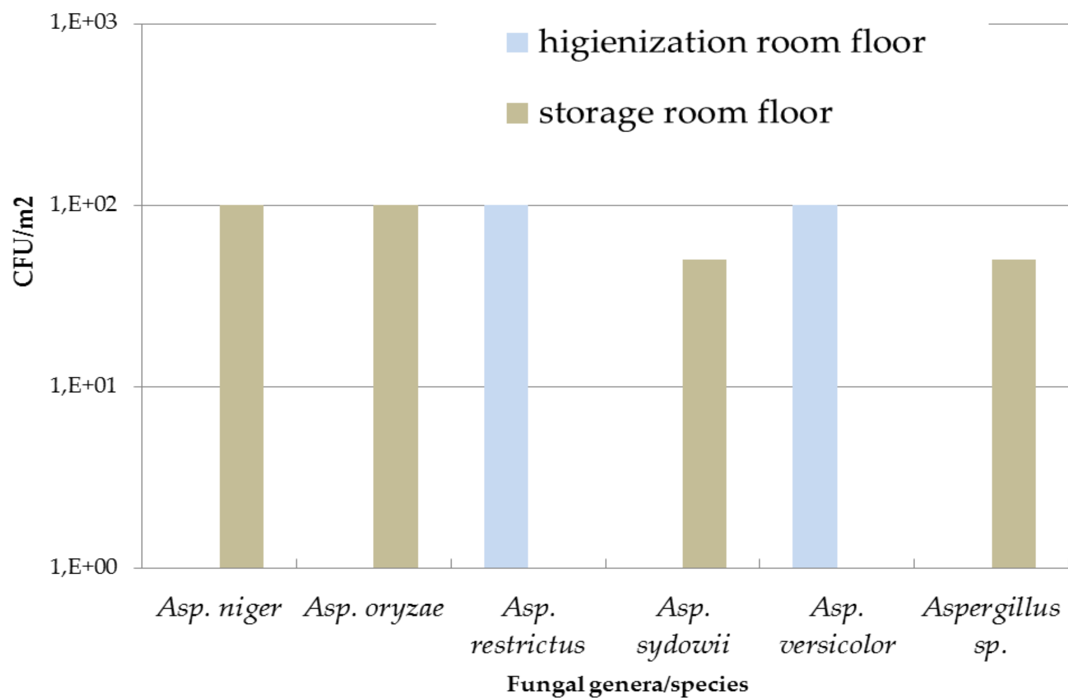


Figure 4.48 - Fungal load and diversity of the *Aspergillus* species on TT surface samples, 2nd season, winter. Please mind the logarithmic scale.

When the samples are document archival cases in the storage room the level of contamination varies between 4500 CFU/m2 in archival case 3 and 33100 CFU/m2 in archival case 1. This represents a very steep increase from the first sampled season (summer) (see Figure 4.46).

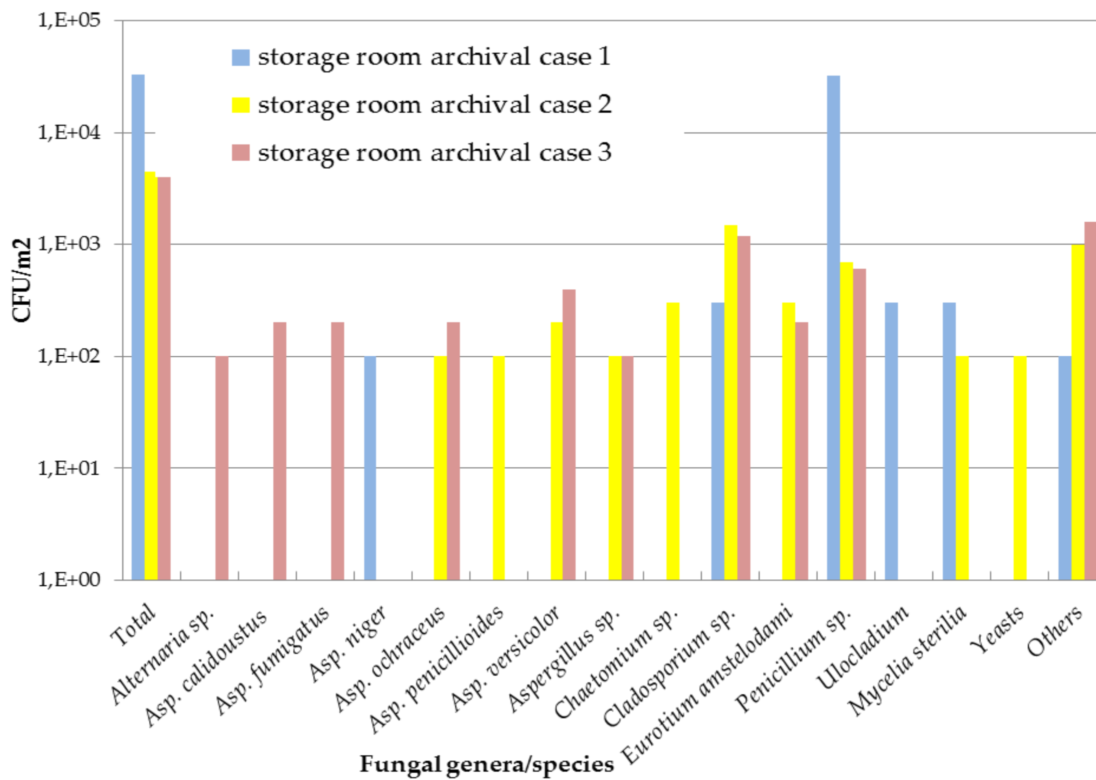


Figure 4.49 - Fungal load and diversity on TT's archival cases, 2nd season, winter. In the x-axis are the fungi associated with paper biodeterioration. Others include: *Acremonium, Arthrinium, Arthrographis, Aureobasidium, Chrysosporium, Geomyces, Geotrichum, Hormographiella aspergillata, Rhizomucor sp., Scopulariopsis, Scytalidium dimidiatum and Wallemia sebi*.Please mind the logarithmic scale.

Neither air nor other surface samples taken at the storage room show such an increase between seasons. The storage room's air sample does present a higher fungal load in the second season (174 CFU/m^3 vs 24CFU/m^3) which may have helped in the DACs contamination. The water cleaning activities possibly responsible for the high *Penicillium* and yeasts prevalence in the surfaces in season 1 may have also played a role in the higher contamination verified in the document archival cases when the second season was performed since cellulose based materials due take more time than the inorganic surfaces to respond to alterations in thermo hygrometric conditions (Valentin, 2007). The possible scenario would then be:

1. The storage room floor is washed with water before the first samples are collected.

2. When the sampling occurs yeasts have taken over previous flora on the storage floor. This effect is not readily noticed in the air samples since this room has forced ventilation.
3. When the second season takes place the air sample is higher in terms of fungal load with a higher prevalence of *Penicillium* sp and yeasts. Aerosolization of the fungal flora present in the floor might be partially responsible for this result since the outside air composition is very different in this season.
4. The inorganic surfaces regain a variety of fungal genera but show the same level of contamination as before (10^3magnitude) while the document archival cases show a much higher level of contamination than before. In fact, when analysing the water activity levels in both seasons, these are considerably higher in the second season (0,552, 0,560 and 0,547 vs 0,669, 0,67 and 0,667, respectively) in the document archival cases (see Chapter 6 for details).

In terms of the species found in all of the sampled surfaces (both seasons considered), the presence of potential biodeterioration agents was found to be:

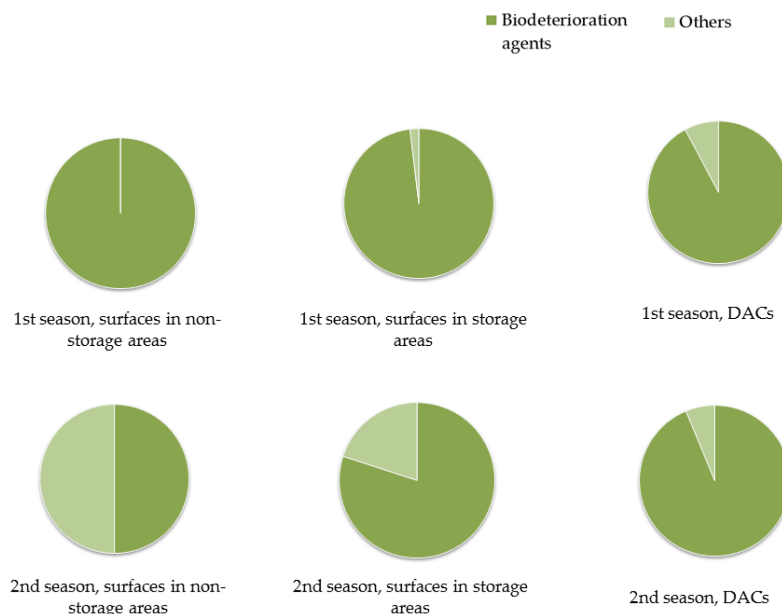


Figure 4.50 - Fungal population distribution in all surfaces tested in TT archive's for both considered seasons.

All the surfaced areas in the summer season (1st season), as previously described, show a very particular fungal flora characteristic of moist environments and that can correlate to cleaning activities involving water. *Penicillium* sp. and yeasts were found in all surfaces where culture growth was verified.

The second season can be considered to depict a more "normal" contamination scenario for both storage and non-storage inorganic surfaces. When the water presence is less intense, the normal fungal flora tends to replace the one that thrived during high moisture periods. The DACs seem unaffected by the fungal flora present but that is not true because the difference not so much the fungal flora present but the quantities in which they are present, 10x higher in the second (winter) season.

In the second season yeasts were present in one of the archival cases and on both samples taken from the trays in which books are carried. The document cover was positive for *Candida parapsilosis* (confirmed by both D2 sequence analysis and ITS2 fragment length correspondence with the AFCE database).

The *mycelia sterilia* contained in one of the document cover was, unfortunately, too contaminated to reisolate and no positive identification was obtained for this sample. For the other sample, the presence of *Fusarium sp*. was confirmed. Two DNA regions were sequenced but the results were discordant.

Both trays contained only yeast like fungi and these were composed solely of *Candida guilliermondii* in one of the trays and a mixture of both *C. krusei* and *C. glabrata* in the second tray. Both these identifications were performed by using the AFCE methodology but to this last sample DHPLC was also applied. Before *Candida krusei* was introduced in the AFCE database the yeast sample mixture collected from the tray presented a dubious result in the fragment analyses as one of the peaks could be easily attributed to *Candida glabrata* but the other yeast presented a ITS2 length not yet contemplated in the database. Genomic D2 region amplification was performed and the amplified fragments were submitted to a temperature behaviour study in the DHPLC. In Figure 4.51 is presented the chromatogram obtained with 61°C and 63°C.

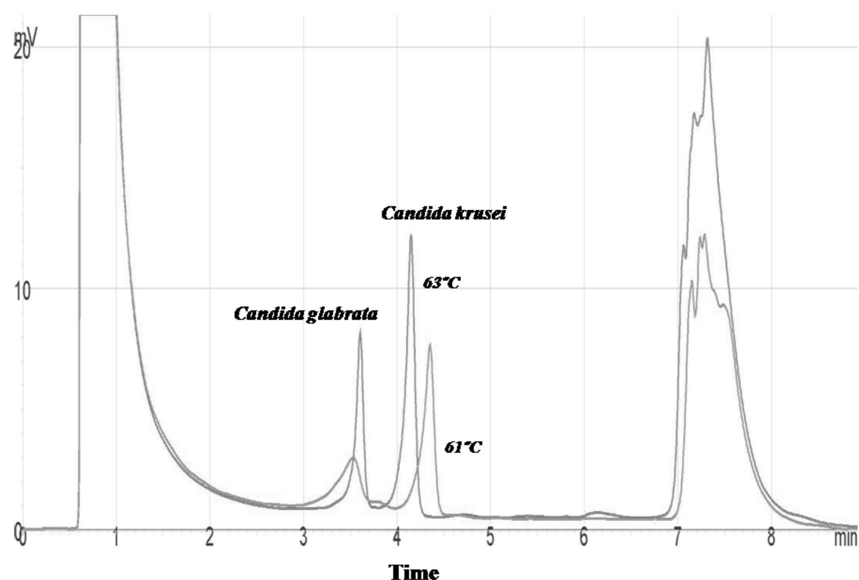


Figure 4.51 - DHPLC chromatogram of a mixture of two yeasts.
The run was performed at 61°C/55%B and 63°C/54%B. at a 0.9ml/min rate flow. The first peak, collected at minute 3.5 at 63°C corresponds to *Candida glabrata* and the second peak, collected at minute 4.1 again at 63°C corresponds to *Candida krusei*.

At 63°C (when the resolution was best), the peaks were collected at 3.5 minutes and 4.1 minutes. After sequencing the authors could attribute the first peak to *Candida glabrata* and the second peak to *Candida krusei*. The ITS2 fragment size obtained in the first study was then attributed to this yeast and inserted in the database for further use.

4.2.2.5 Global Analysis on Surface Samples

After presenting the surface samples results it is possible to verify that the universe of fungi present in the surfaces closer to the documents is much wider than what was known. Table 4.4 compiles these results:

Table 4.4 - Fungal genera/species found on surface samples (archival cases, book bindings, shelves, floor and trays) after the present study and compared to the other three existing studies on the subject.

Fungi	Organic surfaces - document archival cases or book bindings	Inorganic surfaces - shelves/floor/tables/trays
***Acremonium sp.* Link**	Maggi et al. (2000); Pinheiro et al (2015b)	Pinheiro et al (2015b)
Acremonium murorum	Zielińska-Jankiewicz et al. (2008)	
***Alternaria sp.* Nees**	Maggi et al. (2000); Pinheiro et al (2015b)	Maggi et al. (2000); Pinheiro et al (2015b)
***Alternaria alternata* (Fr.) Keissl.**	Maggi et al. (2000); Pinheiro et al (2015b)	Maggi et al. (2000); Pinheiro et al (2015b)
Alternaria arborescens		Pinheiro et al (2015b)
Alternaria dianthicola	Pinheiro et al (2015b)	
Arthrinium sp.	Pinheiro et al (2015b)	Pinheiro et al (2015b)
***Arthrinium phaeospermum* (Corda) M.B. Ellis**	Maggi et al. (2000)	
Arthrographis sp.		Pinheiro et al (2015b)
***Aspergillus sp.* P. Micheli ex Haller**	Maggi et al. (2000); Pinheiro et al (2015b)	Maggi et al. (2000); Harkawy (2011); Pinheiro et al (2015b)
***Aspergillus calidoustus* Varga**	Pinheiro et al (2015b)	
***Aspergillus candidus* Link**	Pinheiro et al (2015b)	Pinheiro et al (2015b)
***Aspergillus flavus* Link**	Maggi et al. (2000); Pinheiro et al (2015b)	Pinheiro et al (2015b)
***Aspergillus fumigatus* Fresem**	Maggi et al. (2000); Zielińska-Jankiewicz et al. (2008); Pinheiro et al (2015b)	Pinheiro et al (2015b)
***Aspergillus glaucus* Link**	Pinheiro et al (2015b)	
***Aspergillus japonicus* Saito**	Maggi et al. (2000)	
***Aspergillus nidulans* Eidam (G) Winter**	Pinheiro et al (2015b)	
***Aspergillus niger* Tiegh**	Krysińska-Traczyk (1994); Maggi et al. (2000); Zielińska-Jankiewicz et al. (2008); Pinheiro et al. (2015b)	Maggi et al. (2000); Harkawy (2011); Pinheiro et al (2015b)
***Aspergillus ochraceus* K. Wilh**	Maggi et al. (2000); Pinheiro et al (2015b)	Maggi et al. (2000); Pinheiro et al (2015b)
***Aspergillus oryzae* (Ahlb.) Cohn**	Pinheiro et al (2015b)	Pinheiro et al (2015b)
***Aspergillus penicillioides* Speg.**	Pinheiro et al (2015b)	Pinheiro et al (2015b)
***Aspergillus restrictus* G. Sm.**		Pinheiro et al (2015b)
***Aspergillus sydowii* (Bainier & Sartory) Thom & Church**	Pinheiro et al (2015b)	Maggi et al. (2000); Pinheiro et al (2015b)
***Aspergillus terreus* Thom**		Pinheiro et al (2015b)

Fungi	Organic surfaces - document archival cases or book bindings	Inorganic surfaces - shelves/floor/tables/trays
***Aspergillus ustus* (Bainier) Thom & Church**	Zielińska-Jankiewicz et al. (2008)	
***Aspergillus versicolor* (Vuill.) Tirab**	Maggi et al. (2000); Pinheiro et al (2015b)	Maggi et al. (2000); Pinheiro et al (2015b)
Aureobasidium sp.		Pinheiro et al (2015b)
***Aureobasidium pullulans* (De Bary) G. Arnaud ex Cif., Ribaldi & Corte**	Pinheiro et al (2015b)	
***Beauveria sp.* Vuill.**	Pinheiro et al (2015b)	Pinheiro et al (2015b)
***Botrytis sp.* P. Micheli ex Haller**		Pinheiro et al (2015b)
***Botrytis cinerea* Pers.**	Maggi et al. (2000)	Maggi et al. (2000)
***Candida famata* (F.C. Harrison) S.A. Mey. & Yarrow**		Harkawy (2011)
***Candida glabrata* (H.W. Anderson) S.A. Mey. & Yarrow**		Pinheiro et al (2015b)
***Candida guilliermondii* (Castell.) Langeron & Guerra**		Pinheiro et al (2015b)
***Candida krusei* (Castell.) Berkhout**		Pinheiro et al (2015b)
***Candida parapsilosis* (Ashford) Langeron & Talice**	Pinheiro et al (2015b)	
***Chaetomium sp.* Kunze**	Maggi et al. (2000); Pinheiro et al (2015b)	Maggi et al. (2000); Pinheiro et al (2015b)
***Chaetomium globosum* Kunze ex Fr.**	Maggi et al. (2000)	Maggi et al. (2000)
***Chalastospora gossypii* (Jacz.) U. Braun & Crous**	Pinheiro et al (2015b)	
***Chrysonilia sp.* Arx**	Pinheiro et al (2015b)	Pinheiro et al (2015b)
***Chrysonilia (sitophila)*(Mont.)Arx**		Pinheiro et al (2015b)
***Chrysosporium sp.* Corda**	Pinheiro et al (2015b)	Pinheiro et al (2015b)
***Cladophialophora sp.* Borelli**		Pinheiro et al (2015b)
***Cladosporium sp.*Link**	Maggi et al. (2000); Pinheiro et al (2015)	Maggi et al. (2000); Pinheiro et al (2015b)
***Cladosporium cladosporioides* (Fresen.) G.A. de Vries**	Maggi et al. (2000); Zielińska-Jankiewicz et al. (2008)	Maggi et al. (2000)
***Cladosporium cucumerinum* Ellis & Arthur**	Maggi et al. (2000)	Maggi et al. (2000)
***Cladosporium herbarum* (Pers.) Link**	Krysińska-Traczyk (1994); Maggi et al. (2000); Zielińska-Jankiewicz et al. (2008)	Maggi et al. (2000)
***Cladosporium macrocarpum* Preuss**	Maggi et al. (2000)	
***Cladosporium sphaerospermum* Penz.**	Maggi et al. (2000)	Maggi et al. (2000)

Fungi	Organic surfaces - document archival cases or book bindings	Inorganic surfaces - shelves/floor/tables/trays
***Corynascus spedonium* (C.W. Emmons) Arx**	Pinheiro et al (2015b)	
***Cryptococcus sp.* Vuill.**	Pinheiro et al (2015b)	
***Cryptococcus albidus*(Saito) C.E. Skinner**	Pinheiro et al (2015b)	
***Cryptococcus laurenti* (Kuff.) C.E. Skinner**		Pinheiro et al (2015b)
***Curvularia sp.* Boedijn**		Pinheiro et al (2015b)
***Debaryomyces hansenii*(Zopf) Lodder & Kreger**		Pinheiro et al (2015b)
***Emericella sp.* Berk.**		Pinheiro et al (2015b)
Emericella nidulans* (Eidam) Vuill *(anamorph: A. nidulans)	Maggi et al. (2000)	Maggi et al. (2000); Pinheiro et al (2015b)
***Epicoccum purpurascens* Link**	Pinheiro et al (2015b)	Pinheiro et al (2015b)
***Eurotium sp.* Link**	Pinheiro et al (2015b)	Pinheiro et al (2015b)
***Eurotium amstelodami* L. Mangin**	Pinheiro et al (2015b)	Pinheiro et al (2015b)
***Eurotium chevalieiri* L. Mangin**		Pinheiro et al (2015b)
***Eurotium herbarium* (F.H. Wigg.) Link**		Pinheiro et al (2015b)
***Eurotium rubrum* Jos. König et al..**	Pinheiro et al (2015b)	
***Exophiala sp.* J.W. Carmich.**	Pinheiro et al (2015b)	Pinheiro et al (2015b)
***Fusarium sp.* Link**	Pinheiro et al (2015b)	Pinheiro et al (2015b)
Fusarium (annulatum?)	Pinheiro et al (2015b)	
Geomyces sp.		Pinheiro et al (2015b)
***Geomyces pannorum* (Link) Sigler & J.W. Carmich.**	Pinheiro et al (2015b)	
***Geotrichum sp.* Link**	Pinheiro et al (2015b)	
***Geotrichum candidum* Link**	Krysińska-Traczyk (1994)	
***Gliocladium sp.* Corda**		Pinheiro et al (2015b)
***Graphium sp.* Corda**		Pinheiro et al (2015b)
***Gymnascella sp.* Peck**	Pinheiro et al (2015b)	
***Hormographiella aspergillata* Guarro, Gené & De Vroey,**		Pinheiro et al (2015b)
***Humicola grisea* Traaen**	Pinheiro et al (2015b)	
***Mucor sp.* Fresen**	Pinheiro et al (2015b)	Pinheiro et al (2015b)
***Mucor racemosus* Bull.**	Krysińska-Traczyk (1994)	
Mycelia sterilia	Maggi et al. (2000); Pinheiro et al (2015b)	Maggi et al. (2000); Pinheiro et al (2015b)
Nectria sp.	Pinheiro et al (2015b)	

Fungi	Organic surfaces - document archival cases or book bindings	Inorganic surfaces - shelves/floor/tables/trays
***Nigrospora sp.* Zimm**		Pinheiro et al (2015b)
***Paecilomyces sp.* Bainier**	Pinheiro et al (2015b)	Pinheiro et al (2015b)
***Paecilomyces variotti* Bainier**	Maggi et al. (2000); Zielińska-Jankiewicz et al. (2008)	Maggi et al. (2000)
***Penicillium sp.* Link**	Krysińska-Traczyk (1994); Maggi et al. (2000); Zielińska-Jankiewicz et al. (2008); Pinheiro et al (2015b)	Harkawy (2011); Maggi et al. (2000); Pinheiro et al (2015b)
***Penicillium brevicompactum* Dierckx**	Maggi et al. (2000)	Maggi et al. (2000)
***Penicillium chermesinum* Biourge**	Maggi et al. (2000)	
***Penicillium chrysogenum* Thom**	Maggi et al. (2000); Zielińska-Jankiewicz et al. (2008)	Maggi et al. (2000)
***Penicillium citreonigrum*Dierckx**		Maggi et al. (2000)
***Penicillium citrinum* Thom**	Maggi et al. (2000)	
***Penicillium coprophylum* Berk. And M. A. Curtis**		Maggi et al. (2000)
***Penicillium corylophilum* Dierckx**	Maggi et al. (2000); Zielińska-Jankiewicz et al. (2008)	
***Penicillium cyclopium* Westling**	Maggi et al. (2000)	Maggi et al. (2000)
***Penicillium digitatum* (Pers.: Fr.) Sacc**	Maggi et al. (2000)	
***Penicillium griseofulveum* Dierckx**	Maggi et al. (2000)	Maggi et al. (2000)
***Penicillium italicum* Wehmer**	Maggi et al. (2000)	Maggi et al. (2000)
***Penicillium lividum* Westling**	Maggi et al. (2000)	Maggi et al. (2000)
***Penicillium myczynski* K. M. Zalessky**	Maggi et al. (2000)	Maggi et al. (2000)
***Penicillium oxalicum* Currie and Thom**	Maggi et al. (2000)	
***Penicillium rugulosum* Thom**		Maggi et al. (2000)
***Penicillium verrucosum* Dierckx**		Harkawiy (2011)
***Penicillium viridicatum* Westling**	Maggi et al. (2000)	Maggi et al. (2000)
***Penicillium waksmanii* K. M. Zalessky**	Maggi et al. (2000)	Maggi et al. (2000)
***Periconia cookei* E.W. Mason & M.B. Ellis**	Maggi et al. (2000)	
***Pithomyces sp.*Berk. & Broome**	Pinheiro et al (2015b)	Maggi et al. (2000)
Phaeoacremonium sp. (Phialemonium sp.)	Pinheiro et al (2015b)	Pinheiro et al (2015b)

Fungi	Organic surfaces - document archival cases or book bindings	Inorganic surfaces - shelves/floor/tables/trays
Phialophora sp.		Pinheiro et al (2015b)
***Phoma sp.* Sacc**	Pinheiro et al (2015b)	Pinheiro et al (2015b)
***Phoma glomerata (Corda)* Wollenw. & Hochapfel**		Pinheiro et al (2015b)
***Ramichloridium sp.* Stahel ex de Hoog**		Pinheiro et al (2015b)
***Rhizomucor sp.* Lucet & Costantin**	Pinheiro et al (2015b)	Pinheiro et al (2015b)
***Rhizopus sp.* Ehrenb**		Pinheiro et al (2015b)
***Rhizopus arrhizus*Went & Prins. Geerl. (formerly R. *oryzae*)**	Pinheiro et al (2015b)	
***Rhizopus nigricans* Ehrenb.**	Zielińska-Jankiewicz et al. (2008)	
***Rhodotorula glutinis* (Fresen.) F.C. Harrison**	Pinheiro et al (2015b)	
Scedosporium sp.		Pinheiro et al (2015b)
***Scopulariopsis sp.* Bainier**	Pinheiro et al (2015b)	Pinheiro et al (2015b)
Scopulariopsis (fusca)	Pinheiro et al (2015b)	Pinheiro et al (2015b)
***Scytalidium sp.* Pesante**	Pinheiro et al (2015b)	Pinheiro et al (2015b)
Scytalidium (dimidiatum)		Pinheiro et al (2015b)
***Stemphylium sp.* Wallr.**		Pinheiro et al (2015b)
***Torula herbarum* (Pers.) Link**	Maggi et al. (2000)	Maggi et al. (2000)
***Trichoderma sp.*Pers**	Maggi et al. (2000); Pinheiro et al (2015b)	Maggi et al. (2000); Pinheiro et al (2015b)
***Trichoderma aureoviride* Rifai**		Maggi et al. (2000)
***Trichoderma hamatum* (Bonord.) Bainier**	Maggi et al. (2000)	Maggi et al. (2000)
***Trichoderma harzianum* Rifai**	Maggi et al. (2000)	Maggi et al. (2000)
Trichoderma (longibrachiatum)	Pinheiro et al (2015b)	
***Trichoderma viride* Pers.**	Krysińska-Traczyk (1994)	
***Trichothecium sp.* Link**		Pinheiro et al (2015b)
***Ulocladium sp.* Preuss**	Pinheiro et al (2015b)	Pinheiro et al (2015b)
***Ustilago sp.* (Pers.) Roussel**	Pinheiro et al (2015b)	
***Wallemia sebi* (Fr.) Arx**	Pinheiro et al (2015b)	
Yeasts	Pinheiro et al (2015b)	Pinheiro et al (2015b)

From the 131 fungi colonizing the organic surfaces (DACs in this study, book bindings in Maggi's study) 61 of them (47%) were identified by the present study. Only 14 (11%) were common between this and the study performed by Maggi.In the inorganic surfaces, 11% were common between the two mentioned studies and 65% had never been identified before in this particular setting.

As mentioned by Maggi et al. (2000) and firstly stated by Florian (1997) "*dust may be a source of nutrients for some insects or fungi and may form a microenvironment on surfaces as it prevents normal airflow over them and the large surface areas of the small dust particles will absorb moisture*". It is important to know what thrives beneath the dust since a shift in the microclimatic conditions (temperature, water content or Water activity) may create the optimal conditions for germination.

Figures 4.52 and 4.53 present the fungal species/genera distribution in the sampled surfaces - document archival cases and other surfaces (floor, shelves and tables). In the first figure (4.52) are presented the fungi for which damage potential was proven in paper samples (*Alternaria* sp., *Aspergillus* sp., *Chaetomium* sp. , *Mucor* sp., *Myrothecium* sp., *Penicillium* sp., *Rhizopus* sp., *Stachybotrys* sp., *Trichoderma* sp., *Trichothecium* sp. *Ulocladium* sp. *Cladosporium* sp., *Fusarium* sp. *Paecilomyces* sp. *Eurotium* sp and yeasts) while the second figure depicts the still considered less harmful genera.

Considering the fungi already associated with damage and the way these are distributed between the two considered surfaces, this study allowed the identification of 49 genera/species, of which the *Aspergillus* is the most diverse. This diversity is particularly observed in the document archival cases surfaces. Coinciding between the two surfaces are 26 genera/species. The considered"other surfaces" show the presence of 39 fungal genera/species vs the 34 encountered in the document archival cases. Yeasts are the main reason for this difference since they were only encountered in the *washable* surfaces. Mycelia sterilia was not considered as an entry.

Regarding the considered less harmful genera, 41 genera/species were identified but only 15 were coincidental between the two surface types. The fungal flora encountered in the documents archival cases is less diverse with 24 entries in Figure 4.53.

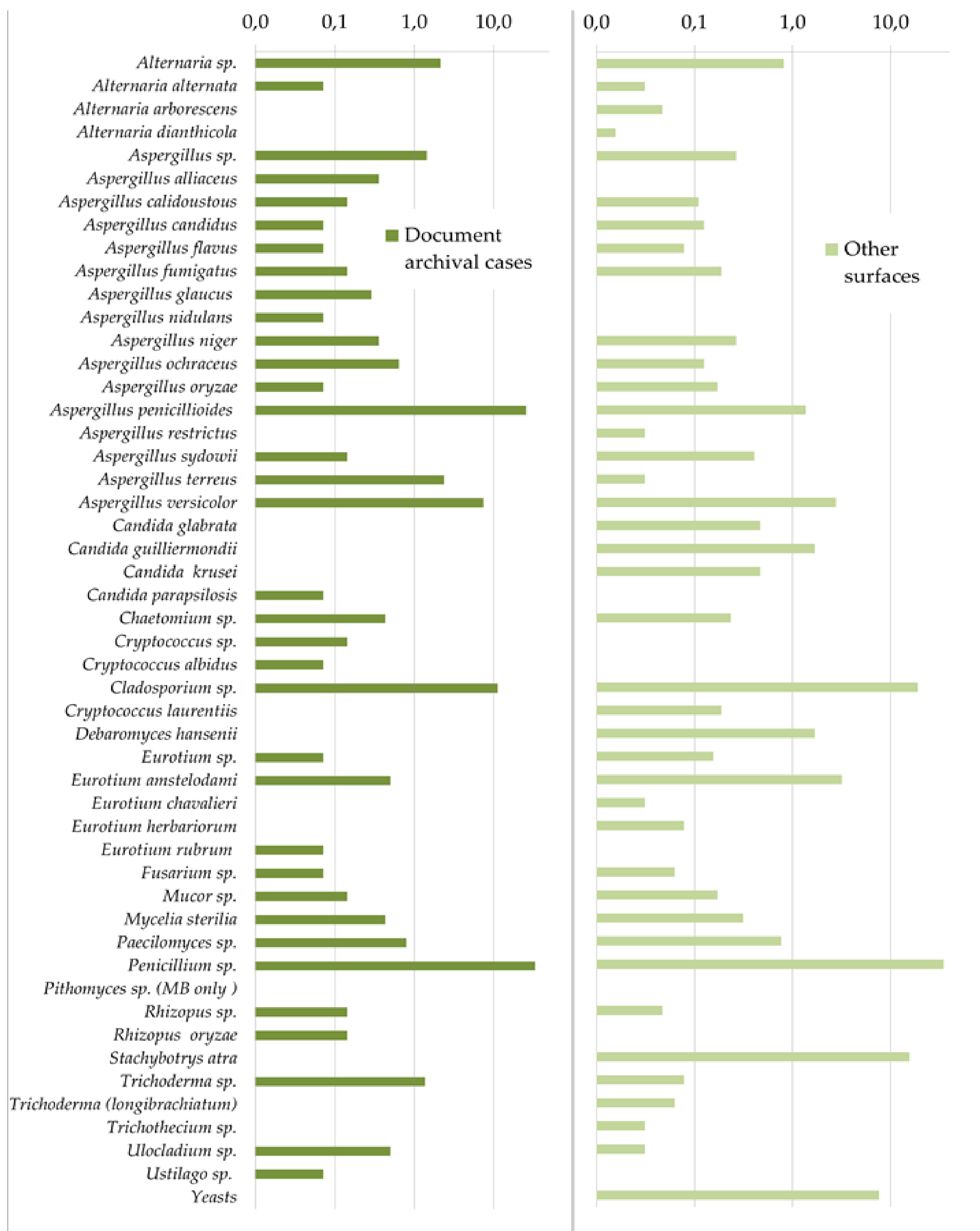


Figure 4.52 – Frequency (%) the fungal flora associated with paper damage according to fungal/yeast (genera and/or species) on surface samples.

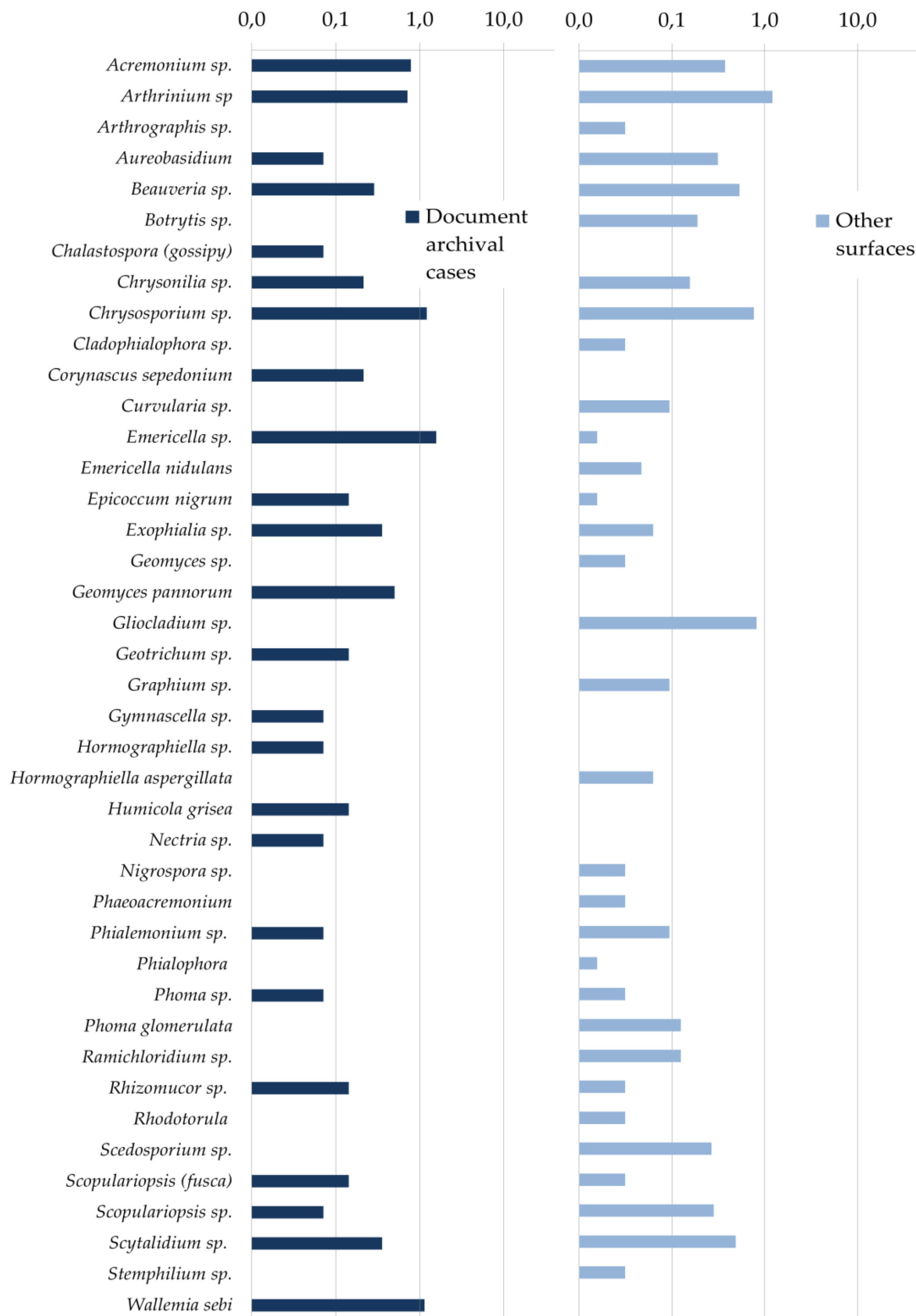


Figure 4.53 – Frequency (%) of the fungal flora yet to be associated with paper biodeterioration according to fungal/yeast (genera and/or species) on surface samples.

In average, 9.7 and 9.8 CFU/24cm² were determined in the two sampling seasons performed by Maggi et al. (2000) which included both shelves and book bindings. In the shelves, the average fungal load was 9 CFU/m² which would roughly correspond to $3.6x10^3$ CFU/m². Only one shelf sample was taken from the AHU archive, in storage room 2 and in the summer (2nd) season. The total CFU/m² in this sample is much higher than Maggi's average value. The IHRU sampled shelves present a more relatable average value. The average value determined in IHRU was 1825 CFU/m2 with a minimum of zero colonies and a maximum of 5750 CFU/m².

Figure 4.54 presents the average (and standard deviation values) for the sampled surfaces total fungal counts for each of the archives and each of the seasons separately.

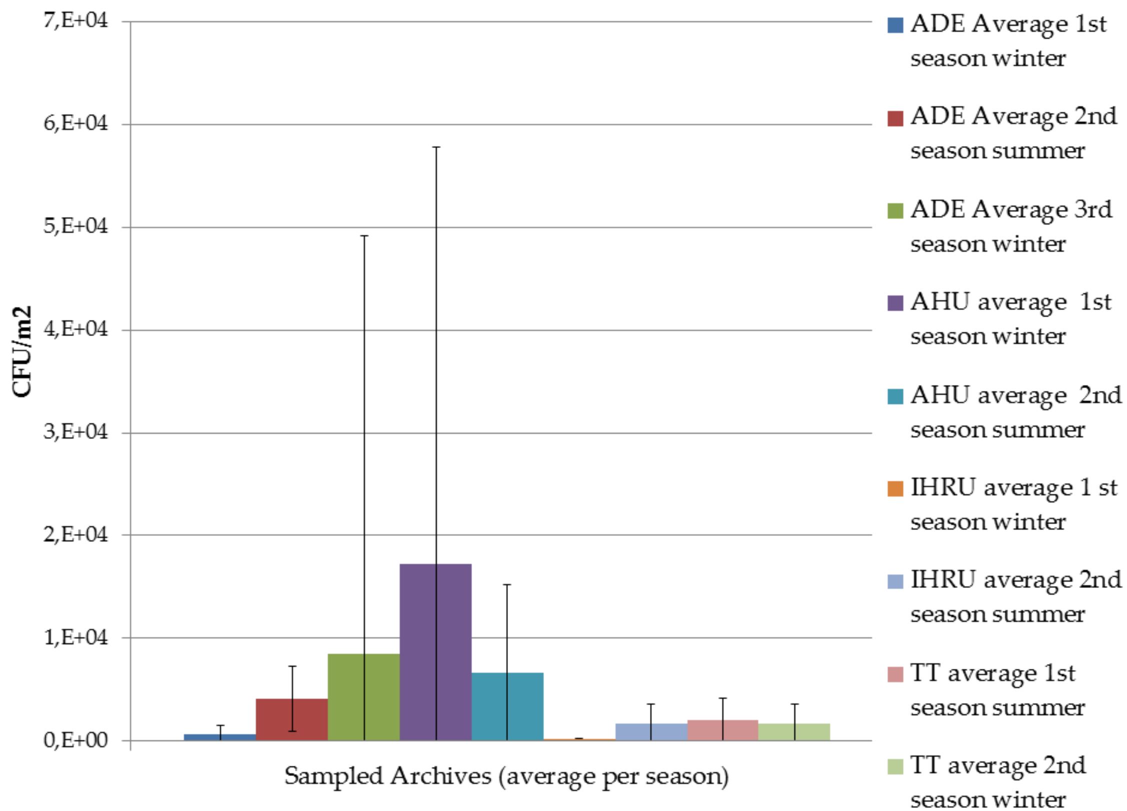


Figure 4.54 - Surface samples average and standard deviation values for fungal contamination.

The IHRU and the TT archive show the lowest values, while the ADE archive shows to have been greatly affected by the construction work on the last season. The AHU archive shows heavy contamination due to the leaking pipe, especially in the first season when the ceiling closer to the leakage was sampled. The problem persisted in the second season but samples were not taken directly from the ceiling, just the surrounding surfaces, hence the smaller value.

In the study performed by Harkawy et al. (2011) the maximum count was 100 CFU/100 cm^2, which translates into 1 x 10^6 CFU/m^2. The only surface with such a high value was a direct surface sample of the area affected by the leaking pipe, in the ceiling of storage room 1. Since no similar issues are mentioned in Harkawy's study, one of the possible reasons for such high value lies in the methodology chosen. The dilution method used by Harkawy might partially explain this comparatively high result since suspending dust samples in liquid and making further dilutions before plating normally leads to higher concentration estimates of culturable fungi in house dust (Macher 2001). On the downside, microorganisms present in small numbers and as single units may be represented less well (Macher, 2001). Average values are not revealed in Harkawy's study.

In the study performed by Zielińska-Jankiewicz et al. (2008) the surface levels varied between $4x10^3$ and $8x10^3/m^2$, exception made to the most frequently and numerous *Cladosporium cladosporioides* and *Paecilomyces variotti* which crossed the 10^7 CFU/m2 level. In the present study this level was not crossed for any of the species but most of the analysed Portuguese archives show higher levels of contamination (minimum zero CFU/m^2 and maximum 1000000 CFU/m^2).

Figure 4.55 presents the average fungal load for the sample document archival cases:

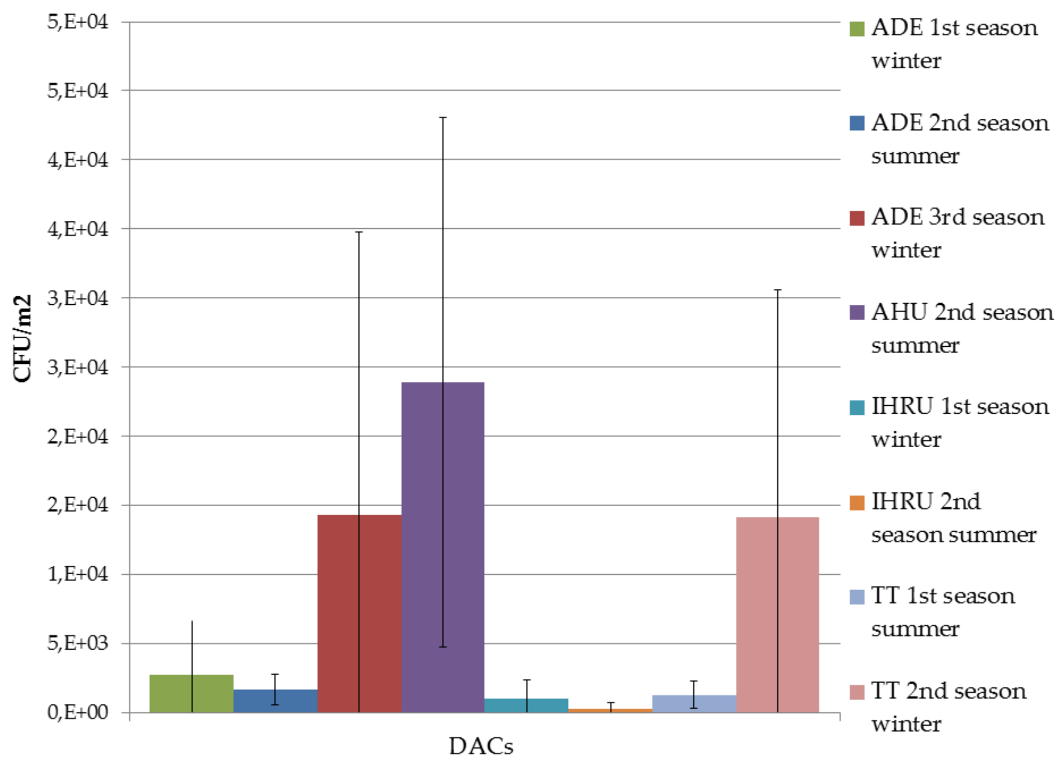


Figure 4.55 – Document archival samples average fungal contamination. Only one season was sampled in the AHU archive.

In the ADE archive, the document covers also show a much higher level of contamination in the last season. AHU document covers were only sampled in the last season so no direct comparison can be made between seasons. However, comparison with the remaining archives places the responsibility of the increased levels in the leaking water pipe in storage room 1. The IHRU archive shows a very low level of contamination as happens with the TT archive in the first season. As exposed before, second season's levels of contamination are probably due to the cleaning activities or any previous incidents involving increased moisture level.

Maggi's (200) study included archival bindings and in these the average fungal loads were circa 10CFU/24 cm^2, corresponding to 4000 CFU/m^2. These would also be the values obtained in the selected archives if external conditions had not shifted in all

but one of the studied settings: dust and debris accumulation in the ADE archive and water/moisture issues in the AHU and TT archive.

Still regarding fungal load, air and surface samples showed low levels of dependence. This is expected as populations are controlled through different ways: controlled air intake and filtration for the air samples and cleaning procedures for the surface samples. The largest contribution of air contamination for the surface's contamination was determined in the TT archive with 30%.

4.2.3 Sampled Documents

Apart from the cases where visible growth is noticed, most of the damage associated with fungal causes appears in the form of chromatic changes in the paper. This effect may last long after the fungus has been there since many of its metabolites (see Table 2.6) are coloured. Along with the air and surface samples, small altered paper areas were also sampled to ascertain the presence of fungi. In all of these samples two protocolos were applied: conventional culturing methods and molecular biology protocols (see Chapter 3).

4.2.3.1 ADE Archive

Several samples were taken from documents in the ADE archive. Most of the documents chosen show visible deterioration as depicted in Figures 4.56 and 4.57 and all but one are from the XIX century:

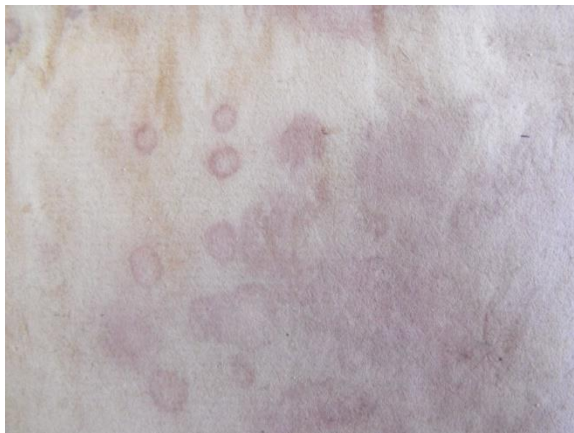


Figure 4.56 – Sampled area from a document from the ADE archive. These pinkish areas did not returned a positive culture but *Fusarium oxsyporum's* presence was identified using molecular biology protocols.

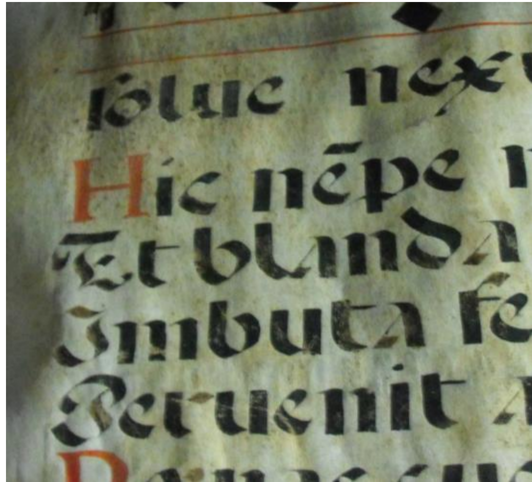


Figure 4.57 - Sampled area from a document (XVIII century) from the ADE archive. These green areas did not returned a positive culture but *Trichoderma viridae* presence was identified using molecular biology protocols.

Five different documents were selected and eight samples were removed from documents visibly stained or altered. From these samples, one showed positive growth (*Aspergillus glaucus*) while six did not show any growth in the two media tested and one presented mycelia sterilia. The molecular biology protocols (using the ITS2 and D2 region) confirmed the presence of *Aspergillus glaucus* in sample Nº2 but did not returned any result in three of the samples with negative growth. The remaining four negative growth samples were positive for *Fusarium oxysporum* (D2 region only), *Epicoccum purpurascens* (light brown stain, D2 and ITS2 amplification), *Aureobasidium pullulans* (dark brown area, D1/D2) and *Trichoderma viride*. The samples with *mycelia sterilia* were also submitted to molecular analysis and these returned the presence of *Chaetomium bostrychodes*.Some of the control areas also showed fungal growth. The results for the control areas are included in the Tables.

Table 4.5 summarizes the results obtained in the ADE archive.

Table 4.5 – Analysis performed on the altered documental samples in the ADE archive.

Docu-ment	Sample	Visible deteri-oration	Culture Result	Control area	Molecular Biology
A	Document area 1	pink area	-	-	*Fusarium oxysporum*
B	Document area 2	grey	+; *Aspergillus (glau-cus)*	-	*Asp. glaucus*
C	Document area 3	pink	-	-	-
C	Document area 4	purple	-	-	-
D	Document area 5	light brown area	-	+ *(Penicillium sp.)*	*Epicoccum purpurascens*
D	Document area 6	dark brown area	+; *Mycelia sterilia*	-	*Chaetomium bostrychodes*
D	Document area 7	dark brown area	-	-	*Aureobasidium pullulans*
E	Document area 8	green area	-	+ *(Penicillium sp).*	*Trichoderma (viridae)*

Fusarium oxysporum has been associated with the formation of purple spots and , according to Sczepanowska et al. (1992) this stain may be completely removed by the long-term application of 1,4-dioxane. Stain production by F. oxysporum is reduced in the absence of light and, as such, storage in the dark should inhibit stain production by this fungus in particular (Sczepanowska et al. (1992). The specificity of the treatment and prevention strategy would not have been possible without proceeding to species identification and highlights the importance of not treating all fungi as equals.

According to the consulted sources, *Aspergillus glaucus* had not been yet identified in any chromatically altered areas. Aspergillus sp. has been associated with damage but not this species in particular. According to Samson et al. (2004) it does produce protease (Table 2.7).

Epicoccum purpurascens has been identified by Corte et al (2003) in similar altered areas and produces protease, amylase and cellulase (Table 2.7).

Chaetomium bostrychodes has not been previously associated with altered areas (Table 2.6). The identification resulted from the use of sequence analysis of the D2 since it was not possible to amplify other genomic regions. *Aureobasidium pullulans* has been identified by Zotti et al (2007) and is able to decompose pectins, dextrins, oligossacha-

rides and starches. It produces a black melanine that, in time, turns green (Michaelsen et al, 2010). *Trichoderma viridae* has been identified by Lourenço et al (2005) and produces cellulase and pigment (Table 2.7). Besides its implication in paper conservation, it is considered a potentially toxinogenic fungi.

4.2.3.2 AHU Archive

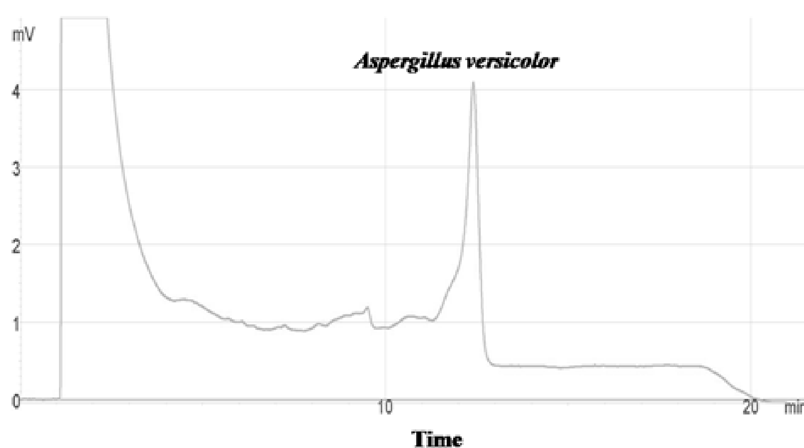
Regarding document samples, the results are presented in Table 4.6. The eight-sampled documents in this archive were all from the end of the 19th century.

Table 4.6 - Analysis performed on the altered documental samples in the AHU archive.

Document	Sample	Visible deterioration	Culture Result	Control area	Molecular Biology
A	**Document area 1**	stain from document cover)	+; *Chrysosporium sp*	.	*Chrysosporium carmichaelli*
B	**Document area 2**	yellow stain	-	-	*Candida oleophila*
B	**Document area 3**	Brown stain	+; *Aspergillus candidus* (13), *Aspergillus sp.* (14); *Penicillium sp.* (1).	Aspergillus sp.+ Penicillium	*Penicillium sp.*
B	**Document area 4**	Yellow stain	+; 15 Penicillium 25 Aspergillus sp.	-	Aspergillus sp.
C	**Document area 5**	black	+; 6 Aspergillus candidus (6), Aspergillus sp. (18);	+ *Aspergillus sp.*	-
D	**Document area 6**	brown	-	-	-
D	**Document area 7**	green	-	-	-
E	**Document area 8**	brown	-		*Asp. versicolor*
F	**Document area 9**	brown	-	-	-
G	**Document area 10**	green	+ *Aspergillus terreus*	+ *(Cladosporium sp.)*	*Aspergillus terreus*
H	**Document area 11**	purple	-	-	*Eurotium halophilicum* (anamorph: Asp. halophillicus)

Chrysosporium carmichaelli is a potentially keratinophylic fungi and was amplified from a positive culture. The documents are mostly manipulated without gloves and the presence of this fungi is a health risk. It had not been identified before in documental case studies. *Candida olephila* was obtained from a documental sample with a negative culture. There is still much to know on the possible effects of yeasts on paper documents. No ITS2 amplification was obtained for this yeast and, therefore, it could not be included in the AFCE database. *Penicillium sp*. and *Aspergillus sp*. were identified in samples three, four and five from positive cultures but no accurate species (high homology) was found upon sequencing. Samples six and seven and showed negative cultures and no identification was possible using molecular biology protocols.

Figure 4.59 correspond to a swab sample collected from a document showing several green/brown patches (sample 8). The cultures showed no growth in any of the media tested but the molecular biology protocol definitely showed the presence of fungal DNA (though with low homology). At partially denaturing temperatures and after peak collection and sequencing it was possible to identify *Aspergillus versicolor* as a contaminant of this document.



Figue 4.58 - DHPLC chromatogram o the sample depicted in Figure 9, after peak collection (minute 13) and now under partially denaturing temperature (59°C) and 55%B, 0.5 ml/min flow rate. Only one peak appeared and the sample was collected again (minute 12/13) and sequenced. The result was *Aspergillus versicolor*.

Aspergillus terreus was identified in culture and confirmed by molecular biology protocols. It is able to degrade cellulose and phenolic compounds (Michaelsen et al. 2010d) and produce pigments (Nol et al, 2001).

Eurotium halophilicum has only been identified once before in document samples by Michaelsen et al (2010a, 2010b). It is a obligate xerophilic (with minimum water activity levels of 0.64), displays a very slow growth rate and is able to outgrow other xerophilic species. It is a possible candidate for the formation of foxing spots (Michaelsen et al, 2010a). It is unable to grow at aw levels above 0.94 and even in optimal conditions presents a very slow growth rate (Arora et al., 1991). In our case, it was present in a paper from a photographic album from the XIX century.

4.2.3.3 IHRU Archive

The same document (XX century blueprint) was sampled three times, in three distinct areas. Two of them resulted in negative culture and DNA negative extraction and/or amplification. The remaining sample revealed a positive culture by *Penicillium sp.* Table 4.6 summarizes the results.

Table 4.7 – Analysis performed on the altered documental samples in the IHRU archive.

Document	Sample	Visible deterioration	Culture Result	Control area	Molecular Biology Result	
A	**Document area 1**	Grey/green areas	-	-	-	-
A	**Document area2**	Grey/ green areas	+ *Penicillium sp.*	-	*Penicillium sp.*	
A	**Document area 3**	Grey/green areas	-	-	-	-

Penicillium species are one of the most represented in Table 2.7. Generally, this genera is a producer of cellulase, amylase and protease (Table 2.7). The efforts placed in achieving a species level for the sample Nº 2 were not successful for either the ITS2 region or the D1/D2 region. ITS complete sequence was not attainable.

4.2.3.4. TT Archive

Three document samples were sampled from the TT archive. All of the swabbed samples returned a negative culture. The result was also negative for one of the samples where DNA analysis was tested. Table 4.8 summarizes the obtained results.

Table 4.8 - Analysis performed on the altered documental samples in the TT archive.

Document	Sample	Visible deterioration	Culture Result	Control area	Molecular Biology Result	
A	**Document area 1**	Pinkish area	-	-	+	*Fusarium sp.*
A	**Document area 2**	Green area	-	-	-	-
A	**Document area 3**	pink, black, greenish spots	-	-	+	Inconclusive; DHPLC; *Alternaria sp.*+ *Fusarium sp.*

DHPLC was used in the last sample to attempt the separation of a possible mixture since the first sequence reading returned a low homology result. Figure 4.59 presents two of the tests performed on this sample to achieve separation.

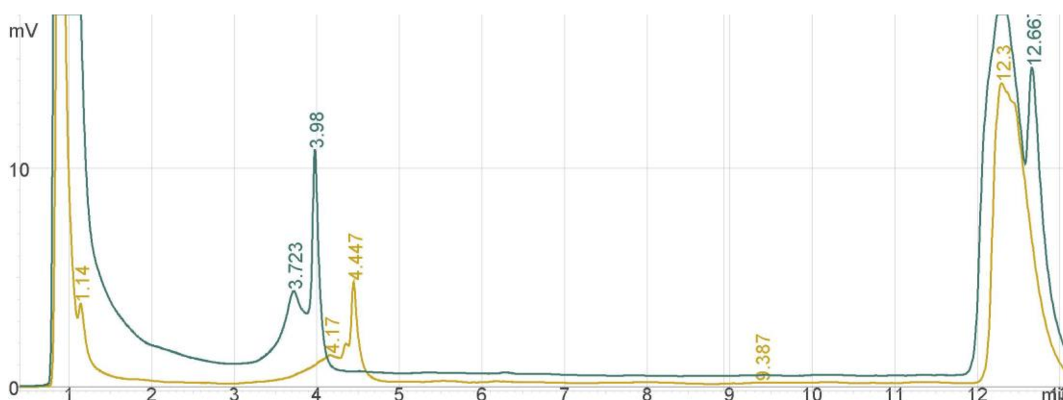


Figure 4.59 – DHPLC chromatogram of the sample under partially denaturing temperature (61.5°C) and 57.2%B, 0.5 ml/min flow rate. It was possible to separate *Fusarium* sp. at 3.7 minutes and *Alternaria sp.* at 3.9 minutes.

The homology results were still low and no species definition was attained for neither *Fusarium* or *Alternaria.* Nevertheless *Fusarium* species are able to produce pigment (red pigment named fusarubin) and are able to degrade cellulose.

Table 4.9 gathers the results from the document case studies: the reviews, the field studies and the results added by the study in the samples from ADE, AHU, IHRU and TT.

Table 4.9 - Fungal genera/species identified in paper samples (cellulose-based samples, documents and books).

The fungi, in these cases, are isolated from small, altered areas and are not quantifiable. The fungi mentioned only in the first column have not been identified in field studies, just mentioned in reviews.

Fungi	Altered Cellulose-based samples: Case studies
***Acremonium* sp. Link (d)(e)**	Lourenço (2005); da Silva (2006),
***Acremonium murorum* (Corda) W. Gams**	Zielińska-Jankiewicz et al. (2008)
***Acrothecium* sp. (Corda) Preuss (e)**	
***Alternaria* sp. Nees (a)(e)**	Rojas et al. (2009); Borrego et al. (2012); Pinheiro et al (2015b)
***Alternaria alternata* (Fr.) Keissl. (c)(d)(e)**	Bacilkova (2006); Mesquita et al. (2009)
***Alternaria solani* Sorauer (d)**	
***Alternaria tenuíssima* (Nees) Wiltshire (c)**	
***Arthrinium urticae* M.B. Ellis**	Corte et al. (2003)
***Aspergillus* sp. P. Micheli ex Haller (e)**	Gambale et al. (1993); Florian and Manning (2000); Corte et al. (2003); Zotti et al. (2007); Rojas et al. (2009); Borrego et al. (2012).
***Aspergillus candidus* Link (e)**	Bacilkova (2006)
***Aspergillus carneus* Blochwitz**	Zotti et al. (2008)
***Aspergillus clavatus* Desm. (a)**	
***Aspergillus flavus* Link (a)(c)(e)**	Nyuksha (1990); Nol et al. (2001); Borrego et al. (2012); Zotti et al. (2008)
***Aspergillus fumigatus* Fresen (c)(d)(e)**	Nol et al. (2001); Lourenço (2005); Mesquita et al. (2009); Zielińska-Jankiewicz et al. (2008)
***Aspergillus glaucus* Link (a)(d)**	Pinheiro et al (2015b)
***Aspergillus japonicus* Saito**	da Silva (2006); Rakotonirainy et al. (2007)
***Aspergillus melleus* Yukawa**	Zotti et al. (2011)

Fungi	Altered Cellulose-based samples: Case studies
***Aspergillus nidulans* (Eidam) G. Winter (d)**	Lourenço (2005); Mesquita et al. (2009); Michaelsen et al. (2009)
***Aspergillus niger* Tiegh. (c; d, a, e)**	Nol et al. (2001); Bacilkova (2006); da Silva (2006); Lourenço (2005); Michaelsen et al. (2009); Borrego et al. (2012)
***Aspergillus ochraceus* K. Wilh (a)(e)**	Lourenço (2005)
***Aspergillus oryzae* (Ahlb.) Cohn**	Rakotonirainy et al. (2007)
***Aspergillus penicillioides* Speg.**	Arai (2000); Michaelsen et al (2010b)
***Aspergillus repens* (Corda) Sacc (a)**	
***Aspergillus ruber* (Jos. König et al.) Thom & Church (a)**	
***Aspergillus sclerotiorum* G. A. Huber**	Zotti et al. (2011)
***Aspergillus sydowii* (Bainier & Sartory) Thom & Church**	Lourenço (2005); Zielińska-Jankiewicz et al. (2008)
***Aspergillus tamarii* Kita (d)**	Nol et al. (2001)
***Aspergillus terreus* Thom (c)(e)**	Michaelsen et al (2010b); Pinheiro et al (2015b)
***Aspergillus ustus* (Bainier) Thom & Church**	Rakotonirainy et al. (2007); Zielińska-Jankiewicz et al. (2008)
***Aspergillus versicolor* (Vuill.) Tirab (c)(e)**	Lourenço (2005); Mesquita et al. (2009); Michaelsen et al. (2009); Michaelsen et al (2010b)Pinheiro et al (2015b)
***Aspergillus wentii* Wehmer**	Bacilkova (2006)
***Aureobasidium sp.*Viala & G. Boyer (e)**	
***Aureobasidium pullulans* (De Bary) G. Arnaud ex Cif., Ribaldi & Corte (e)**	Zotti et al. (2007); Michaelsen et al (2010b) Pinheiro et al (2015b)
***Bjerkandera adusta* (Willd.) P. Karst.**	Rakotonirainy et al. (2007)
Botryotinia fuckeliana* (de Bary) Whetzel *(teleomorph of Botrytis cinerea)	Michaelsen et al. (2009)
***Botrytis* sp. P. Micheli ex Haller (e)**	Lourenço (2005)
***Botrytis cinerea* Pers (e)**	Mesquita et al. (2009)
***Botryotrichum* sp.Sacc. & Marchal (e)**	
***Botryotrichum atrogriseum* J.F.H. Beyma (e)**	
***Candida guilliermondii* (Castell.) Langeron & Guerra (a)**	
Candida oleophila	Pinheiro et al (2015b)
***Chaetomium* sp. Kunze (e)**	Corte et al. (2003);Lourenço (2005); Rojas et al. (2009)
***Chaetomium bostrychodes* Zopf (e)**	Pinheiro et al (2015b)
***Chaetomium elatum* Kunze (c)(e)**	
***Chaetomium globosum* Kunze ex Fr. (c)(d)(e)**	Corte et al. (2003);Lourenço (2005); Rakotonirainy et al. (2007); Mesquita et al. (2009)
***Chaetomium gracile* Udagawa**	Corte et al. (2003)

Fungi	Altered Cellulose-based samples: Case studies
***Chaetomium indicum* Corda (a)(c)(e)**	Lourenço (2005)
***Chaetomium murorum* Corda (e)**	
***Chromelosporium carneum* (Pers.) Hennebert**	Mesquita et al. (2009)
***Chrysosporium* sp.Corda (e)**	
Chrysosporium carmichaelli	Pinheiro et al (2015b)
***Cladobotryum* sp.Nees (e)**	
***Cladosporium* sp.Link**	Gambale et al. (1993); Corte et al. (2003); Lourenço (2005); Bacilkova (2006); Michaelsen et al. (2009); Rojas et al. (2009); Borrego et al. (2012)
***Cladosporium cladosporioides* (Fresen.) G.A. de Vries (d)**	da Silva et al. (2006); Zotti et al. (2007); Zielińska-Jankiewicz et al. (2008); Mesquita et al. (2009); Michaelsen et al. (2009)
***Cladosporium elatum* (Harz) Nannf. (a)**	
***Cladosporium herbarum* (Pers.) Link (d)(e)**	da Silva et al. (2006); Zielińska-Jankiewicz et al. (2008); Michaelsen et al. (2009)
***Cladosporium resinae* (Lindau) G.A. de Vries**	Bacilkova (2006)
***Cladosporium sphaerospermum* Penz.**	Corte et al. (2003); di Bonaventura et al. (2003; not conclusive); Zotti et al. (2011)
***Coprinus* sp.Pers.**	Mesquita et al. (2009)
***Cunninghamella* sp. Matr.**	Corte et al. (2003)
***Cunninghamella elegans* Lendn.**	Corte et al. (2003)
***Curvularia* sp. Boedijn (e)**	
***Curvularia lunata* (Wakker) Boedijn (d)**	
***Debaryomyces hansenii*(Zopf) Lodder & Kreger**	Michaelsen et al. (2009)
***Doratomyces* sp. Corda (d)(e)**	
***Doratomyces stemonitis*(Pers.) F.J. Morton & G. Sm**	Zotti et al. (2007)
***Eladia saccula* (E. Dale) G. Sm.**	Rojas et al. (2009)
***Epicoccum* sp. Link (e)**	Corte et al. (2003); Lourenço (2005)
***Epicoccum purpurascens* Ehrenb. (e)**	Corte et al. (2003); Pinheiro et al (2015b)
***Eurotium* sp. Link**	Florian and Manning (2000); Corte et al. (2003)
***?Eurotium amstelodami* L. Mangin (a)(b)**	
***Eurotium chevalieri* L. Mangin *(anamorph: Aspergillus chevalieri)* (b)**	
***Eurotium halophilicum* Chr., Papav. & Benj.**	Michaelsen et al (2010a,b)Pinheiro et al (2015b)

Fungi	Altered Cellulose-based samples: Case studies
Eurotium herbariorum* (Weber ex F.H. Wigg.) Link *(anamorph: Aspergillus glaucus* Link*)	Arai (2000)
***Fusarium* sp. Link (a)**	da Silva et al. (2006); Pinheiro et al (2015b)
***Fusarium oxysporum* Schltdl. (d)**	
***Fusarium semitectum* Berk. & Ravenel (d)**	
***Fusicladium* sp. Bonord.**	Corte et al. (2003)
***Geomyces pannorum* (Link) Sigler & J.W. Carmich**	Zotti et al. (2007)
***Geosmithia putterilli* (Thom) Pitt**	Zotti et al. (2007)
***Geotrichum* sp. Link (d) (e)**	
Gloeotinia temulenta (Phialea temulenta,* Prill. & Delacr.*)	Rakotonirainy et al. (2007)
***Gliocladium catenulatum* J.C. Gilman & E.V. Abbott (d)**	
***Gliocladium roseum* Bainier**	Nol et al. (2001)
***Gymnoascus* sp. Baran (e)**	
***Helicostylum* sp. Corda (e)**	
***Humicola grisea* Traaen (d)**	
***Kockovaella* sp. Nakase, I. Banno & Y. Yamada**	di Bonaventura et al. (2003, not conclusive)
***Melanospora* sp. Corda (e)**	
***Memnoniella* sp. Höhn (d)**	
***Microsporum* sp.Gruby (d)**	
***Mucor* sp. Fresen (a)(e)**	Lourenço (2005); Michaelsen et al. (2009)
***Mucor racemosus* Bull (c)(d)(e)**	
Mycelia sterilia	da Silva et al. (2006); Zotti et al. (2008)
***Myrothecium verrucaria* (Alb. & Schwein.) Ditmar (d)**	
***Neurospora* sp. Shear & B.O. Dodge (d)**	
***Neurospora sitophyla* Shear & B.O. Dodge *(anamorph: Chrysonilia sitophyla* (e)**	
***Oidiodendrium* sp. Robak**	Corte et al. (2003)
***Oidiodendron citrinum* G.L. Barron**	Corte et al. (2003)
***Paecilomyces* sp. Bainier (e)**	Lourenço (2005)
***Paecilomyces variotti* Bainier (a)(d)**	Zielińska-Jankiewicz et al. (2008); Zotti et al. (2008)
***Penicillium* sp. Link (e)**	Gambale et al. (1993); Florian and Manning (2000); Corte et al. (2003); da Silva et al. (2006); Zielińska-Jankiewicz et al. (2008); Mesquita et al. (2009); Rojas et al. (2009); Borrego et al. (2012); Pinheiro et al, 2015b

Fungi	Altered Cellulose-based samples: Case studies
***Penicillium albidum* Sopp**	Bacilkova (2006)
***Penicillium bilaiae* Chalab.**	Lourenço et al. (2005)
***Penicillium brevicompactum* Dierckx (c)(d)**	Corte et al. (2003); Lourenço et al. (2005)
***Penicillium chrysogenum* Thom (c)(d)**	Corte et al. (2003); Bacilkova (2006); Zielińska-Jankiewicz et al. (2008); Mesquita et al. (2009); Borrego et al. (2012); Michaelsen et al. (2009);
***Penicillium citreonigrum* Dierckx**	da Silva et al. (2006)
***Penicillium citrinum* Thom (a)**	Corte et al. (2003); Lourenço (2005); da Silva et al. (2006); Rakotonirainy et al. (2007)
***Penicillium commune* Thom (d)**	Michaelsen et al. (2009); Borrego et al. (2012)
***Penicillium corylophilum* Dierckx**	Lourenço et al. (2005); Zielińska-Jankiewicz et al. (2008)
***Penicillium decumbens* Thom**	Corte et al. (2003); Lourenço et al. (2005)
***Penicillium expansum* Link (a)**	Corte et al. (2003); Lourenço et al. (2005)
***Penicillium fellutanum* Biourge (a)**	da Silva et al. (2006)
***Penicillium glabrum* (Wehmer) Westling (d)(a)**	Lourenço et al. (2005)
***Penicillium griseofulveum* Dierckx**	Lourenço et al. (2005)
***Penicillium oxalicum* Currie and Thom**	Lourenço et al. (2005)
***Penicillium paxilii* Bainier**	Zotti et al. (2007)
***Penicillium restrictum* J. C. Gilman and E. V. Abbott**	da Silva et al. (2006); Zotti et al. (2007)
***Penicillium spinulosum* Thom (a)**	Lourenço et al. (2005); Zotti et al. (2007)
***Penicillium steckii* K. M. Zalessky**	Zotti et al. (2007)
***Penicillium turbatum* Westling**	Zotti et al. (2007)
***Pestalotia oxyanthi* Thüm. (d)**	
***Peziza* sp. Fr.**	Corte et al. (2003)
***Peziza ostracoderma* Korf**	Corte et al. (2003)
***Phialophora* sp. Medlar**	Lourenço et al. (2005)
***Phlebia subserialis* (Bourdot & Galzin) Donk**	Mesquita et al. (2009)
***Phoma* sp. Sacc. (e)**	Corte et al. (2003); Lourenço et al. (2005)
***Phoma pigmentivora* Massee (d)**	
***Phoma pomorum* Thüm.**	Corte et al. (2003)
***Polyporus brumalis* (Pers.) Fr.**	Rakotonirainy et al. (2007)
***Ramichloridium* Stahel ex de Hoog (e)**	
***Rhizopus* sp. Ehrenb. (e)**	Lourenço et al. (2005)
***Rhizopus arrhizus* A. Fisch. (a)**	Michaelsen et al. (2009)
***Rhizopus nigricans Ehrenb.* (d)**	Zielińska-Jankiewicz et al. (2008)

Fungi	Altered Cellulose-based samples: Case studies
Rhizopus stolonifer **(Ehrenb.) Vuill. (c)**	
Rhodotorula sp. FC Harrison (e)	
Rhodotorula aurantiaca	Michaelsen et al (2010b)
Saccharicola bicolor **(D. Hawksw., W.J. Kaiser & Ndimande) D. Hawksw. & O.E. Erikss.**	Rakotonirainy et al. (2007)
Scopulariopsis **sp. Bainier**	Borrego et al. (2012)
Scopulariopsis nigricans **(d)**	
Scopulariopsis brevicaulis **(Sacc.) Bainier**	Bacilkova (2006)
Sordaria **sp.Ces. & De Not. (e)**	
Sporotrichum pruinosum **J.C. Gilman & E.V. Abbott (d)**	
Stachybotrys chartarum **(Ehrenb.) S. Hughes (c)(a)(d)**	
Stemphylium **Wallr. (a)(e)**	
Stemphylium botryosum **Wallr. (c)(d)**	
Stemphylium vesicarium **(Wallr.) E.G. Simmons (d)**	
Talaromyces funiculosus **Thom (a)(c)**	Nol et al. (2001)
Talaromyces helicus **(Raper and Fennel)**	Mesquita et al. (2009); Borrego et al. (2012)
Talaromyces minioluteus **(Dierckx) Samson, Yilmaz, Frisvad and Seifert**	Rakotonirainy et al. (2007)
Talaromyces pinophilus **(Hedgcock)**	Michaelsen et al. (2009)
Talaromyces purpurogenus **Stoll (a)**	Zotti et al. (2011)
Talaromyces ruber **Stoll. (a)(c)**	
Thielavia **sp. Zopf**	di Bonaventura et al. (2003, not conclusive)
Toxicocladosporium irritans **Crous & U. Braun**	Mesquita et al. (2009)
Trichoderma **sp. Pers (e)**	Gambale et al. (1993); Corte et al. (2003); Lourenço (2005); Michaelsen et al. (2009)
Trichoderma citrinoviride **Bissett**	Rakotonirainy et al. (2007)
Trichoderma harzianum **Rifai (a)**	
Trichoderma koningii **Oudem.**	Rakotonirainy et al. (2007)
Trichoderma pseudokoningii **Rifai**	Zotti et al. (2007)
Trichoderma viride **Pers. (a)(c)(d)**	Lourenço (2005); Bacilkova (2006); Pinheiro et al (2015b)
Trichosporum **sp. Vuill.**	da Silva et al. (2006)
Trichothecium roseum **(Pers.) Link (e)**	
Ulocladium **sp. Preuss (a)(e)**	Corte et al. (2003); Rakotonirainy et al. (2007)
Ulocladium botrytis **Preuss**	Corte et al. (2003)
Ulocladium chartrum ***(Preuss)*** **E.G. Sim-**	Rakotonirainy et al. (2007)

Fungi	Altered Cellulose-based samples: Case studies
mons	
***Ulocladium consortiale* (Thüm.) E.G. Simmons (d)**	
***Ulocladium cucurbitae* (Letendre & Roum.) E.G. Simmons**	Rakotonirainy et al. (2007)
***Verticillium* sp. (e)**	
***Verticillium nigriscens* Pethybr. (d)**	
(Other) Yeasts	Corte et al. (2003); Michaelsen et al.(2009)

(a) Included in the review by Sterflinger et al., 2010. (b) Included in the review by Pinzari et al., 2004. (c) Included in the review by Gallo et al. (2003). (d) Included in the review by Valentin et al. (2003). (e) Included in the review by Zyska (1997).

4.2.4 AFCE Methodology Results

The ITS2 length database being created for the yeasts grown in the culture media has to be able to provide a clear identification of the species present and the values cannot be altered by the presence of more than one fungus.

When mixed and run together the reference size was kept allowing for the identification of each of the individual components (see Figure 4.60) with only a minor difference to the individual analysis of each component. Therefore we can conclude that this method can be effectively used to discriminate the individuals in a mixture.

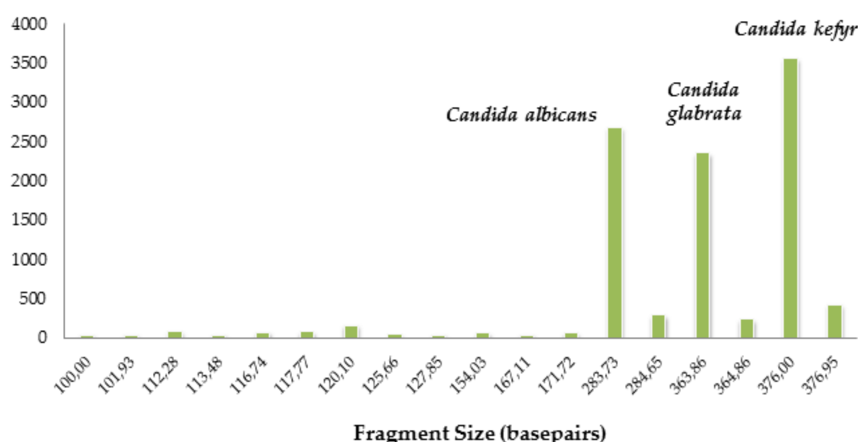


Figure 4.60 Fragment size analysis of a mixture of *Candida albicans* (283,73 bp), *Candida glabrata* (363,73 bp) *and Candida kefyr* (376 bp).
The smallest peaks are stutter bands, common in these analyses and the medium height peaks which have similar sizes to the species peaks are a result of a high concentration of DNA and are also common. Normally, it is considered only the highest peak.

There are very few studies isolating yeasts from surface or air samples in archives. In the study performed by Wiszniewska et al. (2010) on conservation and museum staff, specific IgE to fungi were found in 14 (7%) cases, not only to a selection of filamentous fungi but also to *Candida albicans*.

The study on yeasts is still very incipient but the results obtained by Michaelsen et al (2010a) emphasize their importance since there was positive association between the genera *Candida* and *Cryptococcus* and fermentative activity with carbohydrates on frescoes and textile fibres. Yeasts are known producers of carotenoids and are likely to be associated with foxing stains (Michaelsen, 2010a). More studies are needed to better understand the true role of yeasts. Most of the yeasts species identified using the D2 were also amplified for the ITS2 region in order to create a fragment size database for further identifications. Others - reference strains and environmental samples – were also included in the database (see Chapter 7, clinical studies).

Figure 4.61 presents the AFCE methodology database so far.

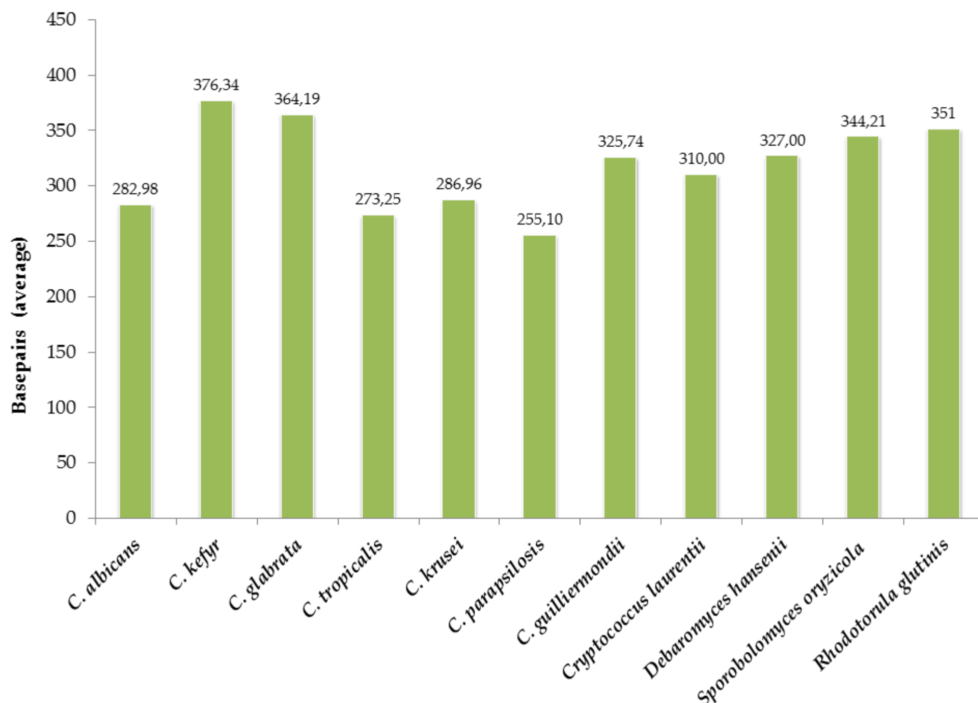


Figure 4.61 – Fragment length sizes from the yeasts found in the sampled DAC's

(Candida parapsilosis, C. glabrata, C. krusei and C. guilliermondii, Rhodotorula glutinis, Debaryomyces hansenii and Cryptococcus laurentiis) and other environmental samples included in the AFCE database.

The standard deviation obtained so far is still under one basepair (0.96bp). Further tests on the reliability and limitations of this method are being performed (Antoniazzi et al, 2014). In the future, the development of this method for the identification of yeasts present in a mixture can be used for complex matrices other than paper or the surfaces tested here.

4.3 Conclusions

One of the research problems posed in Chapter 1 was the choice of assessment methodologies to pursuit the analysis of fungal contamination in Archives. The protocol developed and presented here – which makes use of traditional culturing methods and two distinct molecular biology approaches – proved capable of delivering important results both in terms of human health and paper conservation. The two culture media used in the presented study were essential to identify different fungi in surface samples since the xerophilic species (such as *Wallemia sebi* or *Eurotium sp*.) appeared preferentially on DG18. The MEA is useful to identify the mesophilic fungi and should not be discarded. Still on methodology, molecular biology protocols must be discussed as these were valuable tools. Traditional culturing media only allow the viable fraction to grow and for this to happen the adequate conditions must exist. That's the reason why fungi like *Eurotium halophilicum*, identified in otherwise clean sample depended on the DNA based protocols to be identified. This species in particular is extremely xerophilic requiring only minor amounts of free water to develop.

The molecular biology protocols relied on two methodologies. For DHPLC, certain combinations of DNA from different fungal species may need adjustment of conditions for optimal separation. However, as more samples were processed, the 50°C/40%B sizing program followed by a DSSF program at 59°C/55%B approach seemed to be the most fruitful for most separations. From this point on further temper-

atures (61°C or 63°C) can be tested. The process of comparing both regions of interest – ITS2 and D2 – is underway. As for the AFCE, the database being created and tested can be of great use for the identification of *Candida* and other yeasts. As such, the usefulness of this protocol goes beyond its current application in the air quality evaluation in archives.

Methodology wise, it is relevant to highlight the need for uniformity and the selected methodology, as any other, is as valuable as it is interchangeable. An attempt was made to cover all requirements: two types of samples to account for the differences in the settling behaviour of the fungal elements (the results should be presented in terms of CFU/m3 and CFU/m2); traditional culturing methods since these are still the universal way to assess fungal contamination; two types of media culture to account for water activity differences between fungi; direct swabbing on the mediaplates and not dilution, for instance, as this method can hide the presence of the least represented genera; at least two sampling seasons to account for seasonal variations and to pinpoint any problem that may be occurring. Molecular biology methods are constantly evolving and, as such, it is not so easy to present a methodology. The ITS2, however, should be the region to explore as more and more investigation is made based on it.

Knowing and quantifying the fungal flora present in archives and librairies is extremely important to assess the risk and to devise mechanisms to diminish it. In Portugal, the study by Nunes et al (2013) and the just presented results constitute the only (known) attempts to characterize the fungal contamination in Portuguese archives. In regard to fungal load, the four archives analysed delivered higher levels of contamination than the ones obtained by Nunes et al. (2013). When compared with international results (see Table 2.3) the obtained results in the four Portuguese Archives are lower than many similar settings abroad.

A comparison was also made between the air fungal load results and the existing guidelines for paper conservation. According to these guidelines, only IHRU and TT (with mechanical ventilation) performed satisfactory, if considering the lowest level established by the Dutch guidelines (25 CFU/m^3). However, it was not possible to

achieve any results for the IHRU storage room in the second season and the TT storage room showed high levels of contamination by *Penicillium* spp. in the second season. It was already mentioned that the presence of *Penicillium* spp. can be an indicator of moisture problems. This seems to indicate that mechanical ventilation systems, when working properly can assure low levels of contamination. It is also true, however, that when these systems fail and the room dependes solely on them for ventilation the problem is aggravated since it is not possible to naturally ventilate the room.

The Dutch guidelines predict the possible presence of a source when the air fungal count is located between 25 and 100 CFU/m^3. In the ADE archive, for instance, the source of higher contamination came from the construction taking place in the entrance to the first floor of the archive and the impact on the fungal load was strong enough to push the values pass the 100 CFU/m^3. On the other hand, in the AHU, the levels do not reach the 100 CFU/m^3 and there is definitely a source of contamination in storage room 1.

When trying to define a value for a standard one realises the difficulty of the task. The variety of results obtained for the same settings is overwhelming and hampers the attempts to find a “normal” value. Given the obtained results, and considering the “uneventful” archives, such as the IHRU, optimal values could be represented by a CFU/m^3 below 10. From this value on the variables are just too many. Instead of presenting a static model, it might be useful to track the changes in fungal load between sampling seasons. For archives with natural ventilation and no filtering system acceptable values have to be higher than 10 CFU/m2.

Indoor fungal concentration in warm or hot climates tends to be higher than in cold or moderate climates (Nevalainen and Hyvärinen, 2015) because fungal contamination comes mainly from the outside air. This contradicts some of the results presented in Table 2.4, taken in different areas of the globe, and this study’s contribution to the subject since summer sampling seasons did not steadily translated into higher inside fungal load. However, it should be possible to establish the “normal” fungal load for a given location or even room and interpret the differences from that defined baseline.

The air of the archives was also analysed qualitatively. It showed similarities with the fungal communities found abroad but it also pointed out to the presence of yeasts, which has not been a very common finding in international studies.

Most of the fungal flora found in the air in archives is considered dangerous for paper since damage has been associated with so many of the commonly encountered fungi. However, the relationship between damage and fungi as, in many cases, been made based on the sole presence of the fungi on the paper, book or document. Care must be taken when presenting these associations since this cause/effect is not so easily established. This does not mean, however, that the presence of potentially damaging fungi is not monitored in storage areas and that maximum levels should not be established. The list of fungi to monitor, however, should be narrowed down.

Surface samples can also aid in this monitoring program since house dust concentrations of *Wallemia sebi, Trichoderma viride, Cladosporium sphaerospermum, Eurotium amstelodami* and the combined assay group for *Penicillium spp., Aspergillus spp.* and *Paecilomyces variotii* have been significantly associated with the extent of the moisture damage indoors (de Lignell et al, 2008). If these predictors are coupled with an increase in the air fungal load of *Penicillium* and *yeasts*, then it is possible to anticipate the presence of a source.

Surface samples are, therefore, vital to detect the presence of potentially damaging fungi, assessed in this study not only on the DACs but also on the other surfaces such as tables where the books are set, the shelves where they are kept and the trays that carry them. The knowledge on fungal contamination on surfaces is still very scarce and the presented study increased in almost 100% the number of fungal elements identified in the archive surfaces. Contrary to the existing studies on fungal contamination in the air *vs* the surfaces in archival settings, this study presented a higher diversity in the surface samples.

A great part of the fungal flora found in archival surfaces is also potentially damaging for paper conservation but the same considerations taken for the air samples can be applied here.

The surfaces also brought us information regarding possible cleaning acivities in one of the archives and suffered a large impact with the leaking pipe and construction work that took place in two of the others. The first situation is avoidable but in the last two care must be taken not to submit the documents to such harsh environments. The construction work was responsible for the highest increase in fungal load with values 10x higher than usual. In terms of fungal variety the archive which presented the largest number of species was the ADE, especially in the third season when construction work was taking place.

Environments containing fungal counts between 1-1000 CFU/m^3 are considered low contaminated environments (Nevalainen and Hyvärinen, 2015). However, one must take into account the fact that "total" levels of airborne microbial particles are normally between 10 to 100 times higher when using other than culture –based evaluation methods (Lignell et al, 2008; Nevalainen and Hyvärinen, 2015). This maybe the cause why, even in low mould-contaminated indoor settings, there are occupational diseases such as asthma, fungal allergy, hypersensitivity pneumonitis and other health outcomes (Nevalainen and Hyvärinen, 2015).

In terms of fungal contamination and human health, no potentially pathogenic was found and no indoor space presented values higher than 500 CFU/m3 but the I/O ratio was higher than 1 in many of the analysed rooms. Toxinogenic fungi were present in relatively low amounts since they represented 5% of the total fungal flora identified in the air samples.

The definition of health standards for archival settings goes beyond the scope of this dissertation and for these locations the 2006 legislation still applied by the time this study was performed. However, the experience on applying both limit values and strategies to the obtained data demands some comments:

1. The emphasis on a I/O ratio higher than one disregards visible fungal growth and this can lead to quickly evolving dangerous situation where all the conditions for mycotoxin production are met. As such, visible fungal growth should not be admissible in any building type.

2. The separation between the potentially pathogenic and the potentially toxinogenic fungi is beneficial since these two groups are quite distinct on the risk they pose for different population. The potentially toxinogenic group is better defined in the 2013 legislation and reinforces the need for species identification. This may take us, however, to the problem of the insufficient resolution obtained when just using the culture method. Specific molecular biology markers can be developed to better address the need for species identification.
3. Surface samples should be performed simultaneously as they provide a wider spectra and are, sometimes, the only way to identify potentially toxinogenic fungi.

Some of the remarks made to the changes in the Indoor Air Quality requirements for Office and Service Buildings can be taken into account in indoor air quality in Archives in indoor air quality in Archives when considering the conservation requirements. Instead of addressing the potentially toxinogenic group of fungi, as mentioned above, specific markers can be created for fungi with recognized biodeterioration abilities.

As mentioned in the 2008 editorial from Indoor Air, in a special issue covering the most relevant breakthroughs presented at the Indoor Air Quality Conference held in Copenhagen, dampness can be linked to health problems but it is still not perfectly clear if the association between dampness and health is due to mould (Sundell, 2008). In fact, indoor air, being a "young science", still leaves many questions unanswered. We still not know, for instance, what indoor air components are responsible for triggering asthma or allergies. Volatile organic compounds, reactions between ozone and terpenes or even cleaning systems may play a relevant role in the onset and development of health issues. The next Chapter brings us an insight on the current Indoor Air Quality surveys applied to Portuguese Archives.

5. Indoor Air Quality in Portuguese archives: preliminary results

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5.1. Introduction

Archives and librairies are safe keepers of written heritage but the health of its staff and visitors is often endangered by the environments created in settings. The microbiological component of a given environment, detailed in Chapter 4, is not the only parameter needed to assess indoor air quality.

From 2006 and up until 2013, the Portuguese legislation on indoor air quality (IAQ) referred to the Decree Law n. 78/2006, April 4th for all buildings. This legislation was developed secondly to a European directive on Energy Certification (Directive 2002/91/CE of the European Parliament and of the Council, December 16th, 2002) and was associated with the certification of Building's Energy Systems (Portuguese Decree-Law n. 79/2006, April 4th). Since people spend 70-90% of their time indoors, a good IAQ has become a societies' need and stipulated limits help to reinforce its importance. When this study was performed, the technical certification of a good indoor environment depended on the results of a series of analysis and procedures. The contaminants assessed and the legislated limits mentioned in the 2006 Decree –Law are presented in Table 5.1.

These levels apply to archives, libraries and museums, since these are public spaces but in these locations, however, contaminants levels must be kept low for people's sake but also to guarantee the correct preservation of works of art and cultural heritage. Books and documents are an important part of this heritage and, as mostly organic materials, very susceptible to the presence of chemical and biological contaminants.

Carbon dioxide effects on health result from exposure to high levels of this gas and can go from unnoticed at rest to gradually increasing shortness of breath and increased breathing rhythm. Repeated exposure provokes headaches and with increased levels of exposure comes increased heart rate, sweating, dizziness, shortness of breath, muscular weakness, loss of mental abilities, drowsiness, and ringing in the ears (Baxter, 2000).

Carbon monoxide is an important ambient air pollutant. Both acute and chronic exposure to carbon monoxide are associated with increased risk for adverse cardio-pulmonary events (Chen et al., 2007).

Formaldehyde can be toxic, allergenic, and carcinogenic. At concentrations above 0.1 ppm (parts per million) in air, formaldehyde can irritate the eyes and mucous membranes. Inhaled formaldehyde may cause headaches, breathing difficulties and trigger or aggravate asthma symptoms (OSHA, 2008).

Ground level ozone can harm lung function and irritate the respiratory system. Exposure to ozone and the pollutants that produce it is linked to premature death, asthma, bronchitis, heart attack, and other cardiopulmonary problems (WHO, 2003; Weinhold, 2008).

VOCs are a class of compounds which includes chemical species of organic nature and even though the majority of gaseous pollutants are inhaled and mainly affect the respiratory system they can also induce haematological problems and cancer (Kampa and Castanas, 2008).

Particulate matter (PM), term used for a mixture of solid particles and liquid droplets suspended in the air, is composed of both coarse (PM10, <10um) and fine particles (PM2.5, <2.5um). The former, under 10um diameter, are a result of mechanical disruption, evaporation of sprays, and suspension of dust and in its constitution one can find aluminosilicate and different oxides of crustal elements (Pinheiro et al, 2014b). Exposure to inadequate levels can lead to adverse health outcomes and material damage, artistic and historical objects included (Pinheiro et al, 2014b). Several epidemiological studies have linked both PM10 and PM2.5 with significant health problems (Oberdorster, 2001). Particles with aerodynamic diameter smaller than 2.5um, correspond to the breathable particle fraction capable of penetrating the alveolar region of the lung and have, according to the US Environment Protection Agency (US EPA), a greater association with mortality and morbidity rates than PM10 (Pinheiro et al, 2014b). On a mass basis, small particles generally induce more inflammation than larger particles, due to a relative larger surface area (Donaldson et al., 2001; WHO, 2003). Wheezing,

exacerbation of asthma, respiratory infections, chronic bronchitis and (exacerbation of) chronic obstructive pulmonary disease are some of the problems that can arise from inhalation of these particles (Pinheiro et al, 2014b).

Table 5.1 Parameters used to determine indoor air quality and their legislated limits as stipulated in the Decree Law n. 78/2006, April 4th and the Technical note NT-SCE-02.

Parameter	Maximum Reference Concentration (MR)	Notes
Carbon Dioxide (CO_2)	1800 mg/m^3 (984 ppm)	±10%; takes into account the number of people present and the space's maximum capacity.
Carbon Monoxide (CO)	12.5 mg/m^3 (10.7 ppm)	±10%
Formaldehyde	0.1 mg/m^3 (0.08 ppm)	±20%
Volatile Organic Compounds (VOCs)	0.6 mg/m^3 (0.26 ppm for isobutilene)	±10%
Particulate Matter (PM10)	0.15 mg/m^3	±10%
Ozone	0.2 mg/m^3 (0.1 ppm)	±10%
Radon*	400 Bq/m^3	±10%
Fungi	500 CFU**/m^3	a)No more than 150 CFU of unusual species** b)The presence of *Stachybotris* sp., *Aspergillus fumigatus* and other toxinogenic fungi must not be detected
Bacteria	500 CFU/m^3	Indoor concentration must not be superior to the outside concentration in more than 300 CFU
Legionella	100 CFU/m^3 H_2O	
Temperature	18-22 (can reach 25°C)	
Relative Humidity	50-70%	

*Only determined in granitic areas; **other than *Cladosporium* sp., *Penicillium* sp. and *Alternaria* sp (see Chapter 4).

Fungi have been discussed in the previous Chapters and the guidelines have been presented earlier (see Chapter 2 and Chapter 4). Bacteria in general and *Legionella* in particular, have not been addressed in this study.

Despite the generally accepted need for preservation, the establishment of limits of exposure is still controversial due to the very wide range of existing guidelines and the difficulty of setting one limit when artefacts (books included) are composed of very different materials and show very different susceptibilities. Nevertheless, efforts have been made to try and keep contaminants levels below certain limits. For the contaminants common to air quality and conservation a very brief list of effects and existing guidelines is given in Table 5.2.

Table 5.2 - Parameters and guidelines used for conservation in museums, archives and libraries

<table>
<tr><th>Parameter</th><th>Effects</th><th>Guidelines</th></tr>
<tr><td>Volatile Organic Compounds (VOCs)</td><td>- Lower the degree of cellulose polymerization
- Yellow paper and photographic documents
- Corrosion of metals</td><td>For acetic acid: 400 ppb (Tétreault, 2003)</td></tr>
<tr><td>Particulate Matter (PM10 and PM2.5)</td><td>- corrosion
- soiling,
- abrasion
(Tétreault, 2003; Bellan et al., 2000; Kuprinska et al.,2012; Chianese et al., 2012)</td><td>a) PM2.5: between 0.1 and 10 $\mu g/m^3$ Tétreault, 2003
b) PM10: 30 $\mu g/m^3$, (MIBAC, 2001)
c) Conservation categories (proposed by (Pinheiro et al., 2014b)
<table>
<tr><th>Conservation Categories</th><th>PM2.5</th><th>PM10</th></tr>
<tr><td>A</td><td><1</td><td><10</td></tr>
<tr><td>B</td><td>1-5</td><td>10-30</td></tr>
<tr><td>C</td><td>5-10</td><td>30-75</td></tr>
<tr><td>D</td><td>>10</td><td>>75</td></tr>
</table></td></tr>
<tr><td>Ozone</td><td>- fading of dyes and pigments (indigo family, madder lakes and salmon) (Whitmore and Cass, 1988; Whitmore et al., 1987)
- ozone-induced attack on organic materials such as cellulose (Salmon et al., 2000, Katai and Schuerch, 1966)</td><td>a) 1-13 ppb (Salmon, 2000)
b) average of 1 ppb (Baer and Banks, 1985, Salmon et al., 2000)
c) Dosimeter threshold values at 55% RH are set for 1.15 ppb (Grontoff, 2008)
d) Usually accepted guideline of 1 ppb in storage areas (Tétreault, 2003; Salmon, 2000)</td></tr>
</table>

<table>
<tr><th>Parameter</th><th>Effects</th><th>Guidelines</th></tr>
<tr><td>Relative Humidity and Temperature</td><td>- Hydrolysis
- Mechanical Strain
- Biodeterioration</td><td><table>
<tr><th>Conservation Categories</th><th>RH%</th><th>T (ºC)</th></tr>
<tr><td>AA</td><td>50</td><td>20(±5)</td></tr>
<tr><td>A</td><td>50(±10)</td><td>20(+5, -10)</td></tr>
<tr><td>B</td><td>50 (±10)</td><td>T+10 (but T<30) no limit for winter set-back</td></tr>
<tr><td>C</td><td>>25 and <75</td><td>< 30</td></tr>
<tr><td>D</td><td>< 75</td><td>-</td></tr>
</table>(ASHRAE, 2003)</td></tr>
<tr><td>Fungi</td><td>See chapters 1, 2 and 4</td><td>No guidelines have been proposed. Special cellulolytic species, such as Trichoderma, should be matter of concern.</td></tr>
</table>

Since most museums, archives and libraries cannot afford complex monitoring and air quality measurements in their buildings a simple and short assessment was performed. This snapshot can be extremely helpful in identifying potential problems and should be considered a stepping stone towards more thorough studies. There are no published results on air quality exposure levels (from immediate measurements or long monitoring periods) on Portuguese archives and this constitutes the first snapshot assessment considering both human and conservation needs.

52. Material and Methods

Two of the already described archives were selected for this particular study: AHU, in central Lisbon and IHRU, a modified fortress in a less polluted location. Active sampling, as performed in this study (see Chapter 3 for details) can give the institution a valuable snapshot of the IAQ conditions (Blades et al., 2000) and, because it is cheaper and easier to install than a passive diffusion method, it can help the institution assess the need for a more thorough study.

Several rooms were selected for study: reading room, storage rooms, office rooms and restoration room. Two surveys were performed: one in winter and the other in summer, to account for possible changes between seasons (see Chapter 3 for details).

5.3. Results and Discussion

The analysed parameters levels are discussed for both human health exposure and conservation needs (when relevant).

5.3.1 Carbon Dioxide

The carbon dioxide measured level had to be adjusted for the real and maximum number of occupants according to equation 1.

$$([CO_2]_{MedT} - [CO_2]_{Ext.})x \frac{N_{occup.max}}{N_{occup.}} + [CO_2]_{Ext.} \leq [CO_2]_{MR}$$

Equation 1

No determination was above the maximum reference concentration for this pollutant since the maximum obtained value was 792 ppm. There is no guideline established for conservation since no effects from exposure have been determined.

5.3.2 Carbon Monoxide

A non-conformity is present when the inside values are above legislated (MR=12.5 mg/m^3 (10.7 ppm)). This was not the case for any of the rooms analysed since the maximum value obtained was 2 ppm. As happened with CO_2, there is no guideline established for conservation.

5.3.3 Volatile Organic Compounds (VOCs)

The MR for this wide group of contaminants is 0.26ppm (isobutilene was used as reference). IHRU showed very low readings in all of the rooms analysed but AHU showed values of 2.0 ppm and 2.6 ppm in storage room 1 and 2, respectively. The storage room with the 2.0 ppm value presented, at the time of measurement, visible fungal

growth due to a plumbing problem with water leakage, a problem already mentioned in Chapter 4. Some fungi are capable of emitting volatile compounds which can be read by the device used (Ionization energy below 10.6eV) (Wilkins et al., 2003; Demyttenaere et al., 2003, 2004). In the case of the second storage room, where staff was temporarily allocated, a very intense odour was felt and complaints of headaches and general discomfort started to appear. The staff had to be removed and, in season 2, a second VOC reading was performed with a zero result in both rooms. The authors attribute this value to the possible formation of complex components which cannot be read by the equipment used (Wilkins et al., 1997). Forced ventilation (opening of windows) was performed in storage room 2 and the case is still under study. Air purification equipment was acquired. In terms of health legislation, when the value is higher than advised, the individual components should be identified and this information was transmitted to the institution.

For conservation purposes, the existing guidelines are still very raw and require further studies and refinement. As recommended for health purposes, however, individual components should be identified and a complete study performed.

5.3.4 Formaldehyde

A non-conformity is present when the inside values are above legislated (MR=0.08 ppm). This was not the case for any of the rooms analysed since the maximum value obtained was 0.003 ppm.

5.3.5 Particulate Matter

Although there are only legislated limits for PM10, a study on both PM10 and PM2.5 was performed since recent investigations have highlighted the importance of the finer particulate matter (Pope, 1996; Oberdorster, 2001; WHO, 2003). The rooms with the highest human activity should present values below 150ug/m^3 for PM10 and desirably below 75 μg/m^3 for PM2.5 (Pinheiro et al., 2014b). Two measurement seasons were performed and Figure 5.1 shows the concentrations obtained in the working/reading rooms of both archives studied.

For PM2.5, none of the rooms presents levels higher than 75 µg/m3 which may point to a possible overestimation of the proposed limit. In fact, most of the rooms show values below 50 µg/m3 and, as such, this new found limit might be more appropriate for future evaluations.

For PM10, none of the rooms showed values above legislated in any of the readings performed in the winter season. Regarding the AHU all of the ratios I/O were higher than one in the first season. In the second season AHU registered a maximum of 285.52 µg/m3 in the reading room, much higher than legislated. The restoration room presented very similar levels for PM10 in both seasons.

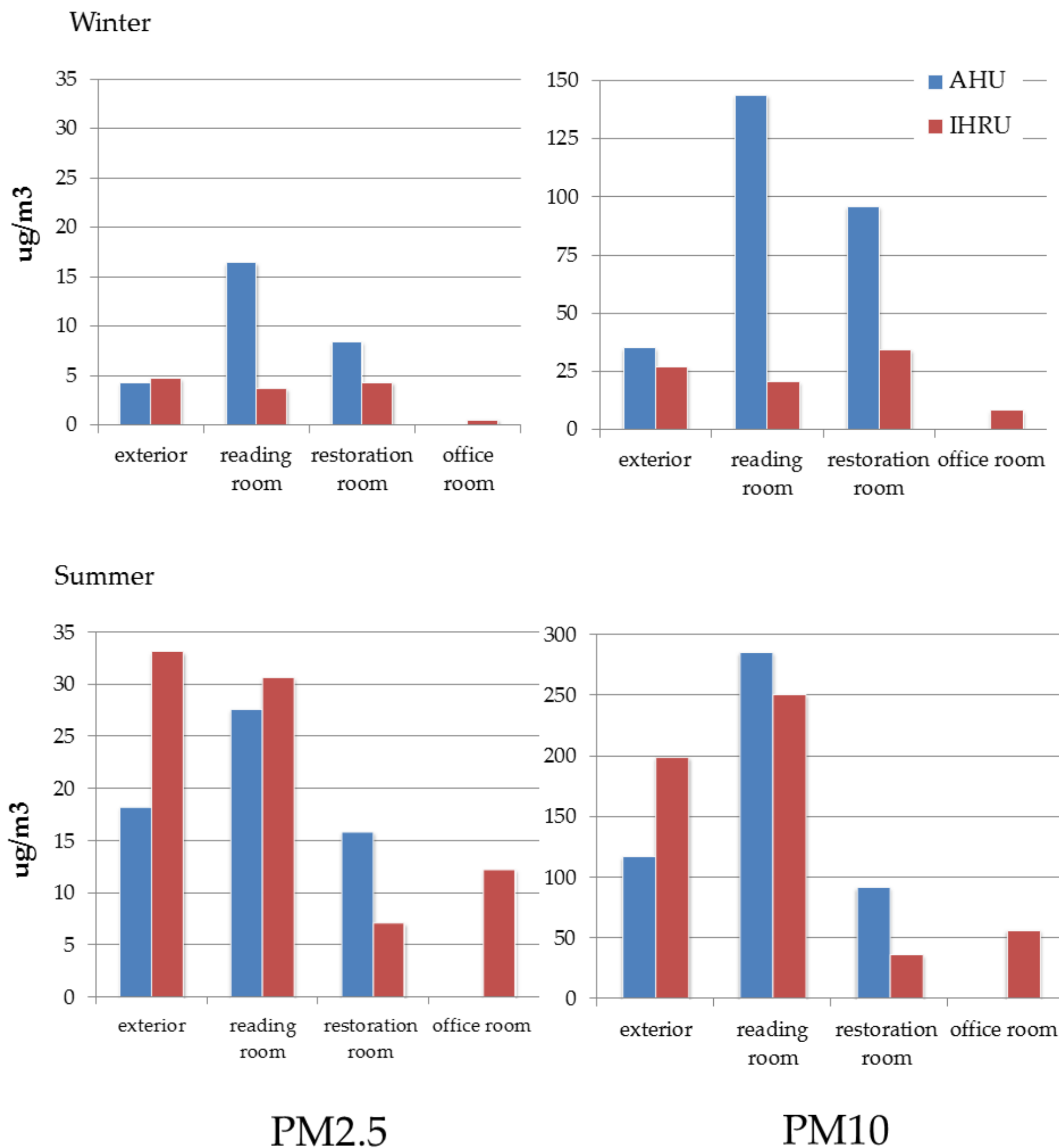

Figure 5.1 - Particulate matter distribution in both archives and seasons. Only the locations where staff or visitors could be found are presented. Please note the different scale on PM10 Summer season.

For IHRU, has had happened with AHU, the highest value (250.79 μg/m3) was obtained in the reading room and was also the only result above the legislated limit.

In general, particulate matter concentrations were higher in the second season and the weather conditions in the winter (precipitation contributes to particulate matter settling) are probably responsible for this asymmetry (Freitas and Solci, 2009).

In terms of conservation, and considering only the storage rooms, the obtained PM10 and PM2.5 concentrations for both seasons and archives are present in Figure 5.2.

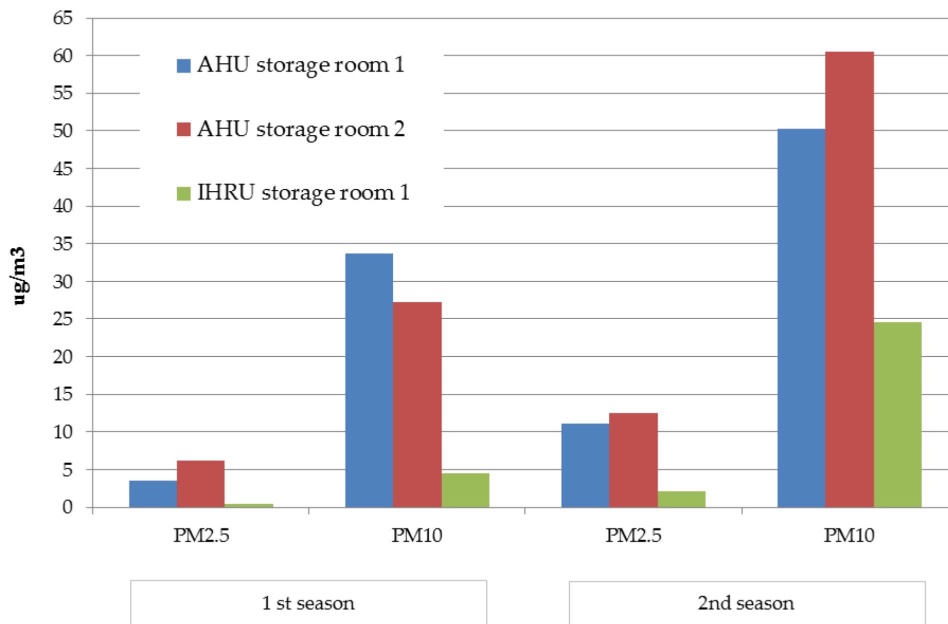

Figure 5.2 - Particulate matter distribution in both archives and seasons. Only the locations where documents are kept are presented.

The authors proposed conservation levels based on the PM10 and PM2.5 concentrations (see Table 5.2) and IHRU presented level A results for both PM2.5 and PM10 in the first season and level B in the second season. For AHU most results fall within the C or D ranges for both particulate matter dimensions studied.

5.3.6 Ozone

For both health and conservation concerns, the ozone levels were higher than recommended in all of the analysed locations. Figure 5.3 illustrates the obtained results.

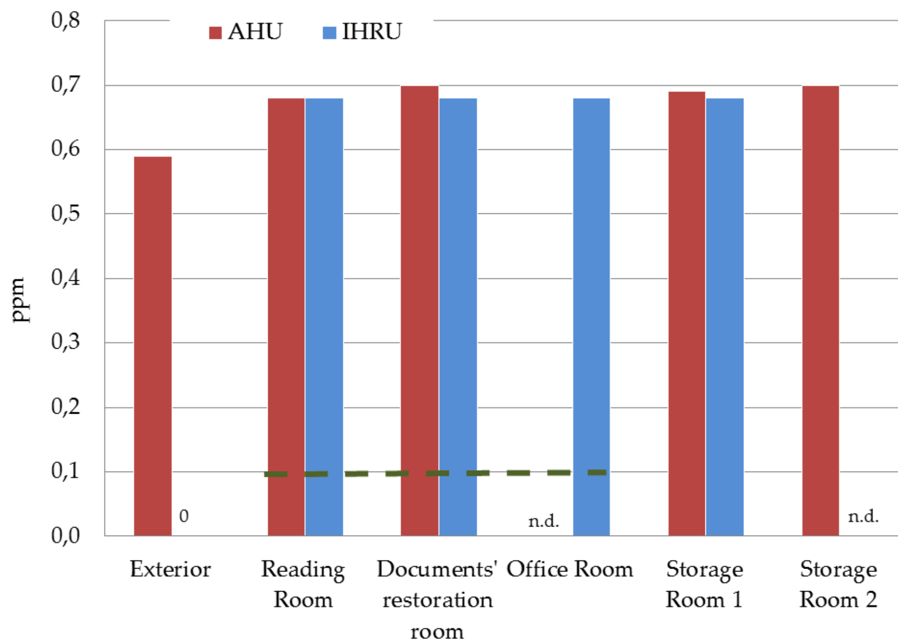

Figure 5.3 - Ozone levels (ppm) for the analysed rooms in both studied archives. Also pointed out is the legislated limit (0.1 ppm) for the ozone level in Portugal. The conservation guideline (0.001 ppm) is much inferior and is not visible in this Figure.
nd= not determined

Ozone levels above 0.1 ppm can eventually induce the onset of respiratory symptoms with decrements in lung function and inflammation of the airways. For certification purposes these values are motif for non-conformity but the law foresees a second measurement and the implementation of corrective measures.

In terms of conservation, the limit is not visible in Figure 5.3 and is severely crossed in both assessed storage rooms. Above 50 ppb paper and organic colorants on watercolour paper and silk change in 1 year and photographic film dyes and images change in six months (Grontoff, 2008). Studies performed by Druzik et al. (1985) in a series of museums, galleries and archives show levels of indoor ozone that for no HVAC system and no filter range between 0.012 and 0.146 ppm, which are much lower than the ones recorded in these settings. Also in Krakow, Poland, the ozone concentrations ranged between 42-21 ppb and the indoor concentrations were always lower than

outdoors (circa 40% lower) even in natural ventilated settings (Salmon et al., 2000).Given the very high results obtained, further studies were advised.

5.3.7 Temperature and Relative Humidity

The results obtained for the temperature and relative humidity are displayed in Table 5.3.

Table 5.3 - Relative Humidity and Temperature determined at each location in both seasons.

Archive	Measurement Location	1st Season (winter)		2nd Season (summer)	
		RH(%)	T(ºC)	RH(%)	T(ºC)
AHU	Exterior	51	10	56	23
	Restoration Room	**47**	**17**	67	22
	Reading Room	**46**	15	62	23
	Storage Room 1	**70**	18	**76**	20
	Storage Room 2	**77**	19	**79**	19
IHRU	Exterior	83	13	81	20
	Reading Room	53	19	66	23
	Restoration room	59	18	**82**	22
	Office room	57	**14**	**74**	21
	Storage Room 1	56	14	60	19

For comfort purposes, levels can range from 18 to 22°C (25°C maximum) for temperature and 50-70% for relative humidity. Despite the legislated sanctioning of this maximum RH level, there should be an attempt to keep the levels below 60%.The non-conformal results are highlighted in a square and the most significant of them refer to incorrect relative humidity: slightly lower than desirable in AHU's working rooms (due to portable heaters used to increase temperature) and higher than desirable in IHRU's working rooms during summer. A relative humidity of 82% places this res-

toration room in considerable danger of mould development in locations where condensation may occur and can potentiate the appearance of microclimates and increased water activity.

For conservation purposes - analysis of the storage rooms in both archives - the values were highlighted in bold underlined and are only above desirable limits in AHU (both seasons) where the levels are very high and conducive of mould development. Taking into account both the relation between T and RH and the fluctuations observed, IHRU performs the best in terms of thermo-hygrometric conditions and is assigned a level A. AHU's high humidity levels - above 75% - place it below level D and at serious risk of mould development. There is also a decrease in the *life time* expectancy for the documents kept at these conditions. Because these are only immediate data continuous monitoring of these values was advised (Pinheiro et al., 2014b).

5.3.8 Fungi

None of the analysed rooms showed counts above 500 CFU/m^3, as legislated. Figures 5.4 to 5.7 show the total number of CFU and the relative proportion of each fungal genera identified in both archives and for the first and second season.

In AHU the maximum number of CFU is registered in the reading room, with 140 colonies (Pinheiro et al., 2011b; Pinheiro et al., 2011a, Pinheiro et al., 2012b). This is also the only room with a I/O above one in the first season.

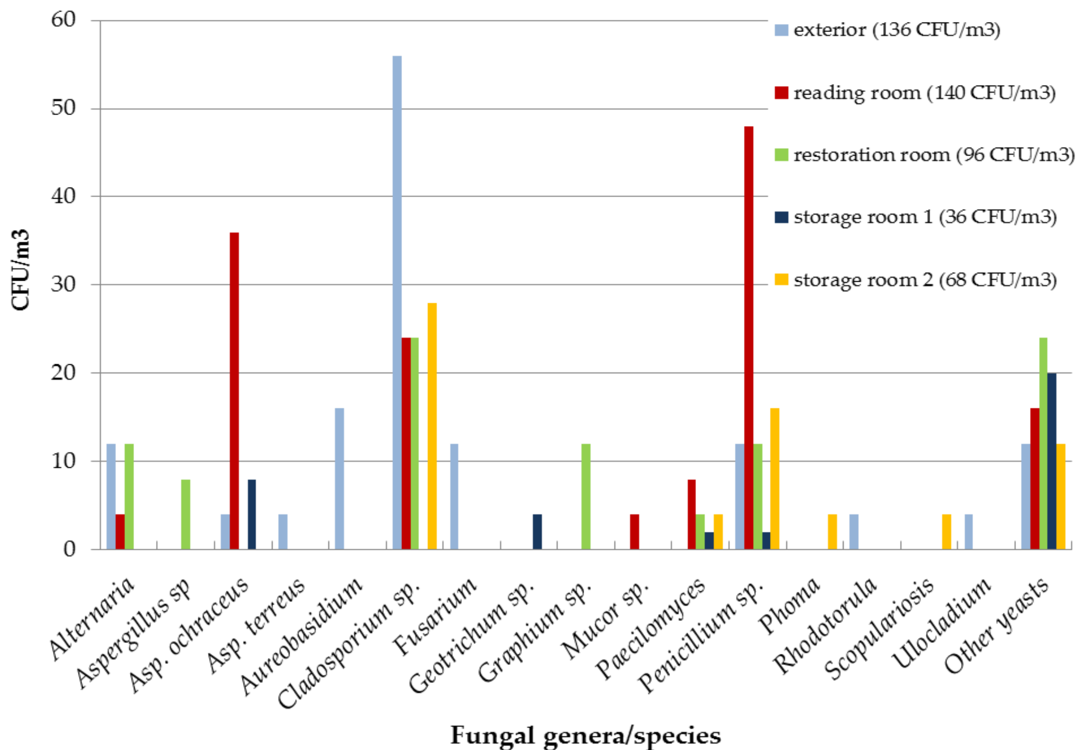

Figure 5.4 Quantitative and qualitative fungal assessment in each of the spaces analysed in AHU, in the first season, winter.

No toxinogenic fungi were found (Aspergillus sp. does not include Asp. *versicolor*, *niger* or *fumigatus*) in the air samples) but *Stachybotrys atra* presence was confirmed when the surface showing visible growth was swabbed (Pinheiro et al., 2011b; Pinheiro et al., 2011a, Pinheiro et al., 2012b). This fungus, due to its characteristics, does not usually appear in the air sample and surface samples (a methodology not mentioned in the NT-SCE-02). Also worth mentioning is the appearance, indoors, of fungi not found outside which suggests indoor contamination (Kemp et al., 2003).

According to the NT-SCE-02, the compliance with all of the following requirements renders unnecessary a second fungal assessment: lack of complaints; absence of obvious sources of biological contamination; PM10 concentration below 25% of the maximum reference level (inferior to 37.5 μg/m3) and an I/O ratio for fungal contamination inferior to one. In this particular archive and in the first season assessed: the reading room presented an I/O above one and a PM10 concentration of 143.46 μg/m^3;

and the restoration room also had a PM10 level above 37.5 μg/m³. A second assessment was due. Though not covered by the rules created for the reading and working rooms (public rooms), storage rooms 1 and 2 were also reassessed since fungal growth was visible (a non-conformity by itself if judged by the NT-SCE-02) in storage room 1 and complaints were registered from staff temporarily allocated to storage room 2. Since people can (and sometimes do) work in storage areas is important to apply the same indoor air quality in these areas as well.

The results from the air samples performed in the second season, in summertime, are presented in Figure 5.5.

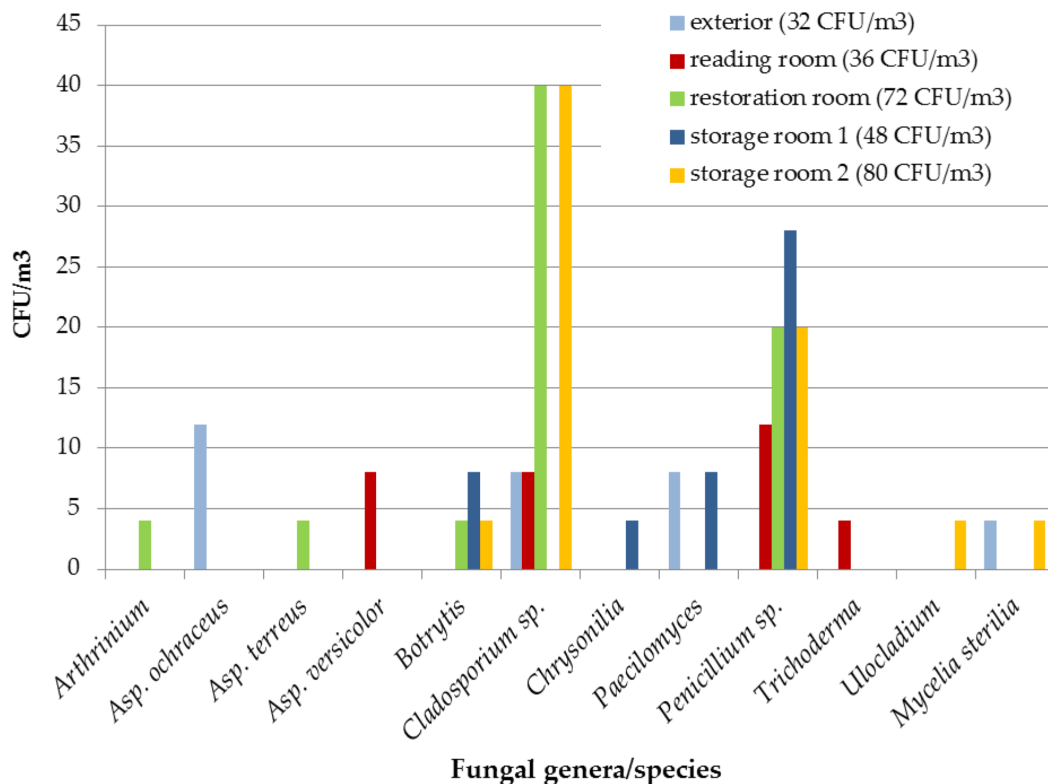

Figure 5.5 - Quantitative and qualitative fungal assessment in each of the spaces analysed in AHU, in the second season, summer.

All rooms showed higher than one ratios for fungal contamination. So, although, the 500 CFU/m³ limit was respected in both seasons this archive still showed motifs for non-conformity: 1) visible presence of fungal growth; 2) higher than one ratios; 3) con-

firmed presence of *Aspergillus versicolor* in the second determination (8 CFU/m3) in the reading room. Recommendations were made to increase the air quality of the given environments. For the documents' conservation, the presence of *Trichoderma* (reading room, second season) is particularly relevant since this is a cellulolytic fungi that can degrade cellulose (Pinheiro et al., 2011b).

In the IHRU archive the number of CFU is also much lower than 500 CFU/m^3 (see Figures 5.6 and 5.7) in both seasons but again there are fungal species indoor which are not found outdoor.

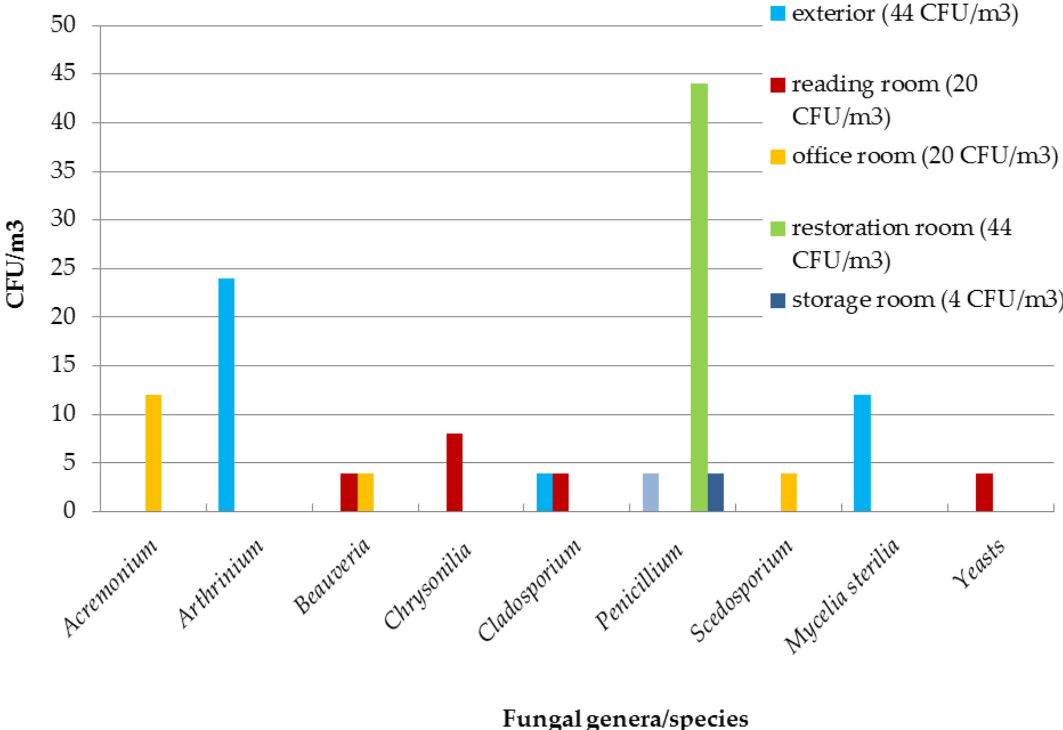

Figure 5.6 - Quantitative and qualitative fungal assessment in each of the spaces analysed in IHRU, in the first season (Winter).

In the first visit there was no visible fungal growth and no higher than one I/O ratios. A more thorough analysis also ruled out the presence of toxinogenic fungi. All conditions were met for a good IAQ.

As mentioned before, since no complaints were made or evident sources of contamination registered, the second monitoring season could then be scheduled only for the restoration room, the only room with a PM10 higher than 37.5 μg/m³.

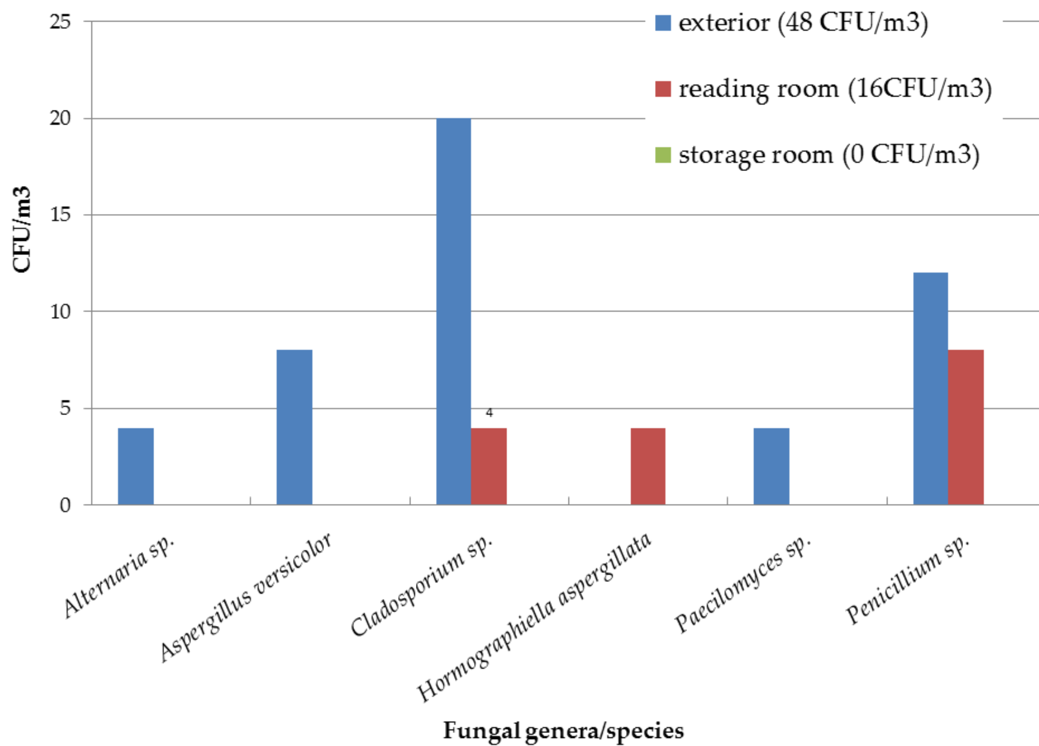

Figure 5.7 - Quantitative and qualitative fungal assessment in each of the spaces analysed in IHRU, in the second season (Summer).

For academic reasons a complete analysis was performed also in the second visit but regrettably the media used for culture incubation in the office and restoration rooms were not stable and the fungal count and identification could not be performed for these two rooms. For the locations assessed, no fungal count was obtained in storage room and the reading room presented levels lower than in the first season and within limits. No potentially toxinogenic fungi were identified indoors.

As far as the study allowed, a good air quality was maintained from the first visit to the second.

In terms of fungal load, and contrary to several studies, there was no in-crease in the summer vs the winter samples as happens in librairies (Bueno et al.,2003) and in other settings (Ali-Shtayedh et al.,2002, Panagopoulou et al., 2002; Medrela-Kuder, 2003; Martins-Diniz et al.,2005, and Ramachandran et al.,2005.)

The influence particulate matter can have on fungal contamination was also evaluated. Figure 5.8 plots the data from these two variables.

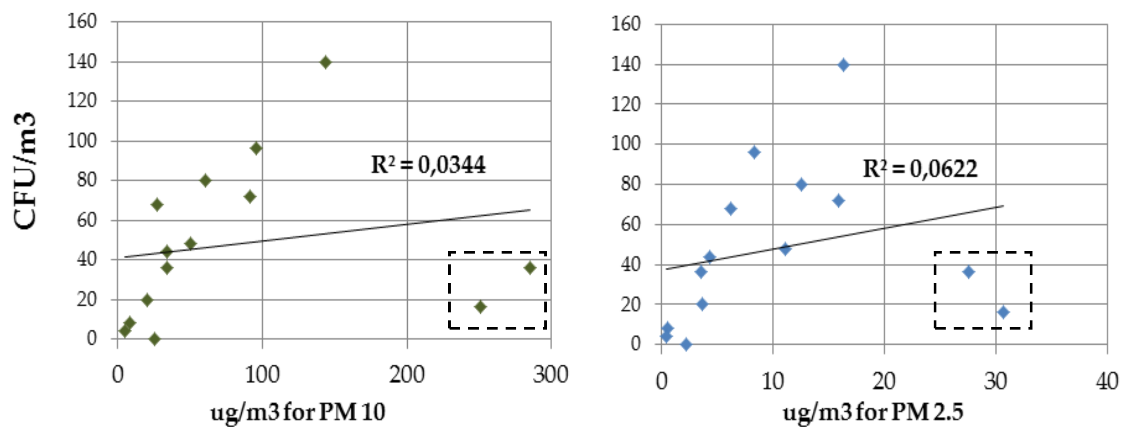

Figure 5.8 - Influence of particulate matter (PM10 and PM2.5) on fungal contamination.

Particulate matter, regardless of its size, does not seem to play a strong role on fungal contamination. However, if only "normal" values are to be considered (excluding the extremely high levels of particulate matter found on both reading rooms, in the second season, highlighted in a dotted square in Figure 5.8), then the relationship between these two variables is strong as shown in Figure 5.9.

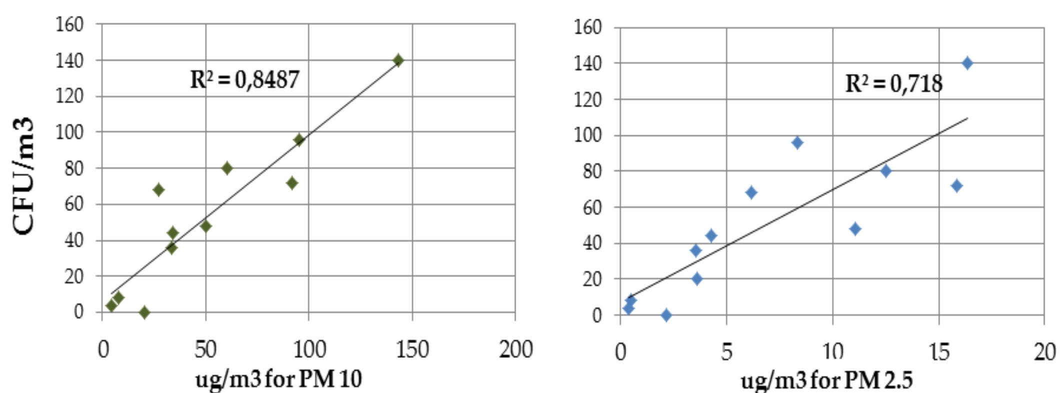

Figure 5.9 - Influence of particulate matter (PM10 and PM2.5) on fungal contamination (adjusted).

These outliers can be justified as these rooms have windows and air drafts or movement of chairs can induce higher immediate particulate matter readings, which can return higher than usual results. Without considering these outliers, the PM10 and PM2.5 particulate matter influences air fungal contamination with an 84% and 72% contribution, respectively.

5.4. Conclusions

Air quality certification is a recent but very valuable requirement. The authors applied the official certification technical approach to Portuguese archives where the safekeeping of cultural heritage goes hand in hand with the health of staff and visitors. From the analysed parameters, carbon monoxide, carbon dioxide and formaldehyde were kept within stipulated limits. Fungal counts, although kept within limits in both archives, were only a confirmation of a good IAQ for one of the archives, since the other presented visible fungal growth (where *Stachybotrys atra* was identified) and higher than one I/O ratios. The authors stress the importance of a surface swab to identify this particular toxinogenic fungus a procedure not foreseen in the technical note. For conservation purposes, the presence of a cellulolytic (*Trichoderma* sp.) fungus is worth mentioning.

Relative Humidity, VOCs, PM and ozone showed values above legislated and justify the implementation of corrective measures. Further studies were also recommended, especially for VOCs, since the technical note also foresees the identification of the individual components when this parameter is above the stipulated maximum concentration.

In terms of conservation, studies on the limit values are still needed but, according to the available international guidelines, some of the analysed parameters (PM, ozone and RH) are also above desirable values. Corrective measures were proposed to these institutions and further studies are also warranted for VOCs since different materials suffer differently from the action of different chemicals. Other chemicals, such as SO_2 and NO_2 assume a particular importance in documents' preservation and their study should be included in future analysis.

Although limited by the monitoring time, this snapshot on the exposure levels registered in Portuguese archives constitutes a valuable tool for the development of more comprehensive studies and sheds some light on the contaminants levels that can affect our valuable written cultural heritage.

As mentioned before, a new legislation was introduced in 2013 (Ordinance n. 353-A/2013, December 4th) for offices and services. Fungal levels and evaluation strategies have shifted and the current perspective was given in Chapter 4. *Aspergillus ochraceus* and *A.terreus*, for instance, are now considered toxinogenic and their presence was registered in the first and second season in AHU, respectively. However, the approach on fungal concentrations mentioned in the former Chapter was not the only parameter to be altered.

Presented next are the current limit values for other parameters considered relevant when assessing indoor air quality:

- CO_2 is now 2250 $\mu g/m^3 \pm 30$. All the results were below the former, lower value.

- CO was 12.5 mg/m^3 and is now 10 mg/m^3. Again, no review on the data is necessary since the obtained levels were much lower.
- Formaldehyde levels suffered no change.
- VOC's levels suffered no change.
- PM10 limit value was 150µg/m^3 and is now 50 µg/m^3, with a 100% margin. This margin means the maximum level is now 100µg/m^3 and this means the AHU presents higher than desirable levels at the first season also, for the reading room.
- PM2.5 limit levels did not exist and are now set at 25 µg/m^3, with a 100% margin, meaning a 50µg/m^3 maximum level. This value is not reached in any of the studied settings but levels lower than 75µg/m3 were proposed by the authors (Pinheiro et al., 2014b).

Regarding Conservation, at the Indoor Air Quality 2012 Meeting, the levels set to be enforced for particulate matter in the Royal Library in Denmark considered only the PM2.5 fraction and the goal is to achieve levels lower than 0.1 µg/m^3, at the lowest end of the values proposed by Tétreault (2003; see Table 5.2) which comes to reinforce the necessity of these particulate matter measurements (Keiser et al., 2012) to assure paper conservation. Besides, given the relationship it establishes with fungal load, it may even work as a predictor of high indoor fungal contamination. Additional studies are needed to confirm this trend.

Other physical parameters, such as water activity, might also be related to fungal contamination since they are intrinsically connected with the ability of fungi to grow and colonize a given surface. Chapter 6 sheds some light on the issue.

6. Water Activity and Fungal Presence

6.1 Introduction

The presence of airborne biological materials, particularly if they are very abundant, may be a cause of deterioration when the characteristics of the substrate and the surrounding environmental conditions are compatible with the ecological and nutritional needs of the microorganisms (Caneva et al., 2003). The nutritional support can be partially assured by the presence of organic dust which settles on the surfaces. Particulate matter, mentioned in the last Chapter contributes to this deposited soiling. But fungi need more to sustain them. Controlling the relative humidity, also mentioned in the last Chapter, is a common preventive measure to prevent microbial growth.

In time, paper will generally offer an environment with low moisture content when stored following the recommendations for libraries and archives which are set around a relative humidity (RH) of 45-55 % at 18-22 °C However, microclimates in books or even within the paper's structure can differ vastly from the overall RH of a room, and many old books contain unsized tissues that interleave the pages and are capable of absorbing water and to retain it over a long period (Michaelsen, 2010a). Water activity or a_w is the partial vapour pressure of water in a substance divided by the standard state partial vapour pressure of water. The water that is available to support microbial life is expressed as water activity (a_w). Pure distilled water has a water activity of exactly one. Adding solutes to pure water lowers the a_w and less free water is available for fungal growth.

Water activity is, therefore, a crucial factor in determining whether or not mould growth is initiated (Nielsen et al., 2003). Micro-climates with very high aw can be generated in a room with an otherwise low RH. For this reason, a measurement of indoor RH alone can be a poor predictor of mould problems (Nielsen et al., 2003) and merely decreasing might not be enough. Also many of the fungi inhabiting archives and libraries are resistant to changes in humidity and temperature (Ponce-Jimenez, 2002a).

There is no definitive water activity limit value for general fungal growth since this is very species-dependant. However, it is over 0.65 that most known fungi thrive

(Rockland and Beuchat, 1987). Fungi can be divided in categories according to their water activity requirements (Samson et al, 2010).

- Hydrophilic fungi require a a_w higher than 0.9. Included in this group are many yeasts and filamentous fungi such as *Stachybotrys chartarum, Fusarium sp.* and *Chaetomium globosum.*

- Fungi that can grow between 0.8 and 0.9 are defined as mesophilic and these include species of *Alternaria*, *Cladosporium*, some species of *Penicillium* and *Aspergillus*. Most of the know fungi are included in this group.

- Fungi that can grow at aw below 0.8 are known as xerophilic fungi. Examples include *Wallemia* species, *Aspergillus restrictus*, *A. repens* as well as some species of *Penicillium*.

Table 6.1 presents some of the commonly found indoor species requirements in terms of minimal water activity (substrate free water).

Table 6.1 - Minimal water activity for some of the fungal species present indoors (Florian 2002; Samson et al, 2010). Asp. fumigatus presents two values according to the two consulted sources, In bold are the fungi (genera or species) found on document archival cases.

Minimal water activity	Fungal species
0.96	*Rhizoctonia solani*
0.90-0.94	*Botrytis cinerea**, **Exophialia sp.**, **Geomyces pannorum, Mucor** (circinelloides, racemosus, spinosus), Neosartorya fischeri, **Penicillium** digitatum, **Rhizopus** stolonifer, Stachybotrys sp., **Trichoderma** sp., Trichothecium roseum.*
0.85-0.89	***Asp.** (clavatus, **fumigatus**), **Alternaria. alternata**, **Cladosporium** (cladosporioides, herbarium), **Epicoccoum purpurascens**, **Fusarium** (avenaceum, culmorum, graminareum, oxysporum, poae, solani, sporotrichoides, verticillioides), **Penicillium** (oxalicum, rugulosum)*
0.80-0.84	***Aspergillus fumigatus**, Bissochlamys nivea, **Penicillium** (citrinum, commune, griseofulvum, expansum, islandicum, roquefortii, verrucosum, viridicatum, wentii)*
0.75-0.79	***Aspergillus** (**candidus, flavus,, niger, ochraceus**, parasiticus, **sydowii**, tamarii, terreus, **versicolor**), **Exophialia** werneckii, **Paeci-***

Minimal water activity	Fungal species
	lomyces *variotti,* ***Penicillium*** *(aurantigriseum, brevicompactum, chrysogenum)*
0.70-0.74	***Aspergillus*** *(**penicillioides**, restrictus, wentii), Chrysosporium xerophillum,* ***Eurotium*** *(**amstelodamii,** chevalieri, repens,* ***rubrum)***
0.69	***Wallemia sebi****, Asp. halophillicus*
0.64	***Eurotium*** *echinulatum*

To the author's knowledge, the existing studies on a_w are very scarce and no study has yet been performed in Portuguese archives or documents.

In order establish a comparison between fungal contamination and water activity, the surfaces where fungal communities were determined were also analysed for water activity.

6.2 Methodology

Water activity is not directly measured. It is the equilibrium relative humidity (ERH) that is reported: the material is enclosed in a small container where it is left to equilibrate, and the relative humidity in the air above the material is measured. The ERH is reported as a percentage, which is the a_w decimal multiplied by 100. A Rotronic Hygroplam Water Activity measure device was used to assess this parameter on sampled surfaces.

Figure 6.1 - Water activity measurement on a document sample.

A triplicate measure was performed for each sample.

6.3 Results and Discussion

The measured water activity in varied surfaces from the chosen archives ranged from 0.440 in document archival cases to 0.710 in one of the sampled tables.

Table 6.2 presents the data for the surfaces tested:

Table 6.2 – Water activity levels in the surfaces sampled. Also presented are the immediate relative humidity values in the room where the surfaces are located.

Location		Surface	Water activity	RH
ADE	storage room 1	table	0,576	72
	storage room 1	DAC 1	0,601	72
	storage room 1	DAC 2	0,589	72
	storage room 2	DAC 1	0,510	65
	storage room 2	DAC 2	0,534	65
	storage room	floor	0,567	72
	storage room	table	0,562	72
	storage room	DAC	0,588	72
	storage room	DAC	0,591	72
	storage room	DAC	0,578	72
	reading room	floor	0,541	61
	reading room	table	0,514	61
	storage room 2	floor	0,503	66
	storage room 2	DAC	0,551	66
	restoration room	floor	0,511	65
	restoration room	table	0,519	65
Location		Surface	Water activity	RH
AHU	restoration room	floor	0,529	67
	restoration room	table	0,519	67
	storage room 1	floor	0,578	76
	storage room 1	DAC 2	0,670	76
	storage room 1	DAC 1	0,640	76
	storage room 1	DAC 3 (below	0,671	76

Location		Surface	Water activity	RH
		leak)		
	storage room 2	floor	0,673	79
	storage room 2	shelf	0,671	79
	storage room 2	DAC	0,681	79
	reading room	table	0,554	62

Location		Surface	Water activity	RH
IHRU	office room	table	0,580	57
	office room	floor	0,581	57
	office room	table	0,527	57
	storage room	floor	0,468	56
	storage room	shelf	0,469	56
	storage room	DAC	0,513	56
	storage room	DAC	0,530	56
	reading room	floor	0,565	66
	reading room	table	0,572	66
	storage room	floor	0,481	60
	storage room	DAC	0,534	60
	storage room	DAC	0,548	**60**
	storage room	shelf	0,476	60
	office room	table	0,710	74
	restoration room	shelf	0,624	82

Location		Surface	Water activity	RH
TT	restoration room	floor	0,480	52
	restoration room	table	0,460	52
	restoration room	DAC	0,450	52
	restoration room	DAC	0,440	52
	restoration room	DAC	0,440	52
	higienization room	DAC	0,530	53
	storage room	DAC	0,551	58
	storage room	DAC	0,561	58
	storage room	DAC	0,550	58
	working room	floor	0,690	52
	storage room	floor	0,681	77
	storage room	DAC 1	0,670	77

	storage room	DAC 2	0,670	77
	storage room	DAC 3	0,671	77
	higienization room	floor	0,652	71
	restoration room	table	0,561	63

The obtained values were compared with the relative humidity measurements and the fungal load obtained for each of the surfaces mentioned in Table 6.2.

6.3.2 Water activity and Relative Humidity

Ever since the concern for the presence of water near documents has begun that conservation science has been measuring relative humidity in the environment surrounding these objects (Camuffo and Bertolin, 2012). The contribution relative humidity provides for the water activity level is well illustrated in Figures 6.3 and 6.4. The first one takes into account all the surfaces where water activity was measured while the second one includes only the document archival cases.

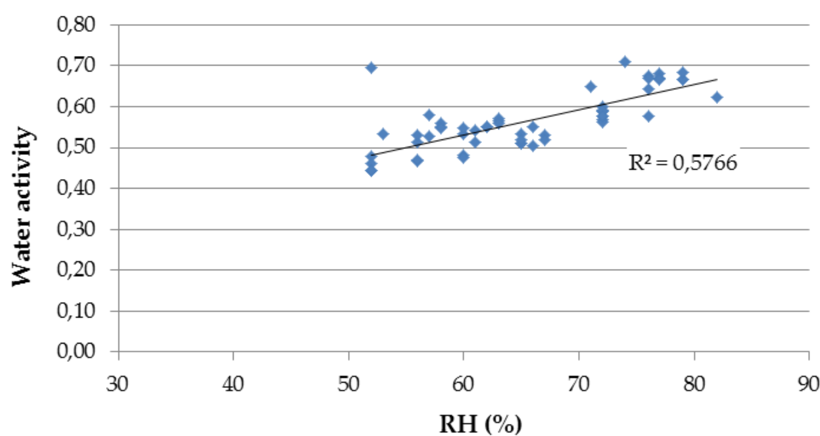

Figure 6.2 – Relative humidity levels and the water activity measured in the different surfaces in all the archives analysed.

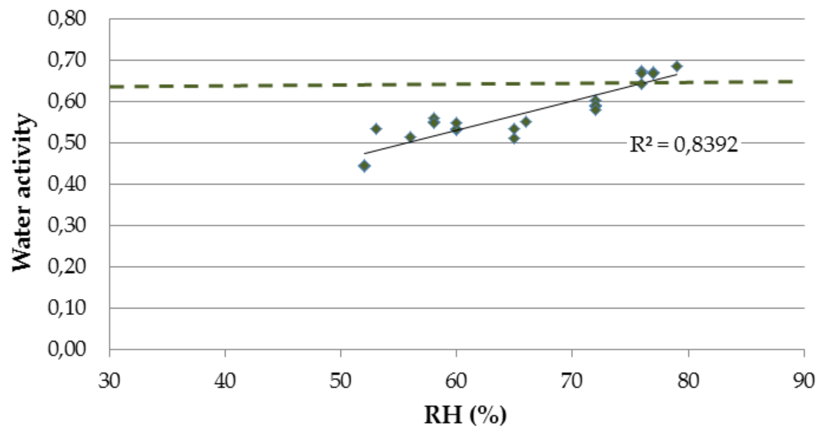

Figure 6.3 - Relative humidity levels and the water activity measured considering only the document archival cases.

In both of them, the RH contribution for the water activity values is considerable. and higher in the document archival cases. This was an expected result since the document archival cases of organic origin and therefore establish an equilibrium with the humidity of the air. Still, the water activity on other surfaces was measured as the microenvironment that is created on these inorganic surfaces can also act like a substrate for fungal growth

One of the possible reasons behind increased contribution is cleaning activities. Floors, tables and shelves are cleaned more often and every time this happens there is a disruption on the equilibrium relative humidity establishes with the surfaces and the particulate matter that lays upon them. More studies are needed to address this matter.

Noteworthy is the fact that many of the performed RH readings are above 70% and the water activity levels determined in the document archival cases are conducive of fungal growth (AHU's and TT's document archival cases)(see Table 6.1).

6.3.3 Water activity measurements and fungal load

The relationship between water activity and fungal load was studied and the impact water activity shows on fungal load is dismissible as R^2 values are very low (maximum $R^2 = 0.102$; data not shown).

6.3.4 Risk of Condensation

Temperature is also recorded when measuring the water activity as the dichotomy determines whether or not condensation will occur. The temperatures measured on the archival cases when assessing water activity were registered in a psychometric chart (Figure 6.9). In red are the extreme values recorded and all the obtained results are inside the area delimitated by these points. In the x-axis is the temperature and curves represent relative humidity or, in this case, equilibrium relative humidity.

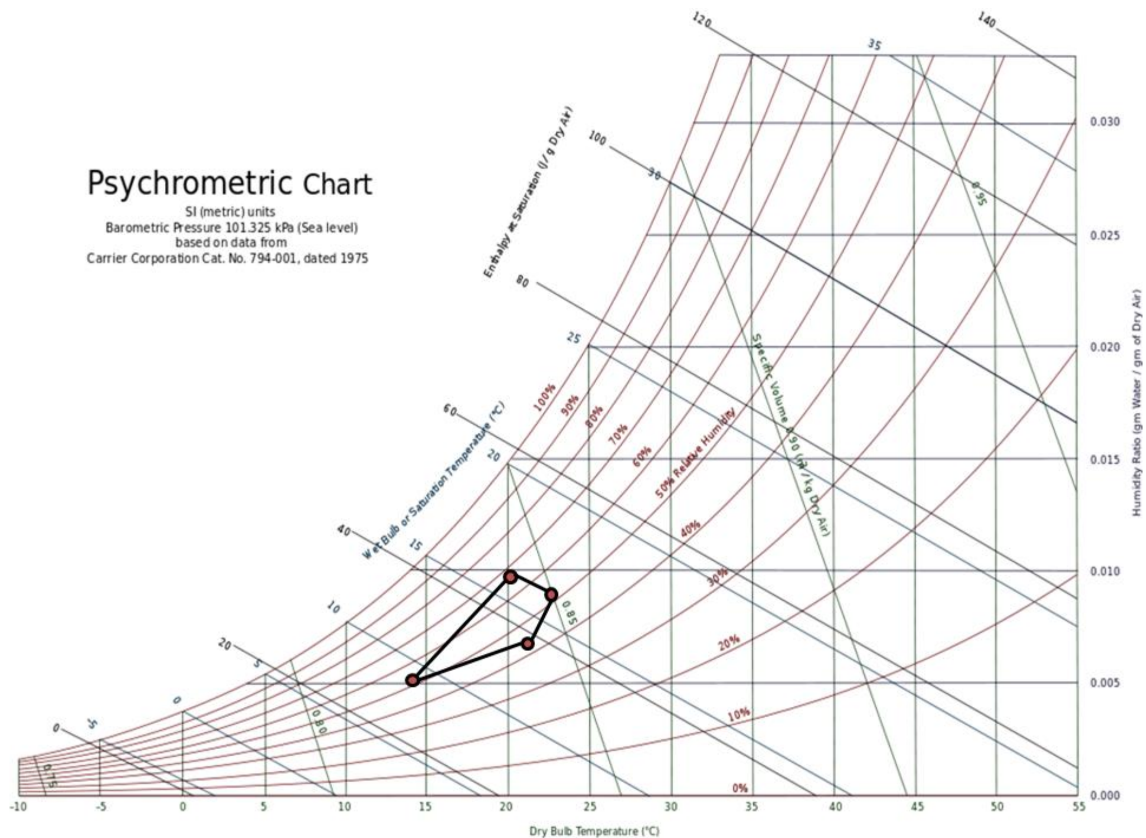

Figure 6.4: Psychometric chart. Water activity values and corresponding temperatures were superimposed on the chart and are delimitated by the red dots. (Psychrometric chart obtained from http://en.wikipedia.org/wiki/ Psychrometrics#mediaviewer)

The risk of condensation, when fungal growth is strongly favoured, is much more likely to occur if water content increases, as can happen in case of a plumbing problem, for instance.

6.3.5 Water activity and Document Samples

Table 6.3 presents the data relative to the water activity and temperature measured in the altered documents sampled. The water activity in the neighbouring areas was also measured and is presented as control.

Table 6.3 - Water activity levels and corresponding temperature for the analysed document samples.

	Water activity		Fungal presence	
	Sample (a_w; T°C)	Control (a_w; T°C)	Culture	Identification
ADE	0,604; 16,7 °	0,607; 17,2°	-	*Fusarium oxysporum*
	0.618; 16,6	0,599; 16.9°	+	*Aspergillus (glaucus)*
	0,632; 16,5°	0.644; 17.0°	-	-
	0,701; 16,4ª	0.603; 17.0°	-	-
	0,655;16,2 ª	0,677; 16,7°	-	*Epicoccum purpurascens*
	0,609; 16,1°	0,612; 16,4°	+ *(mycelia sterilia)*	*Chaetomium bostrychodes*
	0,611; 16,1°	0,613; 16,1°	-	*Aureobasidium pullulans*
	0,590; 18,4°	0,601; 18,2°	-	*Trichoderma viridae*
AHU	0,602; 21.8°	0.612; 21.9°	-	-
	0,601 21.7°	0.600; 21.7°	-	-
	0,558; 22.0°	0.608; 22.5°	-	*Asp. versicolor*
	0,625; 21.9°	0.662; 21.7°	-	-

	Water activity		Fungal presence	
	Sample (a_w; T°C)	Control (a_w; T°C)	Culture	Identification
	0,673; 22.5°	0.611; 21.8°	+	*Aspergillus terreus*
	0,627; 21.9°	0,633; 22.2°	-	*Eurotium halophilicum (anamorph: Asp. halophil-licus)*
IHRU	0,445; 17.2°	0,501; 17.4°	-	-
	0,603; 17.8°	0,589; 17.0°	+	*Penicillium* sp.
	0,442; 17.4°	0,445; 17.2°	-	-
TT	0.410; 21.9°	0,480; 17.2°	-	*Fusarium (merismoides)*
	0.443;22.3°	0,501; 17.2°	-	-
	0.483; 22.1°	0,485; 17.2°	-	*Alternaria* sp.+*Fusarium* sp.

No positive correlation was determined between the values obtained for the sampled documents (affected areas or control areas) and the presence or absence of fungal growth. No positive association was also obtained regarding these values and a positive DNA amplification. The number of samples is reduced and further studies are needed to infer on these relationships.

The association between water activity and relative humidity was also performed for the document samples analysed. Figures 6.5 and 6.6 translate this relationship: first in the control areas from the visibly damaged documental surfaces and second in the chromatically altered regions.

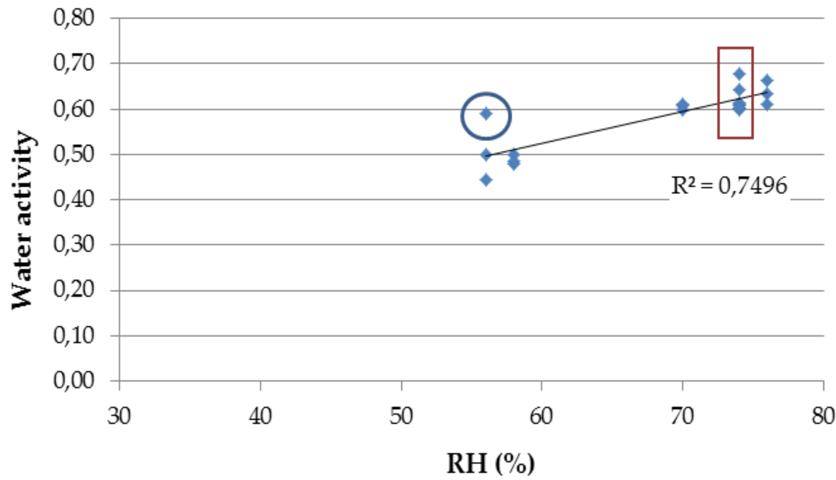

Figure 6.5- Relative humidity levels and the water activity measured in the altered document samples tested (control areas)

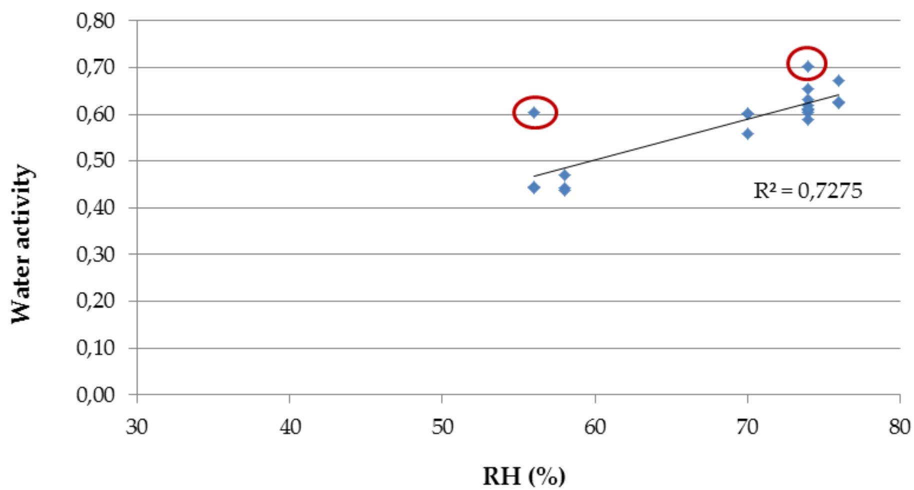

Figure 6.6 - Relative humidity levels and the water activity measured in chromatically altered document samples tested.

The relative humidity measured in the rooms where these samples were taken maintains its influence on the measured water activity (when compared to Figure 6.3) but it is less marked in these samples, especially in the chromatically altered areas.

Despite the expected positive correlation, one still has to keep in mind the formation of microclimates and the time it takes for organic matter to adjust to the sur-

rounding moisture content and temperature. The samples marked under a circle in both Figures 6.5 and 6.6 are an example of a higher than expected water activity for the relative humidity experienced in the room where the sampled documents are kept. Also relevant is the fact that, for the same environmental relative humidity, multiple values of water activity were measured, some of these conducive of fungal growth.

No significant differences were determined in the altered areas *vs* the controls and there does not seem to be a pattern in the way water activity changes according to whether cellulose has suffered some sort of biodeterioration. If we assume that the altered areas are a result of fungal activity than the water activity in these areas must have been higher than the control areas in a certain moment in time… However, if fungal metabolites were secreted by the fungi than it is possible that the water activity is lower in these places comparing to the control areas.

These metabolites may also be the reason behind the difficulties in extracting DNA from most of the samples. More studies, with more samples, are needed to better understand the mentioned hypothesis.

6. 4 Conclusions

Water activity is a valuable tool to address the danger of contamination from biological elements. It has been mostly applied to the food industry but its principles apply to any substrate where fungal growth can occur. The relationship between water activity and fungal growth on paper documents is taking its first steps in the field of Cultural Heritage and very few studies are available for comparison. The measurements performed in the samples taken from these four archives highlight the need to consider the formation of microclimates as different archival cases and documents display different water activity levels even when stored at the same relative humidity conditions. Though a_w values are generally lower than the relative humidity in most of the cases, the exceptions place the paper documents and archival cases in danger of fungal growth.

7. Further Applications for the DHPLC method: Fungi and Yeasts in Cultural Heritage and Clinical Studies

General Introduction

One of the purposes of any PhD project is to bring novelty and increase our scientific knowledge. Besides providing information on the fungal communities present in archives and the quality of the conditions provided for both documents and humans, this project also settled on the development of a new application for a just recently developed technique for DNA analysis. Applying the DHPLC, formerly used just for the detection of mutations, to the study of fungal communities present in cultural heritage became pivotal in the attempt to elevate this project. After its development, opportunity came to test this recent technique in other fields beyond our written legacy.

The first of the case studies presented next is also in the field of cultural heritage. It is the case of a gilded wood carved ceiling decoration in a church, where the DHPLC complemented the results obtained with the traditional culturing method approach.

The second case is in the field of clinical diagnosis where the introduction of DHPLC allowed the resolution of all mixed samples and proved to be a valuable aid in the creation of an ITS2 fragment size database for yeasts identification. As we have seen in Chapter 4 and unlike previous studies had shown, yeasts also constitute an important fraction of the fungal contaminants present in archives and have been associated with biodeterioration and allergen sensitization. Therefore, the molecular biology protocol based on fragment length analysis was also optimized for the study of mixed yeasts samples and the resulting methodology was applied in the present study.

In both fields, the DHPLC was applied successfully.

7.1 Identification of a fungal community on gilded wood carved heritage

Reprinted (with minor changes) as a journal article:

Pinheiro, A.C, Oliveira B.P, Veríssimo, C., Brandão, J., Rosado, L., Jurado, V., Macedo, M.F. (2012). Identification of a fungal community on gilded wood carved heritage. Journal of Cultural Heritage, 14 (1), 76-81.

7.1.1 Introduction

Cultural heritage can sometimes be targeted by extremely destructing microorganisms. In the Portuguese city of Aveiro, the gilded wood carving of the XVIII century St. António and S. Francisco church sacristy was severely attacked by a fungal structure resembling a mushroom in odour and appearance and which grew alongside the wood support reaching a thickness of 3 mm (Pinheiro et al., 2011d).

The fungus was first detected after a water leakage situation in the ceiling and masonry wall surrounding the carved wood structure. The affected area presented an intense rusty colour and fungal spores spread across the room reaching areas located more than two meters away. When moistened, the dusty spores were able to dye the surfaces where they stood. The area affected assumed a darker appearance once the leakage problem was solved and, at this point, hyphae were already penetrating the masonry behind the wood structure.

The dimensions and fast growth of this fungus demanded immediate attention. It threatened the wood carving integrity and had already begun to take its toll on the gilded lining.

The purpose of this study was to identify the fungus responsible for the visible biodeterioration of this gilded wood carved frame and in order to do so, molecular biology and classical culturing methods were applied:

a) the classical method of colony growth in a nutritive agar media followed by macro and microscopic observation of fungal morphological features. This is the standard laboratory procedure performed to an environmental sample.

b) the molecular biology methodology which uses DNA to identify the main fungal species present. In case the first method failed (due to media inadequacy, for instance) this second method would provide an identification.

Due to the commercial impact of wood decaying fungi around the world, several methods have been used to support visual identification of wood-decaying fungi: wood acoustic testing, isolation and culture of fungi and chemical stains, radiography and visual or microscopic inspection of samples are some of the simplest while molecular methods such as SDS-PAGE of intracellular proteins and immunological methods are more sophisticated (Oh et al., 2003). DNA polymerase chain reaction (PCR)-

based methods avoid the need for isolation of fungi and offer great sensitivity for the detection of fungal decay agents at an incipient stage. Sequences of fungal rRNA have been successfully used for species identification by PCR amplification coupled to restriction enzyme analysis (Jasalavich et al., 2000) or through the design of species specific primers (Schmidt et al., 2000) or sequence specific probes (Oh et al., 2003).

Regarding sequencing with universal primers, Chapter 3 presented the two sequencing targets in the rRNA operon that have been used for identification of fungal unknowns: the D1/D2 region of the large ribosomal subunit (LSU) and the internal transcribed spacer regions (ITS1/ITS2) (see Chapter 3 for details).The use of ITS1, ITS2 or both these regions have several supporters (Hinrikson et al., 2005)(de Baere et al., 2002). Opposing the choice of these regions is the still handicapped sequence database available (Hinrikson et al., 2005) (Sujita et al., 2003), a problem not so evident (for the time being) when using the D2 region from the 28S large subunit. As explained before, the D2 region showed to be much more easily amplified and was selected to perform the fungal identification in this case guarantying also the possibility to use the DHPLC methodology if needed. The methodology used in this particular case differs from the genera methodology presented in Chapter 3 and, as such, is presented next.

7.1.2 Materials and Methods

To correctly assess the fungus present in the gilded woodcarving a sample of its mycelia was sent to the INSA's Mycology Section of the Laboratory of Zoonosis and Systemic Infections of the Department of Infectious Diseases.

To minimize the presence of contaminants, the sample was collected directly from the damaged wood with a sterile scalpel and sent to the lab also in a sterilized plastic container.

Following standard procedures, the sample was inoculated in two Malt Extract Agar (MEA) plates and incubated at 27°C for 7 days (Pinheiro et al., 2011d). After incubation a slide preparation of each colony was made and the fungal structures were dyed with lactophenol blue and observed under a 400x magnification. Atlases were used for the identification (de Hoog et al., 2000; Larone et al., 2002; Samson et al., 2004).

The protocol establishes the use of MEA but because this could not be the most suitable for the isolation of the fungus responsible for the heritage deterioration, a mo-

lecular biology approach was also tested. For DNA extraction three fungal suspensions were prepared:

a) Suspension of a fragment retrieved from the inner part of the mycelia sent to the lab in 200 µl TNE (1 ml 1M Tris-HCl, pH8 + 2 ml 5M NaCl + 0.2 ml 0.5M EDTA, pH8 + 96.8 ml H_2O) (sample A).

b) Colony fragment from MEA plate 1 suspended in 200 µl TNE (sample B)

c) Colony fragment from MEA plate 2 suspended in 200 µl TNE (sample C)

These two samples (B and C) were considered assuming the culprit fungus would grow and be identifiable in culture.

A fourth sample (D), which consisted of a dry fragment of the fungal mycelia originally sent to the lab and directly used in the extraction process, was added to this study. All of the samples were extracted with the High Pure DNA Template Kit (Roche) but glass beads were added to the D sample and intense vortexing prior to the DNA extraction protocol. Fragments (0.25cm^2- 0.5 cm^2) used for DNA extraction in samples a) and d) were removed from the inner part of the mycelia sent to the laboratory for analysis together with a negative control sample (200 µl TNE). This was done from the inner part of the sample in order to assure that no other contaminant would be found since its presence would make it difficult to obtain a clean DNA sequence for identification.

After DNA extraction all samples were amplified for the D2 region and the in-house primer pair used for fungal amplification were the NL-3mt and NL4 (Pinheiro et al., 2011d). The PCR, purifying and sequencing protocols are described in the methodologies section.

Six months after the initial detection of the fungi, a Rotronic Hygroplam Water Activity measure device was used to assess this parameter on the affected wood surface.

7.1.3 Results

7.1.3.1 Classical culture approach

The two MEA plates were analysed after incubation. In both plates the structures observed corresponded to *Penicillium* sp a common fungus which does not show any

of the characteristics indicated in the introduction section. The use of one single media and temperature was probably not suited for the growth of the fungus responsible for the deterioration and was just isolating a common airborne contaminant. As only one sample was sent to the lab, the results obtained on the plate could only give the authors an idea (although biased by the selective conditions of the media used) of the environment surrounding the affected wood.

7.1.3.2 Molecular biology approach

Positive amplification was obtained for samples A, B, C and D. No band was present in the negative controls. Before sequencing, and to assure a single DNA product, a series of DHPLC tests were performed.

The first DHPLC run was performed with the injection of 5 μl at non-denaturing temperatures (50°C/40%B) to verify the quality of the amplified product and to determine right away if any of the amplified products could show already a size difference (not observable in the gel) large enough to allow for peak collection and individual assessment of all peaks. This was, however, not the case, since all chromatograms showed a single peak. Samples B and C peaked slightly earlier than samples A and D (see Table 7.1).

Table 7.1 - DHPLC peak retention times of the four samples amplified for the D2 LSU region. This run was performed at 50°C and at 40% B eluent at a 0.9 ml/min flow rate. The peaks are sharper and higher in samples A (fragment suspension) and D (fragment) and wider and shorter in samples B (plate culture) and C (plate culture). These last two samples also peak slightly earlier than samples A and D.

Sample	Peak Retention Time (min)	Peak Height (mV)	Peak Start (min)	Peak End (min)
A	14.3	7.10	14.0	14.7
B	13.8	3.90	13.2	13.9
C	13.7	2.00	13.4	13.8
D	14.4	6.51	14.0	14.5

After this first test, a partially denaturing temperature was applied to all samples tested. Each of the samples was submitted to two different programmes: 61°C/54.2% B, 0.5ml/min flow rate (slope 0.9%B/min) and 63°C/57%B, 0.9 ml/min flow rate (slope 3.4%B/min). Samples A and D showed a single peak at both temperatures tested (see Table 7.2) while samples B and C showed similar and more complex chromatograms (see Figure 7.1).

Table 7.2 Samples A (fragment suspension) and D (fragment) showed the same behaviour at both sets of conditions and both presented one single peak. Sample D was chosen for further studies.

Sample	Peak Retention Time (min)	Peak Height (mV)	Peak Start (min)	Peak End (min)	Temperature (°C) / Gradient (%)
A	8.27	4.80	7.62	8.50	61/54.2
	3.00	2.03	2.71	3.20	63/57.2
D	8.33	8.30	7.47	8.98	61/54.2
	3.00	5.63	2.76	3.21	63/57.2

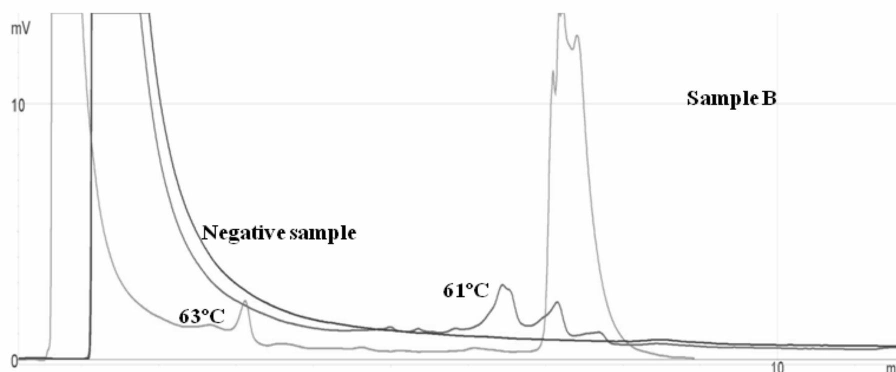

Figure 7.1 - Sample B shows higher complexity than samples A and D as small yet imperfect peaks appear when tested at 61°C. Sample C (not shown) behaved similarly but with lower intensity peaks and, so, these were considered to represent the same fungal community and sample B was chosen for further testing. The first peak in all DHPLC chromatograms refers to the injection peak and does not carry any information regarding the sample being tested.

Given the results, the authors assumed A and D to belong to the same fungus while samples B and C corresponded to a fungal community. Only samples D and B were explored further because these two performed better in terms of peak sharpness and intensity.

Sample D was purified and sequenced. The alignment of the resulting sequence with the Blast NCBI database resulted in *Serpula lacrymans* (Wulfen) P. Karsten (Max Id: 98%, Accession number JF734883) (Pinheiro et al., 2011d).

The B sample was re-amplified for the same region (nested PCR, using the same primers and PCR program as above) and the resulting product was submitted to further studies: at 63, 62, 61 and 60°C (57.2%B) and this last temperature was selected for peak collection at 3.8 and 4.2 min (Figure 7.2)(Pinheiro et al., 2011d).

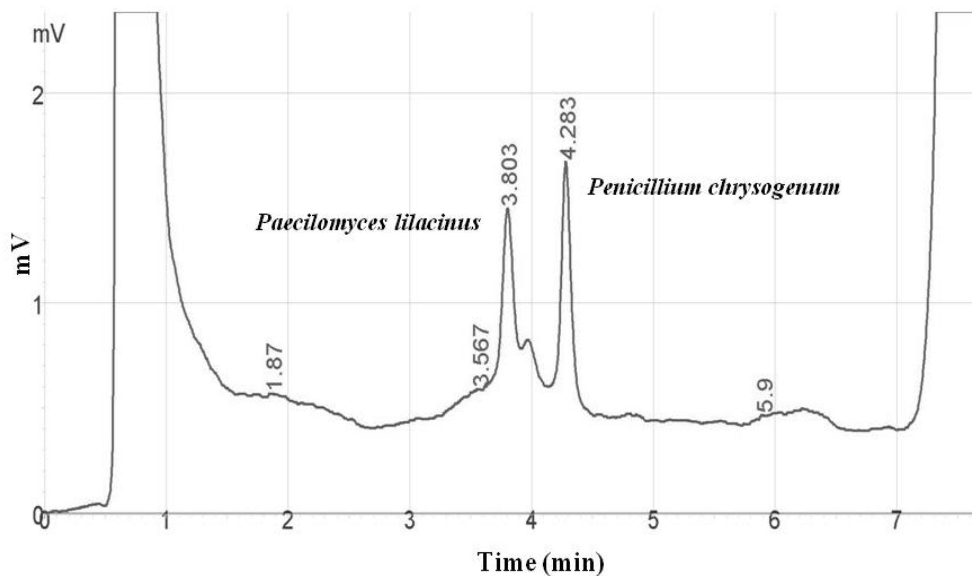

Figure 7.2 - At 60°C/57.2%B, flow rate 0.7 ml/min, sample B showed two distinct peaks which were easily collected for amplification and sequencing. The first peak corresponds to *Paecilomyces lilacinus*, the second peak to *Penicillium chrysogenum*.

These collected peaks (10 μl) were then re-amplified using the same primers, purified and sequenced. After matching the sequences with the GenBank database the results were as follows: *Paecilomyces lilacinus* (Thom) Samson (Accession number JQ734918, Minute: 3.8; Max Id 98.9%), and *Penicillium chrysogenum* Thom (Accession number JQ734919, Minute 4.2; Max Id 98%).

Since a *S. lacrymans* containing fragment was used for inoculation it would be expected to obtain a DHPLC peak corresponding to this fungus also. However, only a small sample of the plate was collected and so, as the S. *lacrymans* mycelia did not develop in the media used, only DNA from the *Penicillium* and *Paecilomyces* was extracted and amplified.

7.1.3.4 Water activity (a_w)

Six months after treatment the water activity (a_w) levels on the once affected gilded wood structure were assessed. Due to the nature of the carving, a_w levels could only be measured in one of the points where fungal growth was observed. At this location

the a_w (or equilibrium relative humidity) level was 0.840 at 15.4°C. On this location the wood was regaining a reddish tone characteristic of *S. lacryman'*s effect.

7.1.4 Discussion

7.1.4.1 Health and Conservation Implications

The analyses performed to the sample sent to the laboratory revealed the co-existence of three fungi: *S. lacrymans*, responsible for the visible deterioration of the gilded wood carving and surrounding masonry and two air contaminants: *P. chrysogenum* and *P. lilacinus*, probably not involved in the deterioration but present in the environment surrounding the wood from which the fragment was collected.

S. lacrymans is usually found in Central and Northern Europe, Japan, Australia and temperate/oceanic climates (Singh 2000) and published data on the occurrence of this fungus in Portuguese built heritage was not found. There are a couple of cases reported in old wooden buildings (Dias, 2008) and a case in a wooden altar piece (Remigio, 2009).

S. lacrymans is responsible for a disease termed "dry rot" which is annually liable for millions spent is recovering wooden structures in temperate regions worldwide (Palfreyman et al., 1995). Wooden altars and roof structures of churches and other cultural heritage sites are known to have been severely attacked by this fungus (Sterflinger, 2010). As in this case, it usually appears when there is a sudden elevation of moisture content, water ingress and insufficient ventilation (Maurice et al., 2011). The leakage that occurred in this church triggered the fungus development. *S. lacrymans* is a common cause of extrinsic asthma (immediate-type hypersensitivity: type I) and has been associated with extrinsic allergic alveolitis (Crissy et al., 1995; Flannigan, 1997). A mask was used by the staff handling this case.

The remaining two fungi are common indoor air contaminants and one of them was partially identified through its morphological features (*Penicillium sp.*). The other one however, would not have been identified this way since, visually, the plate seemed completely covered with a species of *Penicillium*.

Identification of *Penicillium* to the species level is very difficult to attain using only phenotypic features. However, and because the D2 sequence is not discriminative enough for the *Penicillium* genera, the colony morphology made it possible to corrobo-

rate the *P. chrysogenum* result provided by the databases used. Reinforcing this choice was the fact that this species is commonly found on water damaged buildings (Samson et al., 2004) as in this case.

Penicillium sp. is a fast growing fungus which can hinder the growth of other genera and deem them unnoticed to visual inspection. Since only one sample was sent to the laboratory the identification of these two fungal species gives the authors an idea (although biased by the selective properties of the media used) of the environment surrounding the *S. lacrymans* affected heritage. In this case DHPLC made it possible not only to extend point the identification of *Penicillium* to the species level (separating it from the also present *Paecilomyces*) as well as to identify other contaminants not distinguishable on the Petri dish.

To avoid the bias created by the use of a culturing method prior to DNA extraction and amplification, a more direct protocol based on the direct study of a swab suspension is presented in Chapter 3 for document samples (Pinheiro et al., 2011b, see Chapter 3 for details). This method, however, is also not bias-free as the extraction protocol can fail to extract all the components of the mixture and/or retrieve fungi that are no longer viable and, therefore, pose no problem in terms of active deterioration.

7.1.4.2 Treatment and Prevention Strategies

As mentioned, the fungus developed over gilded wood carved patrimony which immediately revoked the classical preventive measure of quickly eliminating the affected area (Carey and Grant, 2002). As recommended by several authors (Singh, 2000; Palfreyman, 2001) the first steps were then directed at the environment surrounding the affected timber and the water source was removed by deflecting the HVAC drainage tube and assessing the floor's surface. The fungal structure removal was initiated after the water source was eliminated. First, the entire wood carved structure was vacuumed and all the visible spores removed from the surfaces where they were deposited.

Regarding biocides, recent legislation has decreased considerably the number of available products to use against *S. lacrymans* and other fungi. *S. lacrymans* shows tolerance to copper and copper citrate (Hastrup et al., 2008; Reinprecht, 2010) but is sensitive to retardants of Fenton depolymerisation and carbamates (Reinprecht, 2010). A fungicide and insecticide solution containing carbamates and triazoles (Xylophene

SOR2®, Dyrup: cypermetrin 0.07%, IPBC 0.05%, propiconazole 0.15% and tebuconazole 0.05%) was injected repeatedly in the fungal structure. This product was chosen as it is the only homologated one by the Portuguese Civil Engineering National Laboratory for the effect.

This particular fungus shows a strong affinity for plaster and masonry from where it removes calcium, sulphur and iron; elements which increase the rate of timber decay (Low et al., 2000). To counterbalance these cations, *S. lacrymans* produces acids – mainly oxalic acid – which have been implicated in the further degradation of stone and other materials (Low et al., 2000). In this particular case, the masonry was indeed affected and some pieces had to be removed for treatment. The same antifungal solution was applied with a brush and syringe wherever possible.

Due to the nature of the conservation work that was being developed in this location, the described intervention was considered a short term solution as immediate remedial actions were needed before any further work could be done. The entire wooden materials will still be subject of further cleaning procedures as the entire sacristy will be totally restored.

To ensure the correct monitoring of the carved wood structure and its surroundings, the installation of a probe data logging system was advised. A warning system could be coupled to the data-logger. According to some authors, by keeping the wood equilibrium moisture content (EMC) below 20-22% it is possible to prevent the fungus growing again and promote its eventual death (Palfreyman et al., 1995; Carey and Grant, 2002).

The authors returned to the church almost six months after the initial analyses to perform water activity evaluation and the a_w levels were 0.84 at 15°C. Using the Hailwood-Horrobin equation, the 0.84 a_w determined at 15°C corresponds to an EMC (Meq) of 17.8%, which is below the previously recommended inferior limit of 20%. However, Palfreyman (2001) also states that while *S. lacrymans* growth is inhibited when the relative humidity levels fall below 86% the viability of the fungus is not lost until these levels reach the 76%. In the location where the a_w level was 0.840, one of the points where fungal growth was observed, the wood is, in fact, regaining a reddish tone, suggesting fungal regrowth. This high water activity is conducive of mesophilic fungal growth (Samson et al, 2010).

Periodic air and surface samples analysis were also advised in order to check whether the cleaning procedures were correct and if there are no damaging fungal species growing on these cultural heritage materials. This is particularly relevant in the case of *Trichoderma* species as these establish a very peculiar relationship with *S. lacrymans* and other species of Basidiomycetes: they can be used as biocontrol agents as they seem to be able to prevent their growth (Score et al., 1994; Verma et al., 2007; Rosa and Herrera, 2009;) but some authors point to the fact that wood already partially decayed by *S. lacrymans* appears to be further deteriorated by these *Trichoderma* species (Palfreyman et al., 1995; Palfreyman 2001).

7.1.4.3 DHPLC as an analytical method

DHPLC is emerging as a relatively easy method to resolve mixed fungal populations. Though it still demands the testing of several parameters to achieve a combination capable of delivering high resolution this is usually achieved quite quickly and after peak collection these peaks can be immediately re-amplified and sequenced. A DHPLC run normally takes about seven minutes to be performed, and a complete study can be performed in less than three days (from DNA extraction to sequence analysis). In this case, only through a method such as this was it possible to identify the second contaminant in the media plate and achieve a species level identification on both *Penicillium* and *Paecilomyces*.

As mentioned before, once ITS amplification is optimized, it should be possible to perform such a study with this alternative rDNA region and complement the D2 results with the ITS analysis. With an important clinical impact, DHPLC is also being implemented by the authors as an alternative method to distinguish yeasts from mixed populations (Pinheiro et al., 2014, submitted to Revista Ibero-Americana de Micologia, see subchapter 7.2).

7.1.5. Conclusions

S. lacrymans is not a common fungus in the south of Europe and, to the author’s knowledge, there are no published cases in Portuguese built heritage.

Although it has an ill reputation, *S. lacrymans* is quite vulnerable to environmental changes and, as such, can be controlled by keeping the environment relatively dry (EMC below 20%, RH below 86%, preferably below 76%). The values determined six months after the leakage problem was solved indicate a borderline a_w of 0.84 at 15°C corresponding to a 17.8% EMC.

To ensure that this is not a recurrent episode the church also has to undergo severe inspection as the plumbing system can present similar flaws elsewhere in the building. Finally, air samples should be performed seasonally to determine the presence of cellulose degrading fungal flora. Now that *S. lacrymans* is a known contaminant in this studied location, the curators and conservation scientists involved are more aware of the nutritional needs of this species and a modified protocol (Schmidt and Moreth-Kebernik, 1991; Palfreyman 2001;) in the culture plates usually used to assess indoor air quality can be included. This modified protocol requires a longer incubation period and so molecular biology protocols such as the one shown here - using DHPLC to resolve a mixed sample of fungal contaminants and partial genome sequencing to confirm an otherwise unculturable sample should also be used in further environmental analysis.

7.2 Denaturing High Performance Liquid Chromatography and Automated Fluorescent Capillary Electrophoresis to resolve mixed Candida sp. Cultures

Pinheiro A.C., Viegas, C., Veríssimo, C., Sabino, R., Brandão, J. & Macedo, M.F. (2014). Denaturing High Performance Liquid Chromatography and Automated Fluorescent Capillary Electrophoresis to resolve mixed Candida sp. Cultures. submitted for publication in *Revista Ibero-Americana de Micologia.*

7.2.1 Introduction

Candida species are fungal pathogens for both immunocompromised and immunocompetent patients (Kothavade et al., 2010). In chapter 4 we have seen them as contaminants in archives where they can induce biodeterioration (Michaelsen et al, 2010a) and be responsible for immunological sensitization (Wiszniewska et al., 2010). In the human body they may be present as colonizing agents but also be responsible for life threatening infections. Since 1990, there has been a shift in the population dynamics for *Candida* species and their relative frequencies - *Candida albicans* has seen its importance fade to other species (C. *parapsilosis*, C. *glabrata* or *C. tropicalis*) (de Baere et al., 2005). Also, infections caused by more than one species may be present in patients with a challenged immune system (Samie and Mashao, 2012).

In laboratory routine, yeasts from clinical samples can be identified using phenotypic characteristics coupled with biochemical methods, such as gallery kits or chromogenic media. The Mycology Laboratory from the National Institute of Health Dr. Ricardo Jorge performs the following ISO 17025 (2005) accredited (IPAC L0425) yeast identification procedure for clinical samples: sample collection, inoculation in Sabouraud broth agar and chromogenic agar, followed by identification using biochemical galleries of colonies that the chromogenic media does not presumptively identify. Though easy to use, when the naked eye is not capable of discerning the number of species in the culture media, the initial suspension may carry more than one yeast species and the result will lack in confidence, be incorrect or even unreadable in the biochemical galleries reading system. Such results are expected also when the yeasts species are not in the database (Pirotta and Garland, 2006). Although this list is quite extensive for *Candida* sp., new species as *C. bracarensis*, *C. orthopsilosis* or *C. metapsilosis* can´t be identified using these systems. Incorrect matches also occur even when the *Candida* species is contemplated in the database and the rate of misidentification can be high (Mota et al., 2012).

Automated fluorescent capillary electrophoresis (AFCE) is commonly used with the ITS2 region of the rRNA (Turenne et al., 1999; de Baere et al., 2002) (see Chapter 3 for details). It is a library-dependent method and a confirmation strategy was adopted to validate the data obtained during the database construction. The D2 region was chosen for this effect as its 300 bp sequence is also exploited for the identification of both moulds and yeasts. It is in fact the primary choice of an important commercial kit of

Fungal DNA Identification (Ninet et al., 2003).For routine testing, its extreme easiness of use is nevertheless impaired by the costs.

The first goal of this study was to establish an alternative molecular biology method for the identification of *Candida* sp. to use whenever the biochemical tests are unavailable, return an invalid result or need further confirmation due to low homology. Some authors (Lo et al., 2001) actually recommend the concomitant use of two methods for yeast's identification. AFCE also offers the advantage of immediate analysis for mixed samples without the need for reisolation. The AFCE method and an in-house 28S rRNA D2 amplification protocol were hence developed and all three (the biochemical and both molecular) compared for yeast identification and validation of this new approach.

When mixed samples appeared and the components were either not all contemplated in the database or resulted in a low homology D2 sequence result, a new method was applied: the Denaturing High Performance Liquid Chromatography (DHPLC). In this method, the amplified mixed sample components are separated, collected and individually sequenced. To successfully perform this separation, the amplified sequence must, however, be small enough for the separation to occur during the chromatography but be long and diverse/non-conserved enough to be useful in sequence analysis. This is the case for the above mentioned 28S rRNA D2 region of the genome. DHPLC has already been proved useful in several settings – clinical and environmental – and for different microorganisms (Goldenberg et al., 2005, 2007; Pinheiro et al. 2011b, 2011c, 2011d) and here it became a valuable assistant in creating the AFCE database.

7.2.2 Methodology

Reference strains (Table 7.3.) and clinical samples (Table 7.4) were used to establish the AFCE method.

Table 7.3 -

Table 7.3 - Reference yeast strains selected to initiate the ITS2 fragment size database for the AFCE method.

• Reference Strain	Yeast Species
ATCC90028	*Candida albicans*
CBS138T	*Candida glabrata*
NQ 9627	*Candida guilliermondii*
NQ9966	*Candida kefyr*
PYCC3341T	*Candida krusei*
PYCC4093T	*Candida lusitaniae*
IMR48[16]	*Candida parapsilosis*

Following current practice, clinical samples (Table 7.4), inoculated in Saboraud with cloramphenicol (0.05%) and incubated for 3 to 5 days were primarily identified with the biochemical galleries API ID32 C (BioMérieux, Marci l'Etoile, France) as described in the brand procedure recommendations.

Table 7.4 Yeast samples selected to create the ITS2 database for the AFCE method. The yeast species was attributed using the API ID 32C, a biochemical assay test (BioMérieux, Marci l'Etoile, France).

Clinical Sample	API 32C	Clinical Sample	API 32 C
1 N		15 O	
2 O		28 O	
4 N		38N	
19 O	*Candida glabrata*	39O	*Candida kefyr*
27 O		120 N	
82 O		152 O	
83 N		153 O	
112 N		79 N	
128 N		164 O	*Candida holmii*
140 N	*Candida albicans*	213 O	
147 N		59 O	*Candida tropicalis*
192 O		145 O	*Candida valida*
118N		971447	*Candida guilliermondii*
125 O	*Candida krusei*	230 O	*Saccharomyces cerevisae*
161N			

To create and validate the ITS2 size length database, DNA analysis followed this first identification step: ITS2 amplification using the primer pairs ITS86HEX-ITS4 and primers NL-3mt/NL4 for the LSU D2 region amplification. Both protocol details are explained elsewhere (see Chapter 3).

The ITS2 amplification protocol was applied to both Table 7.3 and Table 7.4 yeasts. Replicates were created to validate results. The number of bp resulting from yeasts considered in Table 26 were taken as starting points and used as reference for further results.

The D2 rRNA amplification was performed to validate the association between the API32C and the AFCE result. This amplification protocol was only applied to the yeasts in Table 7.4 since the other were reference strains.

7.2.2.1 Sequencing Amplification Protocol

Only the D2 amplified products were submitted to purification and sequencing. The D2 region sequences obtained with the in-house method were compared with the API ID 32C result and the ITS2 size database using automated fluorescent capillary electrophoresis system.

7.2.2.2 AFCE protocols

This analysis was applied to all the yeasts in Tables 7.3 and 7.4.

7.2.2.3 DHPLC protocol

Low homology results with the BLAST database for the D2 rRNA region led to run the DHPLC protocol, using the LSU D2 region amplification products. This protocol was applied to three of the original samples as there was no agreement between the three alternative methods. After an initial test to determine the best conditions for separation of the peaks a second run was performed and the peaks were collected manually. Ten ul of the collected solutions were amplified again for the 28S rRNA D2 region, purified and sequenced as previously described.

7.2.2.4 Methodology Flowchart

The described methodology is schematically presented in Figure 7.3.

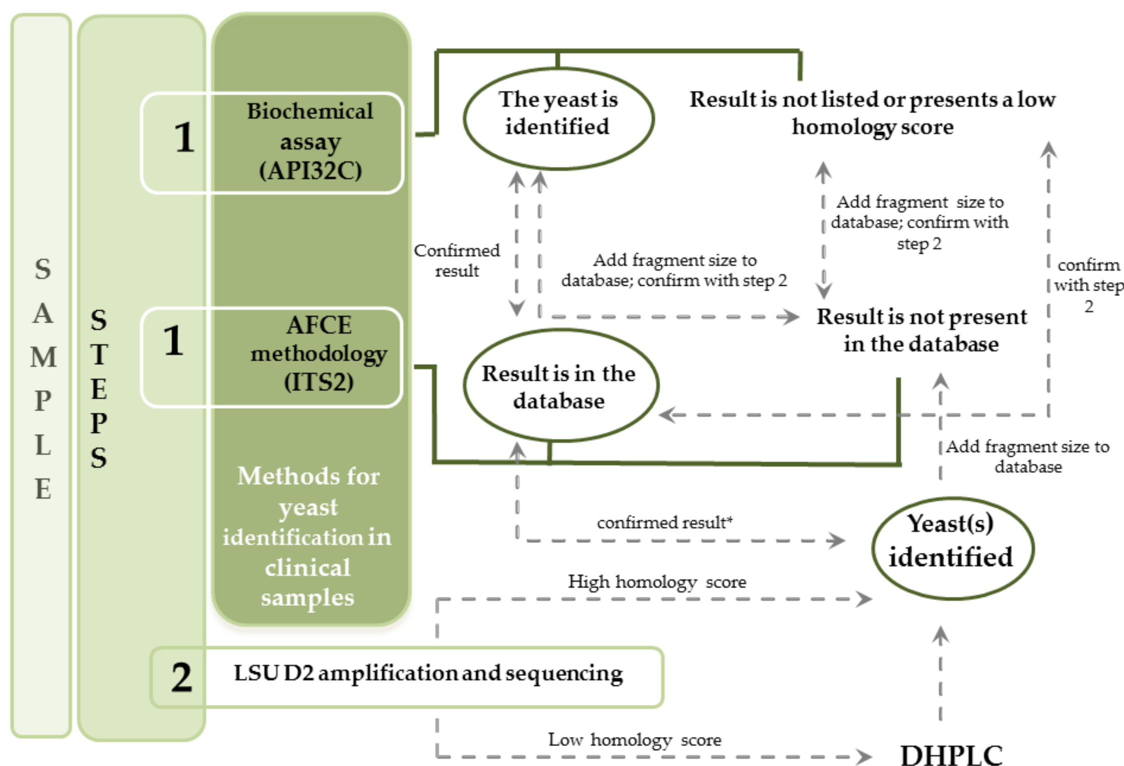

Figure 7.2 - Protocol flowchart for the ITS2 fragment size database creation and analysis of clinical samples.

7.2.3 Results and Discussion

Prompt and accurate detection and identification of yeast species is very important as the virulence and antifungal resistance of *Candida* isolates differs according to the species (Fujita et al., 2001). In order to validate this protocol, three methods were used. Reference strains (Table 7.3) provided the initial ITS2 lengths for the database. Then, the biochemical profile of each of the clinical yeast samples tested (Table 7.4) was compared to the results obtained with the ITS2 fragment size analyses and the sequencing match using the 28S rRNA D2 genomic region (Table 7.5). All yeast samples tested showed a single noticeable amplification product (data not shown). All control samples (DNA extraction and DNA amplification) were negative.

Table 7.5 -Clinical yeast samples identified to the species level using the biochemical method (API ID 32C), the ITS2 amplification using primer pair ITS83hex/ITS4 and sequencing data of the LSU D2 region amplified with primers NL-3mt and NL4

API 32C	Sample	ITS2-hex	NL-3mt/NL4 (%max id)		GenBank Accession Nr.
Candida glabrata	1 N	*glabrata*	*glabrata*	(100)	JX844593
	2 O	*glabrata*	*glabrata*	(100)	JX844594
	4 N	*glabrata*	*glabrata*	(99)	JX844595
	19 O	*glabrata*	*glabrata*	(100)	JX844596
	27 O	*glabrata*	*glabrata*	(99)	JX844597
	82 O	*glabrata+kefyr*	*kefyr*	(95)	(see DHPLC results)
	83 N	*glabrata*	*glabrata*	(100)	JX844598
Candida albicans	112 N	*kefyr*	*kefyr*	(98)	JX844605
	128 N	*albicans*	*albicans*	(100)	JX844600
	140 N	*albicans*	*albicans*	(100)	JX844601
	147 N	*albicans*	*albicans*	(100)	JX844602
	192 O	*albicans*	*albicans*	(99)	JX844603
Candida kefyr	15 O	*kefyr*	*kefyr*	(99)	JX844604
	28 O	*kefyr*	*kefyr*	(98)	JX844606
	38 N	*kefyr*	*kefyr*	(98)	JX844607
	39O	*glabrata*	*glabrata*	(58)	(see DHPLC results)
	120 N	*kefyr*	*kefyr*	(99)	JX844608
	152 O	*kefyr*	*kefyr*	(99)	JX844609
	153 O	*kefyr*	*kefyr*	(99)	JX844610
Candida krusei	118N	*krusei*	*krusei*	(99)	JX844612
	125 O	*krusei*	*krusei*	(100)	JX844613
	146 O	*krusei*	*krusei*	(100)	JX844614
Candida holmii	79 N	*glabrata*	*krusei*	(58)	(see DHPLC results)
	164 O	*kefyr*	*kefyr*	(99)	JX844611
	213 O	*glabrata*	*glabrata*	(100)	JX844599
Candida guilliermondii	971447	*parapsilosis*	*parapsilosis*	(98)	JX844617
Candida tropicalis	59 O	-	*tropicalis*	(100)	JX844618
Candida valida	145 O	*krusei*	*krusei*	(100)	JX844615
Saccharomyces cerevisae	230 O	*krusei*	*krusei*	(100)	JX844616

For one of the samples – 59_O – there was no reference entry in the database so the determined ITS2 fragment size could not be matched. rRNA D2 sequence analysis was performed and the results were compliant with a C. *tropicalis*. With matched API and sequencing results, the ITS2 fragment size obtained was introduced in the database as representative of *C. tropicalis* but only became definitive after a positive result was obtained with a reference strain. Therefore, only 28 samples were considered for the following statistics.

Most of the samples tested (19/28; 68%) showed a full correspondence between the API 32C result and the AFCE method using the ITS2 region length as a marker. All of these matched correspondences were also in agreement with the LSU D2 region sequencing results.

From the 28 samples identified using all three methods, when comparing biochemical tests and AFCE alone, the correspondence was incomplete in one case (82_O) and totally discordant in eight cases (112N, 164_O, 213_O, 145_O, 971447, 230_O, 39_O and 79N).

Amplification of the D2 region was used to decide which result was correct in the case of the eight total mismatches between the API32C and the AFCE method. Using the D2 as tiebreaker, the latter method was proven correct in 75% of the discordant samples: 112N, 164_O, 213_O, 145_O, 971447 and 230_O as the sequence identification scores obtained in these samples are between 98 and 100% (E value very low, data not shown) and correspond, in all cases, to the result obtained with the AFCE.

The remaining two incorrect matches – 39_O and 79N– were submitted to DHPLC partially denaturing conditions since the D2 sequencing resulted in low (<95%) identification scores in both attempts. The authors considered these low scores as an indication of a second DNA present and used the DHPLC to analyse the DNA sequence. Figures 7.4 (for sample 39_O) and 7.5 (for sample 79N) show the results obtained with this resolution method.

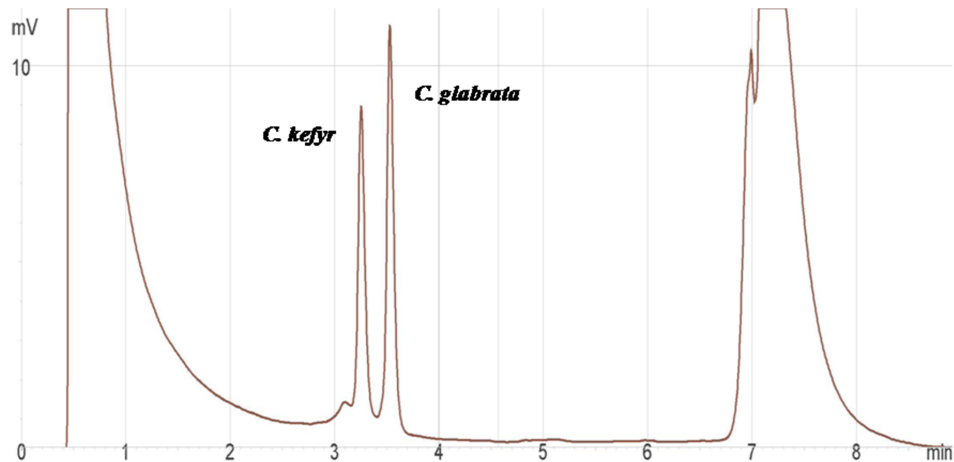

Figure 7.3 - Sample 39_O submitted to partially denaturing conditions at 59°C/57.2%B and a 0.9 flow rate. The DHPLC system allowed the recovery of two peaks which, after re-amplification and sequencing resulted in *Candida kefyr* and *Candida glabrata*.

In the case of the 39_O sample, the API32C reacted to the presence of *Candida kefyr* with a confidence result of 99.9% but the ITS2 pointed to the presence of *C. glabrata.* This sample was, in fact, a mixture of two yeasts – *Candida kefyr* (JX844621) and *Candida glabrata* (JX844622). Since efforts were made to minimize contamination, it is possible that both yeasts were present in the original sample but only one of them (a single colony, as protocol states) was selected from culture to perform the biochemical assay. Since, in the used media, it is nearly impossible to phenotypically distinguish between these two species only one API was performed and a pure culture was assumed. For the AFCE method it is recommended not to remove just one colony but to swab a small area of the media plate. In this case, the ITS2 amplification was preferential to *C. glabrata* but the amplification for D2 rRNA region was achieved for both yeasts present.

Sample 79N was also composed of two yeasts. For this sample the results were in complete disagreement as three different species were obtained with the three different methods used: *C. holmii* using the biochemical method, *C. glabrata* using the ITS2-hex and *C. krusei* (with a low identification score) using the 28S rRNA D2 amplification method.

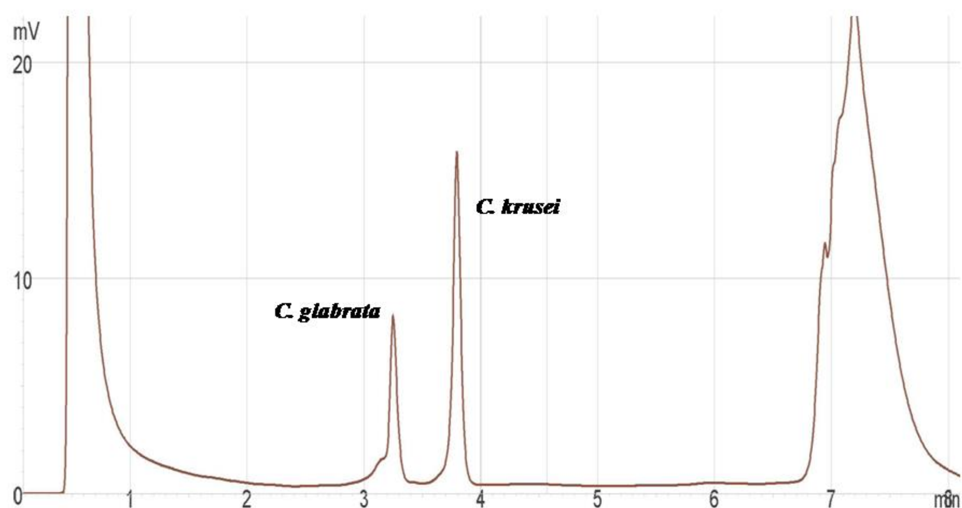

Figure 7.4 - Sample 79N submitted to partially denaturing conditions at 63°C/54%B and a 0.9 flow rate. The DHPLC system allowed the recovery of two peaks which, after re-amplification and sequencing resulted in *Candida glabrata* and *Candida krusei* (Pinheiro et al., 2011c)

D2 sequence analysis (DHPLC included) supported the partial result obtained with AFCE (Pinheiro et al., 2011c).Regarding sample 82_O, the AFCE method revealed the presence of additional yeasts (Figure 7.6).

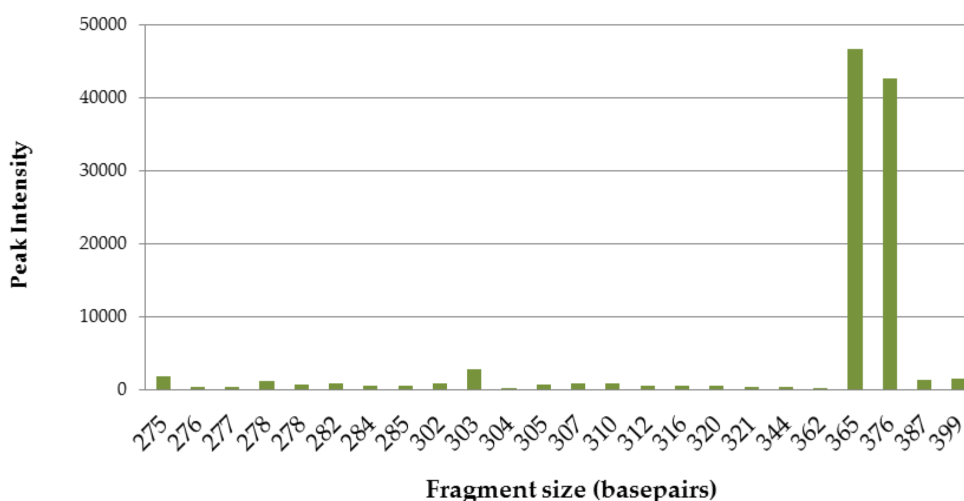

Figure 7.5 - AFCE fragment lengths determined for sample 82_O. Two peaks were obtained at 365 and 376 basepairs.

The maximum identification score for the D2 sequence study obtained was 95%, a value low enough to corroborate the hypothesis of a mixed sample. DHPLC was then used to unveil the components of these mixtures and see if they matched the results obtained with the AFCE method. The DHPLC chromatogram is presented in Fig. 7.7.

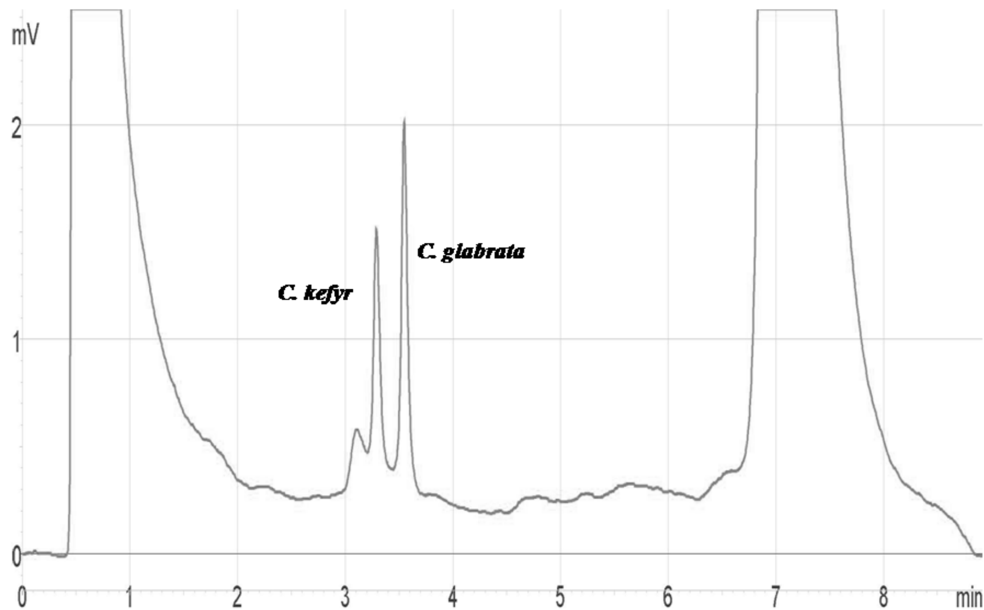

Figure 7.6 - Sample 82_O submitted to partially denaturing conditions at 59°C/57.2%B and a 0.9 flow rate. The DHPLC system allowed the recovery of two peaks which, after re-amplification and sequencing resulted in *Candida kefyr* and *Candida glabrata*.

Using this methodology in this clinical sample allowed different yeast species to be isolated, sequenced and identified: Candida *kefyr* (Acc. No.JX844619) at minute 3.2 and *Candida glabrata* (Acc. No.JX844620) at minute 3.6.

Contrarily to samples 79N and 39_O, the AFCE method alone was able to suggest and identify the second yeast present in sample 82_O. D2 sequencing and DHPLC confirmed the presence of *C. kefyr* and *C. glabrata*. Again, only one colony was removed for the biochemical assay (99.1% confidence), but the swabbed area used for DNA analysis revealed a wider spectrum of yeast.

In no case was the sequencing supportive of the API32C and in total disagreement with the AFCE. AFCE and D2 sequencing showed the same exact result in 89% of

the cases. The study suggests the API method to have delivered incomplete results in two cases (82_O and 39_O) (7%) and incorrect results in seven cases (25%).

Regarding the incorrect results, the API ID 32C only recommends additional testing for *C. norvegensis* and *C. inconspicua*. *Candida holmii* was incorrectly attributed to three samples, and *C. parapsilosis* and *C. valida* to one sample each. Further studies with these species are being performed to ascertain the need for further testing when faced with such results.

More samples have been tested and so far the database includes more than 89 independent isolates of seven species in total (*C. albicans, C. glabrata, C. guilliermondi, C. kefyr, C. krusei, C. tropicalis* and *C. parapsilosis*). The maximum standard deviation (SD) obtained so far is 0.94bp. Comparison between the ITS2 size results obtained and the published databases (de Baere et al., 2002, 2005;Turenne et al., 1999) reveals a slight discrepancy in the bp number, a fact that can be related to the sequencing system's upgrade from the 310 model to the 3130 XL version. More data is needed to infer on the persistency of this trend. Also being tested are known mixtures of yeasts and known concentrations of different yeasts in order to test sensibility and method reproducibility (Antoniazzi, Master Thesis, *in prep*.)

7.2.4 Conclusions

AFCE revealed much more accuracy than the biochemical assay since this last one provided several totally incorrect results (0% vs. 25%, respectively). This method also has the clear advantage of being able to select more than one colony in the same procedure. For API ID 32C method this is not only impossible as it is undesirable.

As more species are added to the database the authors foresee the eventual problem of ITS2 size repetition or a lower than the SD bp difference between two species. To overcome this problem frequency studies for each population or type of sample must be performed. In archives and librairies, where yeasts are now becoming part of the potentially harmful spectra of fungi, this method can be used to provide a species identification.

The DHPLC method proved very effective in resolving the mixed samples. For one of the three samples submitted, the AFCE protocol was able to suggest the presence of one more species of yeast as well as to identify which. On the other two cases

there was only ITS2 amplification for one of the *Candida* species present. DHPLC has also been capable of identifying species in a mixture whenever these were not yet contemplated in the database. It was the case of *Candida krusei* before a reference strain was introduced in the study (Pinheiro et al., 2011b, see Chapter 4).

8. Final Remarks and Future Perspectives

In this study, the presented review gathered studies from across the globe, showing us the fungal communities encountered so far in the air and surfaces of archives and librairies. Despite the changes in nomenclature and in the applied methodologies, the fungal flora is relatively stable in different parts of the world, showing how truly universal some fungal genera and species are. Comparing with other environments, *Chaetomium* sp., *Fusarium* sp. and *Geotrichum* sp. were found to be special contaminants in the air of archives and are normally associated with paper biodeterioration. In Portugal the main air contaminants were the commonly encountered *Cladosporium* sp., *Aspergillus* and *Penicillium* sp, but *Fusarium* sp. and *Geotrichum* sp. were also present. Yeasts, surprisingly, were well represented in the Portuguese air results. The large majority of fungi encountered in the air of the sampled archives already revealed biodeterioration activity in other studies.

Internationally, the fungal load in different archives is also quite relatable although some differences, mostly due to methodology, are noted. In the sampled Portuguese archives the fungal load for air samples reached its maximum during construction work (more than 200 CFU/m^3), was more dependant on outside air in the archives with natural ventilation (ADE and AHU) and more dependant on cleaning activies in the archives with forced ventilation (IHRU and ADE). No significant differences were found between summer and winter seasons.

The fungal communities identified in archives - national and foreign – can and were compared with guidelines and/or directives created to aid us in the task of assuring a healthy environment for both humans and documents. Not so many of these guidelines cross the border between the academic and the legislative world. The frequency on which guidelines appear and disparity of recommended values assures us of two things: how much we need them and how little we know to design them.

Most of the studied archives abroad show contamination levels higher than desirable for both conservation and health safekeeping. The air samples retrieved from the studied Portuguese archives were compared with the existing governmental directive for indoor air quality and with some of the proposed guidelines for paper conservation.

None of the rooms – storage, reading or office areas – crossed the 500 CFU/m^3 but some of the indoor air results were higher than the control sample which reveals indoor contamination. Though relevant - as fungi's origin is mainly from the outside –

this ratio was probably overestimated when the 2013 Ordinance was created. As pointed out in the results section, many of the problems encountered in the analysed archives would go unnoticed if further investigations were only taken when the I/O ratio was higher than one.

In the field of paper conservation, almost all of the archives showed at least the presence of a source of fungal contamination (values above 25 CFU/m3, according to the Dutch Guidelines) and in three of the readings the value surpassed the Italian governmental guidelines. No Portuguese guidelines have been presented so far but the performed readings allowed recommendations to be drafted. In these recommendations the importance of defining species is highlighted. *Penicillium* sp. and yeasts are good indicators of moisture problems and their presence in concentrations higher than normal can represent a valuable help.

For human health, the species one can find in the indoor environment are considered relevant and are discriminated in both existing legislations (2006 and 2013 for offices and services buildings). The obtained results in the archives were viewed under both of them in order to test for improvement between them. No potentially pathogenic fungi were found in this study but potentially toxinogenic fungi were present in almost all of the performed assessments.

With the 2013 Ordinance for office and services buildings, the definition of specific rules for specific building types reveals a tendency which could become an opportunity to define the guidelines for archival settings, librairies and museums.

Whatever the field of expertise, guidelines should include air and surface samples. Be it in conservation or in health, here or abroad, none of them do. Fungal elements are deposited and airbone just to settle once more and the fungal communities can only be truly ascertained if both samples are valued.

As in many other settings, the surface samples presented a more diverse array of fungal genera/species than air samples, a trend not noticed in the few studies on surfaces performed in Archives. The fact that the number of genera/species found in the Portuguese archival surfaces contributed with almost 50% to our common knowledge on the subject, though flattering, is actually a result of the extreme lack of published results in other archives.

Since conservators, general staff and readers use their hands to touch and manipulate the surfaces (books included), it is relevant to point out the presence of a potentially keratinophylic fungi in this study. Only through surface areas can this type of elements be determined.

One would think that assuring air, surfaces, total fungal load and species definitions was enough to establish a methodology. But it is not.

Different sample analysis have their pros and cons. Conventional culturing methods highlight the viability of the fungal flora and are still the most currently used for fungal assessment. Though time consuming and requiring a trained eye, they are widely available and relatively cheap. But they only give you a partial picture. Quality wise, we now know that most of the knowledge on fungal flora is lost when one chooses a culture plate over a PCR tube. Such a world of information is still hampered by the costs and technical expertise needed to apply the DNA based methods. And when the task is assessing fungal contamination for the presence of allergens then the culturable fungal load is still considered the gold standard. It is back to the culture plate.

To gather the benefits of both techniques and beating their setbacks, the logical way is to include them both. The methodology proposed in this dissertation took this into consideration and, as happened with other authors, molecular biology protocols revealed fungi that had not been previously identified in archives or librairies. In documental samples, altered by biodeterioration agents and from where fungal growth was very difficultly attained, molecular biology protocols played a pivotal role and may even be the best (and only) way to determine the possible culprit. Depending on the culprit is the treatment to be applied, as shown in Chapter 4 (documental case studies). Different stains, created by different fungi, demand different treatments and this makes it of the upmost importance to achieve an identification.

Though expensive, molecular biology is an exciting area where breakthroughs are achieved at a high rate. The DHPLC, a methodology just developed a decade ago has seen much of its applications in the field of mutation detection. Population dynamics came next and it was now applied to distinguish fungal elements in mixed samples, a development used not only in the samples taken from the archives but also to other cultural heritage settings and also to clinical samples.

And since yeasts have now carved their way into our air and surfaces fungal communities it became more than appropriate to provide an easy to use tool to define their presence. The AFCE, based on the construction of a solid database, is a quick and easy molecular biology method which can be applied to mixed samples of environmental or clinical yeasts. The database was initiated with reference strains and was enriched with environmental yeasts presented in the studied archives.

Both methodologies – DHPLC and AFCE - would benefit from additional studies. DHPLC was newly adapted to correspond to the goal of separating mixtures and, as any new methodology, lacks the benefits of time and experience a four year study can not provide. It needs to be further tested and improved in order to achieve maximum efficacy as for some of the samples tested it was not able to provide a clear distinction between peaks. The study of ITS region also needs to be optimized, since this has become the sequencing rRNA region of excellence. The D1/D2 LSU region revealed itself as a much easier region to amplify and the reasons behind the difficulties experienced with the ITS should be further explored.

Despite the excellent results obtained so far, AFCE still requires a stronger database. More data are needed on the species currently found in Archives and how these relate to biodeterioration.

The presence of airborne biological materials, may lead to deterioration when the characteristics of the substrate and the surrounding environment are compatible with the nutritional needs of the microorganisms. When maintained at a steady RH of 45-55 % and 18-22 °C, paper tends to adapt and adjust displaying a low moisture environment, ideal to prevent fungal growth. However, microclimates can be formed even between the pages of a book and the water that is available for fungi to grow becomes just about right. Water activity, another term for this unbound water, becomes crucial. A preliminary study on the water activity levels was performed on the various types of surfaces sampled and the results were, in their majority, low enough to hamper fungal growth. The fungal load was compared with the water activity levels but no positive correlation was encountered.

In the paper samples, it is worth noticing the variety of water activity levels registered at the same relative humidity which comes to reinforce the formation of these dangerous microclimates, despite efforts to maintain a low relative humidity.

Due to the reduced number of samples, no significant onclusion can be taken from the comparison between the water activity values determined in the chromatically altered areas and the otherwise *healthy* areas. As mentioned in Chapter 6, more studies are needed on the subject.

As the study was performed the reality of the professionals who work and spend most of their lives in these settings became closer and the health problems reported by staff are just *too* frequent. Indoor air quality studies identify biological contaminants as the possible causing agents but particulate matter and chemical contaminants are not discarded. Some of these chemicals may even be a product of the fungal metabolism, a toxin produced by it or even the remanescent of previous human attempts to eradicate it. In mid-XX century, biocides were proposed as a method to decrease and even stop fungal growth completely. Entire buildings would be closed and fumigated with increasing risks for both documents and people who dealt with these documents (Florian, 2002). Both at the time of the fumigation and even 30 years later…

A study on the physical and chemical parameters usually taken into account in indoor air quality surveys was applied to two of the sampled Portuguese Archives. The results were higher than desirable for relative humidity, ozone, VOCs and particulate matter and a positive relationship was established between this last parameter and fungal load. Complex settings such as archives and librairies, however, demand further analysis and equipment for the analysis of the VOCs as these, responsible for the trademark that is "the book smell" may contain more than *just* the innocuous "traces of vanilla and mustiness" as defined by Matja Strilic to *The Telegraph*.

In all the visited archives at least one of the workers suffered or reported an allergic episode, either eased quickly or still requiring special care. From skin reactions to respiratory ailments, taking care of our written heritage takes a toll on the caretakers. This is, therefore, a pressing matter: to study the implications the environment has on these workers. To the author's knowledge, despite daily complaints amongst those in the field of conservation, no large-scale occupational medicine survey was conducted yet in Portugal.

Regarding both human health and paper conservation, it is important to highlight the message left in one of the archives visited and which, over the years, has suffered incidents that increased its susceptibility to fungal growth. The advent of mechanical controlled environments made it possible for institutions to pursuit a stable

environment and the strict following of rules regarding optimal temperature and relative humidity levels. A few decades have passed and the experience of highly controlled environments has raised questions and concerns. Devising a room or series of rooms in which forced ventilation is the only available ventilation should come with the guarantee that the system will always work and proper maintenance (including cleaning) will never be a question of available budget. When problems arise – and they will – an archive, library or museum – can be faced with a problem of high humidity levels or fungal contamination and no immediate solution for the problem.

In case of an abnormal fungal contamination, health precautions must be taken before analysing the situation. The fungus in question might not be toxinogenic or hyperallergenic but this information is not readily accessible when first encountering an acute fungal infestation. In the event of one, the measures should be to confine it, stop its growth, eradicate it and prevent its reappearance (Florian, 2002). Stringent personal equipment is required before acting (Florian, 2002).

Ventilation is a powerful tool to achieve environmental conditions that decrease the moisture content of historic buildings and thus prevent biodeterioration and is also a useful, simple and safe way of treating infected objects (Singh et al., 2002). According to Valentin et al. (2002), fungal development can be halted by constant ventilation even if this is kept at very low rates. These will, of course, depend on room volume, temperature and RH or a_w in objects but for heavily contaminated objects the authors recommend continued ventilation for 3 months with 4 air changes/hour in a room of 25-50 m^2.

Conservation strategies including maintenance, microclimate control through the monitoring of environmental conditions, inspection of newly acquired heritage objects (Florian, 2002) and the estimation of the number and types of microorganisms in both air and objects are strongly recommended. Both must be sampled as it was observed that cellulose objects required longer exposure time (three times more) in the improved climate to achieve a significant decrease in microbial growth (Valentin, 2007). In case of infestation, an immediate and an after clean-up monitorization should be performed (Florian, 2002).

These measures coupled with low temperatures and ventilation (as a preventive or corrective measure) in case of contamination can be used to avoid microbial germination and the colonisation of organic materials (Valentin et al., 2010; Florian et al., 1994).

Fungal assessment in every setting is a complex and intrincate subject since it envolves one of the most amazing organism in the world. Paper conservation and human health are equally complex and when the three are combined, as in this Dissertation, the notion of what still needs to be investigated is overwhelming. Though modest, this was my contribution on the subject and I hope it can provide in inspiration for others a fraction of what it provided in enjoyment for me.

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