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Fungal biodeterioration of paper: Development of safer and accessible conservation treatments

Dissertação para obtenção do Grau de Doutor em Conservação e Restauro do Património, Especialidade em Ciências da Conservação

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FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Abril, 2016

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Dedicated to my family

Acknowledgments

This PhD was developed at the Conservation and Restoration Department, FCT, Universidade NOVA de Lisboa, (DCR, FCT-UNL); at the Tropical Research Institute (IICT); at the Chemistry Department, FCT-UNL; and at VICARTE Research Unit, UNL.

This multidisciplinary work was supported by Fundação para a Ciência e Tecnologia - Ministério da Educação e Ciência (FCT-MEC), with a doctoral grant (SFRH/BD/72216/2010) and the project UID/EAT/00729/2013; and by Unidade de Ciências Biomoleculares Aplicadas - UCIBIO which is financed by national funds from FCT/MEC (UID/Multi/04378/2013) and co-financed by the ERDF under the PT2020 Partnership Agreement (POCI-01-0145-FEDER-007728).

A PhD thesis is always the result of a multitude of collaborations and interdisciplinary work. I would like to express my sincere gratitude to everyone that contributed to the achievements presented in this thesis.

To my supervisor Prof. Filomena Macedo, for all the guidance, helpful suggestions, companionship and constant encouragement. To my co-supervisor Prof. Eurico Cabrita, for all the support, valuable advice and friendship. To my co-supervisor Dr. Alan Phillips, for all the important inputs and wisdom.

To my thesis advisory committee (Comissão de Acompanhamento de Tese), Prof. António Portugal and Prof. César Laia, for the discussion and valuable advice on the PhD Thesis Plan Evaluation.

To all the kind people who made available the respective techniques and instruments and for all the support in analysing the results: Prof. António Candeias and Dr. Gabriela Carvalho (DGPC), for the artificial ageing chamber; Prof. Cesar Laia (DQ, FCT-UNL), for the DLS analyses; Prof. Fernando Pina and Prof. João Carlos Lima (DQ, FCT-UNL), for the photochemistry analyses and discussion; Prof. João Lopes (Faculty of Pharmacy, Univ. of Lisbon), for the analysis of FTIR results; Dr. José Carlos Rodrigues (IICT), for the ATR-FTIR analyses; Prof. José Paulo Sampaio (DCV, FCT-UNL), for the autoclave; Prof. Márcia Vilarigues (DCR, FCT-UNL), for the EDXRF analyses; Prof. Maria João Melo (DCR, FCT-UNL), for the FTIR analyses and discussion; Dr. Miguel Silva (DCR, FCT-UNL), for the ATR-FTIR analyses; and Prof. Rogério Simões (DQ, UBI), for the support with the Folding Endurance analyses and hospitality in UBI. I also thank all the other people who helped me during the experimental work, as well as their valuable comments.

To all the participants of the questionnaire, Prof. Luís Ramos for the helpful discussions with the statistical analyses of the obtained results and Conservation DistList manager for the questionnaire distribution and information regarding the number of registered users.

To Dr. Ana Cannas and Prof. Conceição Casanova, for all the support in the work developed in IICT and encouragement to start this endeavour. To Professor Telmo Verdelho for the images of the biodeteriorated song book.

To all my colleagues from labs 302/304 in DQ, the people from DCR and the people from VICARTE, for the companionship and support. A special thank you to Aline Aquino, for her friendship and for helping me in those days where two hands were not enough.

To all my PhD colleagues, especially to Ana Margarida Silva, Ângela Ferraz, Élia Roldão, Filipa Pereira, Marta Félix, Mathilda Larson, Susana Belchior and Susana Sá, for all the companionship and shared concerns. A special thank you to Catarina Pinheiro for the text revisions and precious advice during these PhD years.

To Arminda Fortes, Catarina Gonçalves, Catarina Mateus, Laura Moura, Laura Domingues, Manuela Carvalho, Marta Costa, Rogério Abreu, Tiago Rodrigues and all my colleagues and friends in IICT. It has always been a pleasure working with you.

To my dear professor and friend Luis Elias Casanovas, for being an incredible teacher and human being.

To my family and friends for all the distractions from the PhD.

My special appreciation and heartiest thanks goes to my parents, for being an extraordinary example and support in what really matters, and to my husband for all the patience and encouragement in this everlasting journey.

"(…) libraries should be towards the east, for their purposes require the morning light: in libraries the books are in this aspect preserved from decay; those that are towards the south and west are injured by the worm and by the damp, which the moist winds generate and nourish, and spreading the damp, make the books mouldy."

Marcus Vitruvius Pollio (1st century BC)

Part of the content of this PhD Dissertation has already been published in peer-reviewed international journals included in the Science Citation Index (SCI):

Sequeira, S. O., Coutinho, M. L., Lima, J. C., Phillips, A. J. L., Macedo, M.F., Cabrita, E. J., '4-MUF-NAG for fungal biomass determination: scope and limitations in the context of biodeterioration studies'. Jounral of Cultural Heritage (in press). DOI: 10.1016/j.culher.2016.06.002.

Sequeira, S. O., Phillips, A. J. L., Cabrita, E. J., Macedo, M.F. (2016) 'Ethanol as an antifungal treatment for paper: Short-term and long-term effects'. Studies in Conservation (in press). DOI: 10.1080/00393630.2015.1137428.

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

Sequeira, S., Cabrita, E. J. and Macedo, M. F. (2012) 'Antifungals on paper conservation: An overview.' International Biodeterioration & Biodegradation, 74, pp. 67–86. DOI: 10.1016/j.ibiod.2012.07.011.

Abstract

Great part of the History of mankind is registered in the form of documents or works of art on paper support. Paper can be deteriorated due to physical, chemical and biological agents. Within microorganisms, fungi are the major paper biodeteriogens. Throughout history, several toxic methods have been used to prevent and stop fungal deterioration on paper based materials. More recently, a growing concern about environmental and health issues has led to the research on new antifungal alternatives, with lower toxicity. However, the existent antifungal methods and compounds still have drawbacks in terms of efficacy, health hazards, damaging effects on paper, or lack of thorough testing. In this context, the present thesis focused on testing and developing accessible antifungal treatments with low toxicity, which could prevent the long term paper deterioration.

The selection of antifungal compounds was made taking into account the results from a literature review on antifungals used on paper conservation, a survey to paper conservators, and a review of antifungals used in cosmetics and pharmaceutical industries, having as a basic premise their low toxicity. *Aspergillus niger*, *Chaetomium globosum*, *Cladosporium cladosporioides*, *Penicillium chrysogenum* and *Penicillium corylophilum* were selected as test fungal species. Fungal growth on paper was evaluated by measuring colonization areas and biomass dry weight determination.

A formulation containing parabens and calcium propionate (PBs+CP) presented the best antifungal activity on paper samples, followed by a formulation containing clotrimazole and calcium hydroxide nanoparticles (CLT+NPs), and ultimately 70% ethanol (70%EtOH).

Before application on cultural heritage materials, conservation treatments have to be thoroughly tested to assess if they can cause any damage on the treated materials in the short and long term. The effects of the tested formulations on paper were evaluated in terms of pH, colourimetry, folding endurance and molecular alterations, using moist heat artificial ageing. Besides plain paper, paper previously biodeteriorated by *A. niger* was tested in order to evaluate the potential of each compound to prevent further deterioration caused by fungal metabolites. The obtained results on biodeteriorated samples illustrate how tremendously damaging the products excreted by fungi can be in the long term. PBs+CP formulation was the only one capable of preventing long term acidification, loss of folding endurance, and discoloration caused by fungal metabolites, but on the other hand, on plain samples, this formulation caused paper discoloration. CLT+NPs formulation significantly prevented the acidification and loss of folding endurance, although causing a minor discoloration on paper at a long term. 70%EtOH had a mild positive impact in the chemical stabilization of paper and did not cause any paper discoloration.

The information provided in this thesis contributes to a deeper understanding on safer options for preventing and treating paper deterioration by fungi and opens the way for further research in this challenging field of heritage conservation.

Keywords: paper; biodeterioration; fungi; clotrimazole; calcium hydroxide; ethanol; parabens; calcium propionate; limonene; 4-MUF-NAG; calcium oxalate.

Resumo

Grande parte da nossa História encontra-se registada na forma de documentos ou obras de arte em suporte de papel. A deterioração do papel pode ser catalisada por agentes físicos, químicos e biológicos. Os fungos são os principais agentes de deterioração microbiana deste material. Ao longo da história foram utilizados diversos métodos de elevada toxicidade para prevenir e mitigar a deterioração fúngica em património documental. Nos últimos anos, a crescente preocupação com questões ambientais e de saúde publica, levou à investigação de métodos antifúngicos alternativos, de menor toxicidade. No entanto, os métodos existentes continuam a ter lacunas em termos de eficácia, perigo para a saúde, efeitos nocivos para o papel, ou falta de investigação acerca dos seus efeitos e eficácia. Neste contexto, esta tese de doutoramento incidiu na avaliação e desenvolvimento de tratamentos antifúngicos de baixa toxicidade que fossem acessíveis, a nível de aplicação e aquisição, e que pudessem prevenir a deterioração do papel a longo prazo.

A selecção dos compostos antifúngicos foi baseada nos resultados de uma revisão da literatura acerca dos antifúngicos usados em conservação de papel, num questionário direccionado a conservadores de papel e numa pesquisa sobre compostos antifúngicos usados nas indústrias farmacêutica e de cosméticos. As espécies de fungos seleccionadas foram: *Aspergillus niger*, *Chaetomium globosum*, *Cladosporium cladosporioides*, *Penicillium chrysogenum* e *Penicillium corylophilum*. O crescimento fúngico em papel foi avaliado através da medição de área colonizada e determinação da biomassa.

Os melhores resultados de eficácia antifúngica foram obtidos com uma formulação composta por parabenos e propionato de cálcio (PBs+CP), seguida de uma formulação composta por clotrimazol e nanopartículas de hidróxido de cálcio (CLT+NPs) e finalmente por uma solução de etanol a 70%.

Os efeitos das formulações testadas em papel tratado foram avaliados a nível de alterações de pH, colorimetria, resistência a duplas dobras e alterações moleculares, utilizando-se envelhecimento artificial com temperatura elevada e humidade relativa controlada. Além de papel em branco, testou-se também papel previamente colonizado por *A. niger*, de forma a avaliar o potencial de cada tratamento em prevenir a deterioração causada pelos metabolitos fúngicos. Os resultados obtidos nas amostras biodeterioradas ilustram quão nefastos os metabolitos dos fungos podem ser a longo prazo se mantidos no papel. A formulação PBs+CP foi a única capaz de prevenir a acidificação, a perda de resistência mecânica e a alteração de cor nas amostras biodeterioradas, embora tenha provocado amarelecimento nas amostras em branco. A formulação CLT+NPs preveniu a acidificação e a perda de resistência mecânica no papel, embora tenha causado um ligeiro amarelecimento no papel. O etanol a 70% aumentou ligeiramente a estabilidade química do papel e não causou qualquer alteração de cor.

A presente dissertação contribui para um maior conhecimento de opções mais seguras para prevenir e tratar a deterioração causada por fungos em papel e abre caminho para investigação futura nesta desafiante área da conservação de património.

Palavras-chave: papel; biodeterioração; fungos; clotrimazole; hidróxido de cálcio; etanol; parabenos; propionato de cálcio; limoneno; 4-MUF-NAG; oxalato de cálcio.

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Abbreviations

4-MUF	7-hydroxy-4-methylcoumarin
4-MUF-NAG	4-methylumbelliferyl-N-acetyl-β-D-glucosaminide
AHU	Arquivo Histórico Ultramarino (Overseas Historical Archive)
ATP	Adenosine triphosphate
ATR	Attenuated Total Reflectance
A_w	Water activity
CAS	Chemical Abstracts Service
CFU	Colony-forming unit
CLT	Clotrimazole
CP	Calcium propionate
DGLAB	Direcção-Geral do Livro, dos Arquivos e das Bibliotecas
DLS	Dynamic Light Scattering
DNA	Deoxyribonucleic acid
EMC	Equilibrium moisture content
ERH	Equilibrium Relative Humidity
EtOH	Ethanol
EU	European Union
FTIR	Fourier Transform Infrared Spectroscopy
HCl	Hydrochloric acid
IPA	Isopropanol
JP	Japanese paper
LED	Light-emitting diode
LSD	Least significant difference
MC	methylcellulose
MP	methylparaben
NAG	N-acetylglucosamine
NAGase	β-N-acetylglucosaminidase
NPs	nanoparticles
OPP	ortho-phenylphenol
PBs	Parabens
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
PEL	Permissible Exposure Limit
PP	propylparaben
RGB	Red, Green and Blue colour system
RH	Relative humidity
RNA	Ribonucleic acid
SEM	Scanning electron microscope
Solv	Samples treated with solvent
T	Temperature
TWA	time-weighted average
UV	Ultraviolet
WT	Without treatment samples
XRD	X-ray diffraction
ΔE	Colour difference
μ-EDXRF	Micro-energy dispersive X-ray fluorescence spectrometry

General introduction

Great part of the History of mankind is registered in the form of documents or works of art on paper support. To ensure the passage of this legacy to future generations, the preservation of this material is a matter of the utmost importance.

Paper, like all other materials inevitably degrades over time. However, this degradation can be accelerated by endogenous factors like acidity, metal ions, lignin or paper degradation products, and exogenous factors like heat, humidity, radiation (light, UV), oxygen, pollutants or biodeteriogens (Strlic and Kolar, 2005:6).

Fungal microorganisms are the most common biodeteriogens affecting paper based collections, causing severe material and information losses. The organic composition of paper, which represents an abundant carbon source for such heterotrophic microorganisms as fungi, combined with its hygroscopicity, makes paper a highly sensible material to fungal biodeterioration (Tiano, 2002).

Paper is mainly composed of cellulose, a natural polymer consisting of β-D-glucose units linked by glycosidic bonds that is extracted from plants. The kind of raw material, additives and manufacture processes have greatly changed throughout papermaking history.

The physical and chemical processing, to which cellulose has been increasingly subjected along time, has increased its bioreceptivity to fungi (ability of paper to be colonized by fungi). Native cellulose is mainly crystalline with some amorphous sites, whereas the cellulose present in a paper sheet, by having already undergone physical and chemical processing, contains a larger number of amorphous sites along the polymer. These sites are more susceptible to biodeterioration (Gallo et al., 1998; Allsopp et al., 2004:12). Also, the removal of lignin in papermaking processes, while contributing to the increase of the chemical and physical stability of paper, amplifies its bioreceptivity, since lignin increases the resistance of cellulose to microorganisms (Allsopp et al., 2004:12).

In order to reduce ink spreading in such an absorbent material and also to increase the bonding between fibres, paper was usually sized with polysaccharides, proteins or resins, like starch flour, gelatine or rosin, and ultimately alkaline synthetic compounds (Barrett, 1989; Hubbe and Bowden, 2009). Fillers, like clay or chalk were also added to the paper pulp to increase paper opacity (Cappitelli and Sorlini, 2005). These elements may constitute an additional and easier to absorb food source for fungi, and therefore strongly influence the intensity and results of fungal action on paper (Pinzari et al., 2006).

Paper biodeterioration by fungi

Biodeterioration is any unwanted alteration in a material caused by the vital activities of organisms (Allsopp et al., 2004). These organisms, biodeteriogens, are characterized by their ability to use a substrate to sustain their growth and reproduction (Pinzari et al., 2006).

Filamentous microfungi are considered the most important biodeteriogens in museums, libraries and archives due to their capacity to grow at average relative humidity (RH) levels (above 65%), producing an array of deteriorating enzymes and metabolites (Sterflinger and Pinzari, 2012), and the fact that fungal contamination is very easy to occur. Bacteria can also deteriorate paper materials, but since fungi require less moisture to develop, the environmental conditions normally existent in libraries, archives or museums are more prone to the growth of fungi than that of bacteria.

Four stages of fungal biodeterioration were identified by Gallo et al. (2003): Contamination – germination – development – deterioration of materials. In Figure 1.1 a scheme illustrating the lifecycle of conidial fungi on paper is presented. The stage of paper deterioration occurs mainly during the development of hyphae and colonization of the substrate.

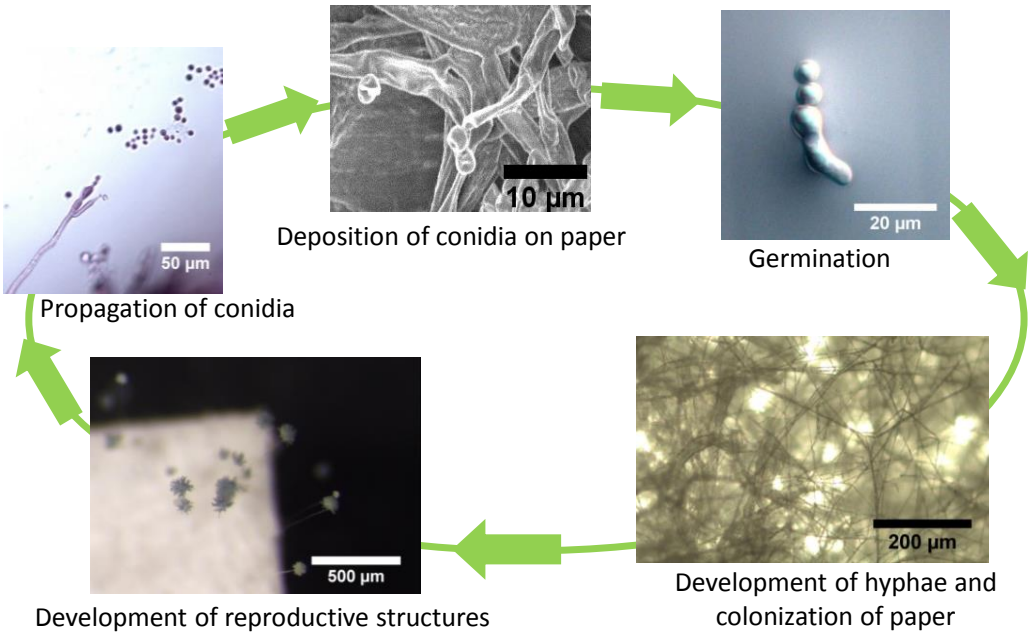


Figure 1.1: Life cycle illustration of conidial fungi developing on paper substrates.

Fungal contamination may be caused by exposure to fungal spores in the air, by contact with contaminated materials or dissemination by vector organisms (e.g. arthropods) (Trovão et al., 2013). Fungal spores may remain viable for several years (Gallo et al., 2003), waiting for the appropriate environmental conditions to germinate.

Besides spores, contamination can also occur from hyphal fragments that can settle on materials and initiate colony formation. However, these elements are more vulnerable to drying and loss of viability (Florian, 2002:41). Since fungal spores and hyphal fragments are ubiquitous in every non-sterilized environment and material, fungal contamination may easily arise in paper (Florian, 2002:28).

Early growth of fungi on paper is not immediately visible to the naked eye (Figure 1.2), and when fungal colonization is detected on paper materials, deterioration has already begun.

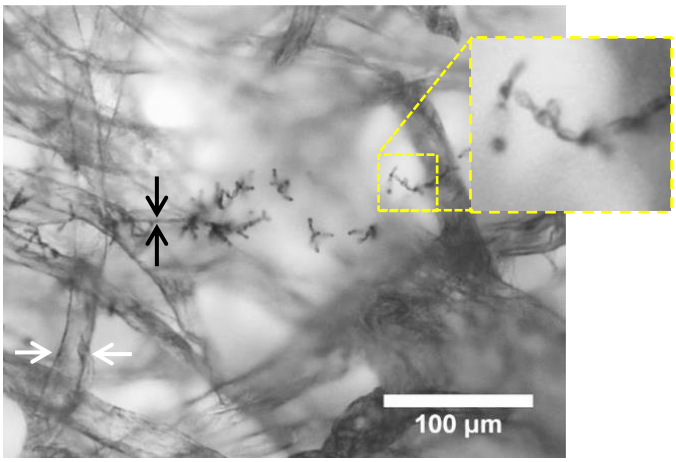


Figure 1.2: Paper with no fungal development visible to the naked eye, when observed under the microscope, where cellulose fibres (white arrows), fungal hyphae (black arrows) and conidia (yellow rectangle) can be distinguished (B).

When paper is colonized by fungi, besides the hyphae penetrating in the paper matrix causing physical alterations, several excreted metabolic products also accumulate in the material. The decomposition of cellulose by cellulolytic fungi is mainly due to the production and secretion of a complex of extracellular enzymes – cellulases - which hydrolyse the cellulose macromolecule into small water soluble sugars which can then be processed by the fungi (Bergadi et al., 2014). Other enzymes, like amylases or proteases decompose paper additives, causing loss of mechanical resistance. Non-enzymatic metabolites, like acids or metal ions also cause accelerated depolymerization of cellulose (Hastrup et al., 2011). Furthermore, the biogenic formation of calcium oxalate crystals promoted by fungi can change the calcium carbonate alkaline reserve in paper and be a source of chemical and mechanical damage (Pinzari et al., 2010).

The deterioration caused by all these fungal compounds will cause a gradual loss of mechanical strength in paper (Ponce-Jimenez et al., 2002). On an advanced stage of deterioration by fungi, paper acquires a felted consistency, with little or no mechanical resistance and its manipulation may result in losses of material and information (Figure 1.3).

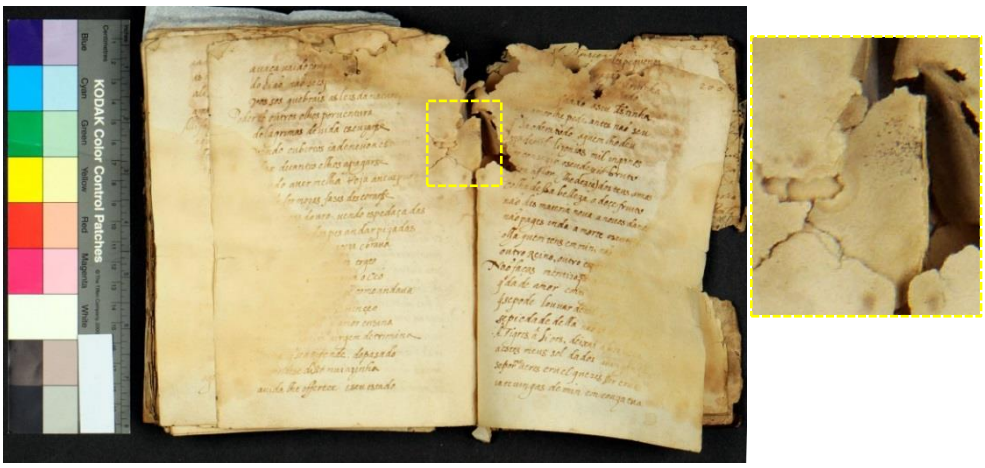


Figure 1.3: Manuscript song book, 16th century, private collection. The felted consistency and coloured fungal metabolites may be observed in greater detail in the magnified yellow rectangle.

The chemical products excreted by the fungi will remain in the paper and continue their deleterious effects, even after the fungi are inactivated. Excreted enzymes are not dependent on the organism for their activity and may remain unaffected, continuing to decompose paper after the microorganism is inactivated (Allsopp et al., 2004:14). Acids and metal ions if not removed from the paper will continue reacting with it. Other excreted products, like glycerine, can increase the moisture content of the contaminated spot and act as conidia activators on a subsequent contamination (Florian, 2002:69). Excreted lipids easily undergo autoxidation and form free-radicals and peroxides, which contribute to the formation of brown discolourations on the substrate (Florian, 2002:68). Therefore it is of utmost importance to remove or neutralize the fungal remains kept within the paper matrix.

Besides chemical and physical damage, fungi often cause aesthetic alterations on paper through the production of coloured pigments, which interfere with the readability and aesthetic value of the object (Figure 1.4) (Florian, 2002:76; Rakotonirainy, Heude, et al., 2007). "Foxing" is a specific type if stains, characterized by small rusty red-brownish spots (Figure 1.4 B) that can be attributed to the action of microorganisms, but also to chemical factors, like iron oxidation within the paper matrix (Choi, 2007).

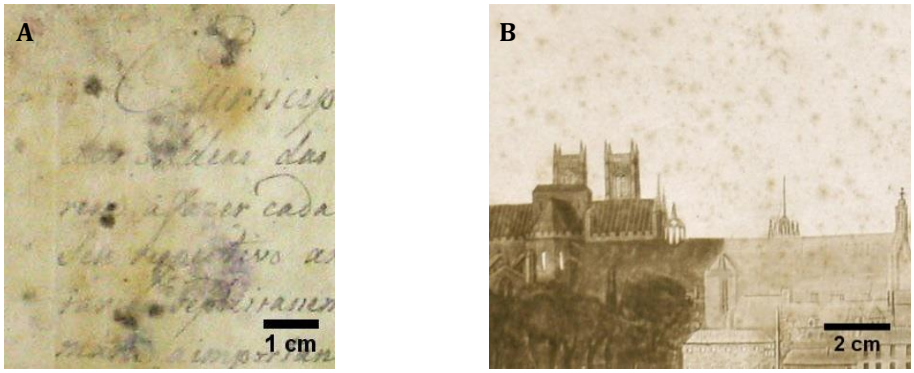


Figure 1.4: (A) Example of coloured fungal stains on an archival document (AHU-DGLAB, Portugal); (B) example of foxing stains on an etching (private collection).

In addition to all these deterioration effects, handling mould contaminated paper objects can constitute a serious health risk, as many of these microorganisms can be pathogenic/toxinogenic (Bennett and Klich, 2003; Pinheiro et al., 2011). Even when the fungi are already inactive, their fungal structures can still contain active allergenic and toxic compounds to humans (Florian, 2002:57), therefore objects affected by fungi should always be handled with protective equipment, such as gloves and masks.

Fungal biodeterioration risk and mitigation

Temperature and humidity are key factors regulating the development of microorganisms. In a material, the existent "free" water which can take part in chemical, physical or biological reactions, is defined as water activity (Allsopp et al., 2004:205). Water activity (a_w) ranges from 0 to 1, and corresponds to the Equilibrium Relative Humidity (ERH) expressed as a decimal (a_w=ERH/100) (Pitt and Hocking, 2009:4).

The amount of water that fungi need for development varies within fungal species. Some xerophilic fungal species are able to germinate at a_w=0.61 (Pitt and Hocking, 2009:354), although the majority of fungi needs a_w levels above 0.80 (Raschle, 2001; Allsopp et al., 2004:207). The a_w needed for the growth of xerophilic species is not rare to occur in many paper depositories (Tiano, 2002). Most bacteria, on the other hand, require a_w levels above 0.95 (Pitt and Hocking, 2009:5).

The a_w of a given material varies with its hygroscopicity (Mandrioli et al., 2003). In the case of paper, hygroscopicity varies with composition (presence of lignin, nature of cellulose, sizings, fillers) and kind of writing media. The higher the hygroscopicity of the paper, the lower is the relative humidity (RH) needed to achieve values of a_w suitable for the development of microorganisms (Gallo et al., 1998).

Measuring RH and temperature on the environment surrounding a material can provide a generic indication of the risk of microbial biodeterioration; however these measurements are usually performed in a determined area of paper repositories, not reflecting the conditions in every niche and corner. Local microclimates with very high a_w can be generated by ineffective ventilation and surface temperature dishomogeneity in a room with an otherwise low RH (Sterflinger and Pinzari, 2012). Also, in already colonized materials, the humidity preserving biofilm will help fungi survive in lower environmental HR (Camuffo, 1998:67).

Lowering the environmental RH, e.g. with the aid of dehumidifiers, is a commonly used approach to decrease a_w in paper. A single sheet of paper on a shelve will react more or less immediately to the changes in the environmental RH and temperature, reaching an equilibrium. However, in an archive, library or museum, paper is rarely housed in this fashion. Generally, several sheets of paper are conditioned together inside boxes or in a book, generally in compact shelves, or inside sealed frames. In these conditions, there is a constant but very slow diffusion of the humidity from the outside to the inside of the paper volume due to a self-buffering effect, and so its response to thermohygrometric changes will be much slower (Garside and Knight, 2011). This premise is even more problematic in case of direct contact of the object with liquid water, in which paper can remain completely wet for days even at low environmental RH.

By keeping the a_w of paper under 0.60, and temperature less than 20°C, with a good air circulation, the development of fungi can be avoided (Arai, 2000; Raschle, 2001; Florian, 2002; Allsopp et al., 2004:207; Valentin, 2010). Nevertheless, the technology and/or building characteristics needed for an efficient climate control are not available to all the heritage repository institutions in the world.

Moreover, microbial infestations can occur even in rooms with climate control, as, like stated before, local microclimates with higher water availability can support fungal activity (Sterflinger and Pinzari, 2012), and emergency situations like floods, leaks or aqueous fire suppression circumstances can take place. In these emergency situations, paper objects are in direct contact with liquid water and fungal development occurs very rapidly and intensively (Figure 1.5).

Figure 1.5: Fungal development on archival documents due to water leak in the roof of a depository room. Image courtesy of Arquivo Histórico Ultramarino (AHU-DGLAB, Portugal).

UNESCO (2007) considers climate change as one of the most serious threats impacting on the conservation of cultural heritage. With climate change, the intensity, frequency and seasonality of extreme events, such as intense rainfall or floods, are projected to increase, together with a rise in global mean temperatures. This will lead to an increased risk of biodeterioration, even in altitudes and latitudes that may not have been previously concerned by such threats (UNESCO, 2007). This increased risk intensifies the need for an urgent development of mitigation strategies to protect our cultural heritage from fungal deterioration.

In the past, several toxic treatments have been applied in paper based collections to prevent and control fungal development, like ethylene oxide, formaldehyde, or thymol (Gustafson et al., 1990; Valentín and Garcia, 1999; Tateo and Bononi, 2006), which are described in greater detail in Chapter 2. Nowadays, the toxic residues left by those treatments, represent a health hazard for the people manipulating the treated materials (Hollinger and Hansen, 2010).

More recently, a growing concern about environmental and health issues has led to the research on new antifungal alternatives, with lower toxicity (Chapter 2). A proper antifungal method for materials should have a broad activity spectrum, good chemical stability, low cost, should not be toxic to humans, and have no adverse effects on the treated material. However, all existent antifungal methods and compounds still have drawbacks in terms of efficacy, health hazards or damaging effects on paper, or have not been tested on the long term effects (Chapter 2). Therefore, these methods/compounds cannot be regarded as safe to be applied in cultural heritage objects. The exploration of safer and effective antifungal treatments for paper objects is consequently an open and challenging field of research.

Objectives

In the scientific field of paper conservation, the main objective is to slow down the deterioration rate of paper as much as possible, by developing methods or compounds, which avoid or minimize the exposure of paper to its deteriorating agents.

Biodeterioration by fungi is a major problem in paper conservation, not just because of the damages it causes, but also due to the lack of effective and safe preventive and curative treatment options. This problem encouraged the development of this thesis, whose main goal is studying and developing remedial antifungal conservation treatments that can be applied when preventive conservation has failed or has not been applied. Remedial conservation includes all directly applied actions, aimed at "arresting damaging processes, and when possible, stabilizing their condition against further deterioration" (CEN, 2011).

This work focused on developing chemical antifungal treatments, with low toxicity and easy to access, which can be used by paper conservators in their own work facilities, do not cause further deterioration of treated paper, and help prevent the damaging effects caused by fungal metabolites.

To achieve this main goal it was necessary to:

- Review the literature of past and most recent present antifungal methods used for paper collections.
- Develop a questionnaire for paper conservators regarding what is currently being applied to control and treat paper biodeterioration by fungi, and what are their major necessities in this research field.
- Select novel antifungal compounds for testing on paper, from the pharmaceutical and cosmetics field, taking into account their chemical properties and health effects.
- Select representative fungal species to test the selected antifungal compounds.
- Study methods for efficient quantification of fungal growth on paper.
- Test the antifungal properties of the selected compounds.
- Test the addition of deacidificant compounds to antifungals in multipurpose formulations aimed at neutralizing the deterioration caused by fungal metabolites.
- Evaluate the safeness of the studied formulations in terms of paper conservation at a long term.
- Evaluate the capacity of the tested formulations in preventing the deterioration caused by fungal metabolites in theshort and long term.

Outline of the thesis

Chapter 1. General Introduction

This introductory chapter covers a brief description on the composition of paper, the causes and effects of paper biodeterioration caused by fungi, and the risk and mitigation strategies of fungal biodeterioration on paper. The framework and objectives of this research and the outline of the thesis are also presented in this chapter.

Chapter 2. The use of antifungals on paper based collections: an overview

Chapter 2 presents a critical review on the history and state of the art of antifungal compounds and methods used in paper collections. It enables the distinction of the best existent methods for inhibiting fungal development on paper, providing a basis for selection of methods deserving further research in this thesis.

Chapter 3. What is the panorama of fungal biodeterioration of paper worldwide? - results of an international survey

Chapter 3 presents and discusses the results of a survey of paper conservation practitioners worldwide, aimed at assessing the current panorama of fungal biodeterioration of paper, namely on how common is this type of deterioration, what methods are actually being adopted for the prevention and recovery of fungal affected paper, and what do conservators consider the most urgent topic to be studied in this field. This chapter provides an overview of current paper conservation practice regarding fungal biodeterioration, reinforcing the objectives of this research.

Chapter 4. Selection of fungal species for antifungal testing

Chapter 4 covers a literature review on the most common fungal species found on paper collections. Based on that inventory, a more informed and representative selection of the five fungal species to test on the following chapters is achieved.

Chapter 5. Quantifying fungal growth on paper

Chapter 5 is divided in two subsections, where different methods for quantification of fungal growth on paper are tested and discussed. The first subsection focuses on the quantification of a specific fungal enzyme, related to fungal development. The second subsection consists on the adaptation of classical measurement methods - size of colonies and biomass dry weight – to paper samples. The optimization of methods for fungal growth measurement achieved in this chapter is then adopted for the evaluation of antifungal compounds in the subsequent chapters.

Chapter 6. Ethanol as an antifungal treatment for paper: short-term and long-term effects

Ethanol, currently one of the most used antifungals on paper collections (see Chapter 3), is studied in Chapter 6 in greater detail, since there is no general

agreement on the efficacy and safeness of this alcohol. Taking into account the selected fungal species in chapter 4 and the fungal quantification methodologies optimized in Chapter 5, the effect of ethanol on the germination of spores and inhibition of fungal development is assessed. The preventive and curative antifungal activity of ethanol on paper samples is evaluated, together with its effect on the chemical and physical properties of paper in the short and long term.

Chapter 7. Study of antifungal and deacidificant formulations to inhibit fungal biodeterioration of paper

In Chapter 7, antifungal and deacidificant formulations are tested, with the aim of developing multipurpose treatments to inactivate fungi and prevent paper biodeterioration. This chapter is divided into 3 sub-chapters:

Chapter 7A. Parabens and calcium propionate

From Chapter 3, a previously developed antifungal and deacidificant formulation using parabens and calcium propionate presenting promising results, is thoroughly tested. The preventive and curative antifungal activities on paper are assessed against a higher number of fungal species. The durability of the antifungal activity and influence of application method are also evaluated. To ascertain the safeness of use of this formulation on paper artefacts, the potential damaging or protective effects on pristine paper and biodeteriorated paper in the short and long term are analysed.

Chapter 7B. Limonene vs Clotrimazole

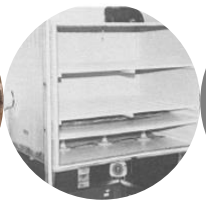
Taking into account the results from the survey presented in Chapter 2, where paper conservators considered the development of new non-toxic antifungals a priority in this field, two antifungal compounds used in the pharmaceutical and cosmetics industries are selected. Clotrimazole and limonene are screened for their antifungal activity on *Penicillium chrysogenum* at different concentrations and the compound achieving the best results is further studied in the following sub-chapter.

Chapter 7C. Clotrimazole and $Ca(OH)_2$ nanoparticles

A formulation of clotrimazole and calcium hydroxide nanoparticles is tested against the fungal species selected in Chapter 4. As in Chapter 7A, the preventive and curative antifungal effect of this formulation is evaluated on paper samples, as well as the potential damaging or protective effects on pristine paper and in biodeteriorated paper in thea short and long term.

Chapter 8. Concluding remarks and future perspectives

In this final chapter, the main results of the entire thesis are discussed and conclusions are taken, along with suggestions for future research possibilities.

2

The use of antifungals on paper based collections: an overview

Published with minor alterations as Sequeira, S., Cabrita, E. J. and Macedo, M. F. (2012) 'Antifungals on paper conservation: An overview.' International Biodeterioration & Biodegradation, 74(0) pp. 67–86.

Throughout history, several methods have been used to prevent and stop fungal deterioration on paper based materials. In this chapter a review of the main chemical and physical methods used to avoid fungal paper biodeterioration until nowadays and also of some new approaches tested recently is presented. The advantages and disadvantages of these methods are discussed as well as their health effects. Studies regarding antifungal compositions, methods of application, performance and effects on the treated materials are also presented with the aim of providing a clear set of conclusions on the topic.

2.1. Introduction

Since ancient times there has been a great concern about the inhibition of biodeterioration of paper items. Tsuen-hsuin (1985:84) cites the 6th century author Chi Min Tao Shu, recommending that "Between the fifteenth of the fifth month and the twentieth of the seventh month, book rolls must be unrolled and rolled three times. This should be done on a clear day in a spacious house which is aired and cool, and books should not be exposed directly to the sun, for it will turn the paper brownish. Rolls heated by the sun quickly attract insects, and rainy and humid days should especially be avoided. If books were cared for in this way, they would last for several hundred years".

The great majority of antifungal methods used to prevent and/or stop biodeterioration caused by fungi in paper conservation have been adapted from other scientific fields (Nittérus, 2000b), like material protection, agriculture or medicine. These methods can go from limiting the access to water by the fungi, to the application of chemical products in the gaseous or liquid state, or physical methods like extreme temperatures, radiation or current. Generally, the physical methods do not have a long term action - since they leave no residues, their microbiocidal action is only immediate. However, the majority of chemical compounds, even in the gaseous state, leave residues that can prolong its antimicrobial effect during a limited period of time.

Limiting the access to water, by lowering the water activity on the substrate, may be the simplest and harmless way to stop fungal growth. After a water-related disaster, this can be achieved by drying, but when dealing with great amounts of humid or wet paper, due to the high hygroscopicity of paper, this is usually a slow process and time is a key factor on the growth of microorganisms.

Chemical microbicides can be introduced already in the manufacture process of paper, as an addition to sizing products, directly to the paper pulp, or as a coating, to prevent microbial development (Paulus, 2004). These compounds can also be added to glues used in paper items.

Traditionally, in the preparation of starch paste - an adhesive easily spoiled by microorganisms when fresh and eaten by insects when already applied in documents - fungicides and insecticides were added occasionally (Jenkinson, 1922; Freitas, 1937). Nevertheless, the majority of antifungal methods have been applied in the disinfection of already formed paper objects, like documents and artworks.

Although antifungals can be applied locally on a single object, most of the antifungal methods for paper items have been used as massive disinfection treatments for collections. These massive treatments have been applied after a mould outbreak, as a preventive measure before incorporation in an institution, or as periodic curative and preventive procedures. Occasionally, the same product was used to eliminate both insect pests and mould.

A proper antifungal method for materials should have a broad activity spectrum, good chemical stability, low cost, should not be toxic to humans, and have no adverse effects on the treated material.

There are several factors that can affect the antimicrobial activity of a microbicide, namely the period of contact, concentration, temperature, pH, presence of organic soiling matter and type of microorganism (Russell, 2003).

Below, the most frequent antifungal methods used for paper items until the present time and also some new approaches tested recently, are described in alphabetical order.

The main goal of this chapter is to make a review on the main chemical and physical antifungal methods used in paper based collections until nowadays. By describing their major advantages and disadvantages, the data gathered in this chapter will be useful to provide a basis for decision making on the prevention and treatment of fungal deterioration of paper, and also a basis for selection of methods deserving further research in this thesis.

2.2. Chemical Methods

Antifungal chemical substances act on the fungi through the interaction between their active ingredients and specific target sites on and in the microbial cell (Paulus, 2004:9). The efficacy of these chemical compounds can be related to their chemical structure and chemical and physical properties (Paulus, 2004:9). The majority of microbicides used for material protection can be characterized according to its mechanism of action as being electrophilically or membrane-actives (Paulus, 2004:10; Fraise et al., 2013:231).

Membrane-active microbicides (e.g. alcohols, phenols, acids, salicylanilides, carbanilides, dibenzamidines, biguanides, quaternary ammonium salts, azoles) act directly on the cell membranes through a lytic effect (Paulus, 2004:10; Fraise et al., 2013:231). These compounds coat the cell wall adsorptively (forming a thin film on its surface), and cause changes in the outer membrane and along the cell wall. By losing integrity, these barriers allow the microbicide to access the cytoplasmic membrane, where the lethal effects occur (e.g. enzyme inhibition, escape of essential components from the cytoplasm) (Paulus, 2004:10).

Electrophilically active microbicides (e.g. heavy metals, halogens, oxidizers, aldehydes, carbamates and isothiazolones) are attracted to components in the microbial cell with high electron density, such as nucleophilic components (Paulus, 2004:14; Fraise et al., 2013:231). By

reacting through electrophilic addition or substitution, these microbicides can lead to the inactivation of critical enzymes, inhibiting metabolism and growth (Paulus, 2004:14; Fraise et al., 2013:231). The higher the electrophilicity of the microbicide, the higher is its antimicrobial efficacy.

The chemical antifungal compounds organized by chemical classes are described below and summarized in Table 2.1.

2.2.1. Alcohols

Alcohols are membrane-active microbicides whose anti-microbial efficacy is universally acknowledged. This efficacy increases with chain length (Paulus, 2004:271) and is mainly caused by damaging cell membranes, denaturing essential microbial proteins and ultimately causing cell lysis (Fraise et al., 2013:36). Aqueous alcohol solutions are more effective against microorganisms than pure alcohol. The highest efficacy is reached with concentrations between 50% and 90% (v/v), depending on the kind of alcohol (Bacílková, 2006).

Alcohols can be effective against vegetative forms of bacteria, fungi and viruses, but their effect against sporulating microorganisms has not been yet proven, and so, they are only considered disinfecting and not sterilizing agents (Bacílková, 2006).

A comparison between the effects of ethanol, isopropanol and butanol vapours on the inactivation of two species of fungi and on solubility of four types of writing inks has been performed by Bacílková (Bacílková, 2006). The results show that butanol vapours eliminated the tested fungi on concentrations between 80 and 96%. Isopropanol and ethanol were effective on a range between 30 and 90%. For the solubility assay, all the alcohols vapours caused feathering of three of the tested inks.

Isopropanol is currently used as an antifungal by paper conservators (see Chapter 3), but although it may have a slightly higher antimicrobial activity than ethanol, it is also twice as toxic to humans (Fraise et al., 2013:37).

As ethanol is the most commonly used alcohol for treating mould contaminated heritage objects, a more detailed review follows.

2.2.1.1. Ethanol

Ethanol (C_2H_6O) or ethyl alcohol is a widely used disinfectant. The first study concerning the effects of ethanol on bacterial cultures dates from 1880 (Bacílková, 2006). Although the specific nature or site of action of ethanol is not fully known, the most widely adopted theory is that ethanol acts through coagulation/denaturation of proteins and membrane damage, with consequent interference with metabolism and resulting in cell lysis (McDonnell and Russell, 1999; Block, 2001:232).

The microbicide effect of ethanol depends on its concentration and formulation composition. The higher effect is reported for ethanol concentrations between 50% and 80% in aqueous solutions, reaching a maximum of efficacy at 60-70% (Nittérus, 2000a; Block, 2001; Fraise et al., 2013:37). This optimal concentration is likely related to the mechanism of action of ethanol and is described in higher detail in Chapter 6.

Ethanol disinfectant solutions are usually applied by spraying, brushing, swabbing, immersion, or by exposing the infected papers to the vapours of this compound.

Bacílková (2006), reports the efficacy of vapours of ethanol solutions on paper samples inoculated with *Aspergillus niger* and *Penicillium notatum*. According to this author, concentrations between 30 and 90% can inhibit fungal re-growth for at least 14 days after the evaporation of the alcohol. Nevertheless, Bacílková (2006), regards that for thicker materials, like books, the treatment will not be as effective.

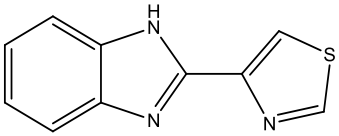
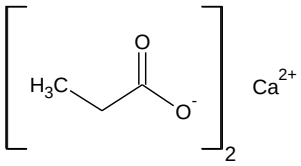
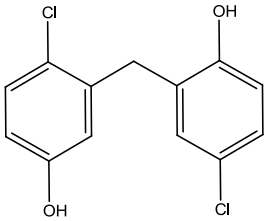
The effectiveness of 70% ethanol solution applied by spray or immersion on paper inoculated with the fungi species *Aspergillus flavus* and *A. niger*, *Chaetomium globosum*, and *Trichoderma viride,* was described by Nittérus (2000a). As reported by this author, 70% ethanol does not have a sporicidal effect, since a re-growth of the fungi occurs within 14 days after the treatment. Ethanol immersion shows higher efficacy than spraying (Nittérus, 2000a), maybe due to a longer contact with the solution before evaporation. An interesting observation was also made: *Aspergillus niger* did not grow on untreated reference samples but it did grow on spray treated samples, which can indicate some spore activation properties of the ethanol solution (Nittérus, 2000a).

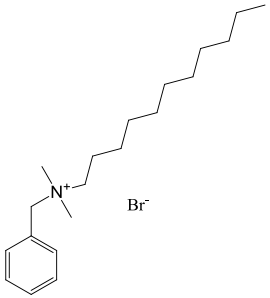
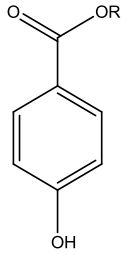
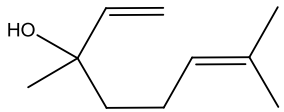
According to Bacílková (2006), alcohols, ethanol included, can cause changes on paper, namely extraction of soluble components, specially by immersion application. Other changes, especially on transparent papers, include loss of gloss, increase in opacity and slight deformation (Bacílková, 2006). Ethanol can also dissolve media, adhesives, and seals, and so, solubility tests have to be performed previously to treatment. Even with vapour application, ethanol can cause feathering of ink lines (Bacílková, 2006).

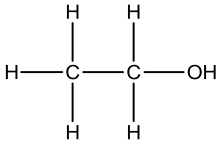
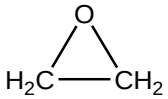
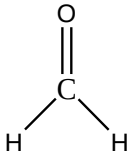
Valentín and Garcia (1999) report that in order to raise ethanol's microbicide activity, it is recommended to add 0.1% of ortho-phenylphenol to the 70% ethanol solution. These authors classify this mixture as the most effective and least toxic of chemical antifungals (Valentín and Garcia, 1999).

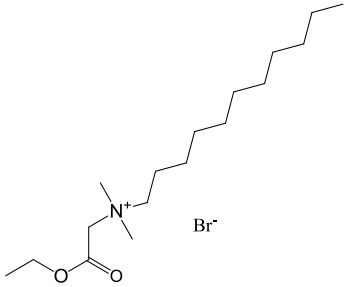
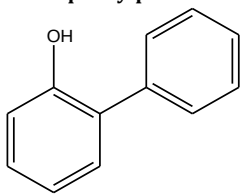
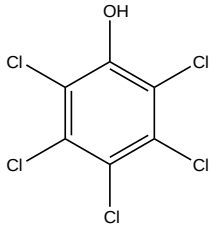
As ethanol is highly flammable it has to be applied and stored away from heat sources. Besides, inhalation can cause respiratory tract irritation and direct contact may cause skin irritation and dehydration, so it must be handled using protective clothing and masks or in a fume hood. The Permissible Exposure Limit (PEL) for ethanol according to the Occupational Safety and Health Administration (OSHA) is 1000 ppm over an eight-hour workday. Ethanol is considered by the World Health Organization as a Group 1 agent (Carcinogenic to humans), but only when ingested in alcoholic beverages (IARC, 2012).

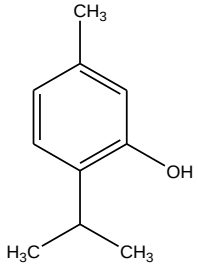
Table 2.1: Summary of the antifungal chemical methods reviewed, in alphabetical order.

Antifungal compound	Advantages	Disadvantages	Effects on paper	First report of use	Use restrictions[a]	LD50 for rats oral route (mg/kg bw)[b]	References
Azole antifungals e.g. Thiabendazole: 	Can be used for aerial disinfection	Fungistatic rather than fungicidal Can leave fatty or powdery films on treated materials	Thiabendazole: Slight decrease on brightness	1944	Different restrictions for each azole compound (see references)	Different values for each azole compound e.g. Thiabendazole: > 2000	(Fabbri et al., 1997; Rakotonirainy et al., 1999; Lamb et al., 2000; US-EPA, 2002, 2005; Gollapudy et al., 2004; Paulus, 2004; Pérez-Rivera et al., 2009; Giavini and Menegola, 2010; EC, 2012)
Calcium Propionate 	Also has deacidifying properties Low toxicity	Fungistatic but not fungicidal	Increase of pH Increase of polymerization degree Minor increase on lightness and yellowness	1930's (1980 on heritage materials)	EU: N/A USA: N/A	3920	(Dersarkissian and Goodberry, 1980; Zappalà, 1990; US-EPA, 1991; Florian, 2002; Paulus, 2004; Suhr and Nielsen, 2004; Neves, 2006; Zotti et al., 2007; Şifa, 2008)
Dichlorophen 	Can be used in alkaline pH Strongly effective against fungi	Causes irritation on skin and eyes	Decrease of polymerization degree Decrease of whiteness Harmful effects on inks	1929	EU: R USA: N/A	1250	(Triolo et al., 1968; Kowalik, 1980; Block, 2001; McBain and Gilbert, 2001; Cox et al., 2004; Paulus, 2004; Yamarik et al., 2004; Escalada et al., 2011; EC, 2012; Gupta and Aggarwal, 2012)

Antifungal compound	Advantages	Disadvantages	Effects on paper	First report of use	Use restrictions[a]	LD50 for rats oral route (mg/kg bw)[b]	References
Dimenthyl-lauryl-benzyl-ammonium bromide 		Low-level disinfectant with no sporicidal activity	Decrease of physical properties, pH, whiteness, and alpha cellulose content	1986: on heritage materials	EU: R USA: R	N/A	(Strzelczyk and Rozanski, 1986; Bello-Gonzalez et al., 2008; EC, 2012)
Esters of p-hydroxybenzoic acid  R= alkyl group	Low toxicity pH range of activity compatible with paper conservation	Mainly fungistatic and bacteriostatic	Minor yellowing Slight decrease on tensile strength Increase of pH Slight increase on percentage of deformation	1920's (1990 on heritage materials)	EU: N/A USA: N/A	2100->8000	(Gustafson et al., 1990; Soni et al., 2002, 2005; Russell, 2003; Paulus, 2004; SCCP, 2005; Neves, 2006; Zotti et al., 2007; Neves et al., 2009)
Essential oils e.g. Linalool: 	Low toxicity	Fungistatic rather than fungicidal Can act as insect attractants Linalool: undergoes autoxidation on air, forming hydroperoxides	Decrease of pH Possibility of causing oxidation by the formed hydroperoxides	N/A	Different restrictions for each essential oil component (see references)	Different values for each essential oil e.g. Linalool: 2790	(Sikkema et al., 1994; Florian, 1998; Karpouhtsis et al., 1998; Sköld et al., 2002; Rakotonirainy and Lavedrine, 2005; Abad et al., 2007; Rakotonirainy, Juchauld, et al., 2007; ECHA, 2012)

Antifungal compound	Advantages	Disadvantages	Effects on paper	First report of use	Use restrictions[a]	LD50 for rats oral route (mg/kg bw)[b]	References
Ethanol 	Evaporates and does not leave toxic residues	Mainly fungistatic, depending on concentration Does not have a preventive antifungal effect	Loss of gloss, increase in opacity and slight deformation (especially on transparent papers) Possibility of dissolving media, adhesives, and seals	1880	EU: R USA: N/A	10470	(Valentín and Garcia, 1999; Nittérus, 2000a; Florian, 2002; Bacílková, 2006; EC, 2012; ECHA, 2012)
Ethylene oxide 	High antimicrobial efficacy Great power of penetration Can be used for mass treatments	Raises the susceptibility of objects to future microbial attack Classified as a category 1 carcinogen	Decrease of mechanical properties and polymerization degree Oxidation Yellowing	1928 (1933 on heritage materials)	EU: R USA: R	330	(Cotton and Roark, 1928; Flieder, 1965; Strassberg, 1978; Ballard and Baer, 1986; Valentin, 1986; Craig, 1986; Valentin et al., 1990; Hengemihle et al., 1995; Ponce-Jimenez et al., 2002; Florian, 2002; Rakotonirainy et al., 2003; US-EPA, 2004; OSHA, 2005; Tateo and Bononi, 2006; Mendes et al., 2007; EC, 2008; IARC, 2008; EU-OSHA, 2009; ECHA, 2012)
Formaldehyde 	Also has sporicidal effect Can be used for mass treatments	At low RH undergoes polymerization and precipitates on materials Low power of penetration It is carcinogenic Causes irritation of the eyes, nose and throat, and contact dermatitis	Cross-linking of cellulose Loss of flexibility Enhance of iron gall ink corrosion	1889	EU: R USA: R	460	(Gallo, 1963; Heim et al., 1968; Valentín and Garcia, 1999; Paulus, 2004; US-EPA, 2008a; EC, 2012; ECHA, 2012)

Antifungal compound	Advantages	Disadvantages	Effects on paper	First report of use	Use restrictions[a]	LD50 for rats oral route (mg/kg bw)[b]	References
Lauryl-dimethyl-Carbethoxymethyl ammonium bromide 	Can be used for mass treatments (nebulisation)	Undergoes acidification with time. Irritates the mucous membrane.	Minor darkening and acidification of paper Depolymerisation of cellulose Decrease of adhesion of inks to paper		EU: R USA: R	N/A	(Heim et al., 1968; Triolo et al., 1968; Strassberg, 1978; Kowalik, 1980; EC, 2012)
Ortho-phenylphenol 	Has a broad spectrum of activity Efficient at low concentrations Can be used in alkaline pH Can be used for mass treatments	Fungistatic but not fungicidal Can leave a very irritating and suffocating odour in the air	Changes in colour Depolymerization of adhesives Accelerated ageing	N/A	EU: N/A USA: N/A	2733	(Triolo et al., 1968; Strassberg, 1978; Haines and Kohler, 1986; IARC, 1999b; Rakotonirainy et al., 1999; Valentín and Garcia, 1999; Paulus, 2004; ECHA, 2012)
Pentachlorophenol 		Carcinogenic, highly toxic	Acid hydrolysis	1936	EU: SR USA: SR	80	(Miller and Aboul-Ela, 1969; Strassberg, 1978; IARC, 1999c; Valentín and Garcia, 1999; Paulus, 2004; Fernández Freire et al., 2005; EC, 2008; US-EPA, 2008b; Gupta and Aggarwal, 2012)

Antifungal compound	Advantages	Disadvantages	Effects on paper	First report of use	Use restrictions[a]	LD50 for rats oral route (mg/kg bw)[b]	References
Thymol 	Can be used for mass treatments (Thymol chambers)	Not fungicidal, only fungistatic Poses a genotoxic risk for humans	Decrease of mechanical properties Yellowing Deterioration of iron gall ink Dissolution of inks	Since ancient Egypt	EU: N/A USA: N/A	980	(Flieder, 1965; Strassberg, 1978; Craig, 1986; Daniels and Boyd, 1986; Haines and Kohler, 1986; Gustafson et al., 1990; Isbell, 1997; Karpouhtsis et al., 1998; Klarić et al., 2007; Buyukleyla and Rencuzogullari, 2009; Napoli et al., 2010; US-EPA, 2010; Coimbra et al., 2011; Numpaque et al., 2011; ECHA, 2012)
Titanium Dioxide (TiO_2 in crystalline form)	May prevent the formation of biofilms	Fungistatic rather than fungicidal Requires UV radiation to exert its microbiocidal activity Classified as possibly carcinogenic to humans	Minor chemical degradation of cellulose Acceleration of organic colorants fading	1985	EU: N/A USA: N/A	> 5000	(Matsunaga et al., 1988; Blake et al., 1999; Huang et al., 2000; Chawengkijwanich and Hayata, 2008; Gavriliu et al., 2009; Harding et al., 2009; IARC, 2010; Afsharpour et al., 2011; Markowska-Szczupak et al., 2011; ECHA, 2012)

[a] Use restrictions concern only the use of the substance as a preservative or biocide for material protection (excluding foods and cosmetics): R=restricted use; SV=severely restricted use; N/A= information not available. This information was gathered from the documents currently available online in the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and the Environmental Protection Agency (EPA) websites, for USA regulations; in the European Community Regulation on chemicals and their safe use (REACH), and in the European Database of Export and Import of Dangerous Chemicals (EDEXIM) websites for EU regulations.

[b] LD50 for rats (mg/kg bw) oral route: Median lethal dose of each compound to kill half the members of a tested population of rats by oral route, measured by mg/kg of body weight.

2.2.2. Alkylating agents

Alkylating agents can combine with amino, carboxylic, sulfhydryl and amino groups in proteins and enzymes in the microbial cell. These compounds are able to inactivate bacteria, fungi and viruses (Russell, 2003).

2.2.2.1. Ethylene oxide

Ethylene oxide [$(CH_2)_2O$], is a colourless flammable gas discovered in 1859 (Ballard and Baer, 1986), although its insecticidal potential was only reported in 1928 (Cotton and Roark, 1928). In 1933, ethylene oxide (EtO) began to be used in disinfection of museum textiles and documents. By that period its microbicidal properties also became recognized (Ballard and Baer, 1986).

The microbiologic inactivation properties of EtO are related to its powerful alkylation reaction of cellular constituents of organisms, like nucleic acids and functional proteins, causing their denaturation. This process affects the normal cellular metabolism, leading to non-viable microbes (Mendes et al., 2007). Due to its effective bactericidal, sporicidal, and virucidal activity EtO is also used as an alternative sterilization treatment for moisture and heat sensitive materials in different fields like in the disinfection of medical devices (Mendes et al., 2007).

Thanks to its high efficacy on the elimination of both insects and microbes this biocide became a very popular fumigant for libraries, archives and museums. This is also related with its great power of penetration and the possibility of use at room temperature and in the gaseous form, allowing the treatment of heat and moisture sensitive objects (Cotton and Roark, 1928; Ballard and Baer, 1986). EtO was generally commercialized as a mixture with other gases, like CO_2 (10% EtO, 90% CO_2) and dichlorofluoromethane (12% EtO, 88% dichlorofluoromethane). These dilutions reduce the explosiveness, flammability and toxicity of the EtO (Craig, 1986). The procedures for EtO fumigation varied almost according to each institution. The fumigations could be performed in vacuum chambers, chambers with normal pressure, or pressurized chambers with pressure varying between 20 mmHg and 400 mmHg (Flieder, 1965; Craig, 1986). The temperatures used in the treatment ranged essentially between 20°C and 37°C, and the exposure times between 3 and 24 hours (Flieder, 1965; Craig, 1986). For example, in France, the standard conditions for the treatment of documents with EtO in 2003, were T= 25°C; RH=50%; 20h; and 500g m^{-2} concentration (Rakotonirainy et al., 2003). Strassberg (1978) refers that the exposure time depended on the gas mixture proportion and the nature of disinfestation, e.g., the elimination of mould requiring twice the exposure time as for elimination of insects.

Before removing the treated materials from the fumigation chamber, air washing cycles should be performed in order to remove as much of the residual EtO as possible, since this compound and also some of its derivatives, such as ethylene chlorohydrin (produced by reaction with chlorine ions) and ethylene glycol (formed by reaction with moisture in the materials) are highly toxic (Tateo and Bononi, 2006; Mendes et al., 2007). In fact, exposure to EtO represents a carcinogenic, mutagenic, genotoxic, reproductive, neurological and sensitization hazard for people (Ballard and Baer, 1986) and is classified as a category 1 carcinogen by the International Agency for Research on Cancer (IARC, 2008).

In each air cycle wash, the chamber is evacuated and air, nitrogen, or carbon dioxide is introduced. Accordingly to the Environmental Protection Agency of the United States (US-EPA, 2004), four to six air washing cycles should be carried out to meet the permissible exposure limit to EtO of 1ppm as an 8 hour time-weighted average (TWA) imposed by the Occupational

Safety and Health Administration (OSHA, 2005). In a study comparing the relative capacity of different library materials to offgas residual ethylene, it is demonstrated that some of the tested materials needed more than 6 air washing cycles to reach 1ppm due to their lower desorption rates (Hengemihle et al., 1995). In the air over wood pulp offset paper and over newsprint paper, even after 8 and 13 air washing cycles, respectively, there was still an EtO concentration of circa 2 ppm. Over photographic film, after 25 air washing cycles, a concentration of 38 ppm EtO was still observed under the tested conditions (Hengemihle et al., 1995).

EtO fumigation treatments have shown to affect the physical and chemical properties of treated paper. Flieder (1965) indicates that EtO treatments can diminish the folding endurance (c. 40%), raise the copper number (4-19%), diminish the polymerization degree (2-8%) and slightly yellow the paper. Ponce-Jimenez (Ponce-Jimenez et al., 2002) reports a slight decrease in the pH of paper after sterilization with EtO.

Moreover, Craig (1986) and Valentin (1986) describe that after sterilization of archive materials with ethylene oxide, they become more susceptible to microbial attack. According to Craig (1986) this effect could be caused by the removal or disruption of the natural flora equilibrium, which would turn the materials more susceptible to colonization by any active microorganism with which it comes to contact. Florian (2002:37) points out that this higher susceptibility may be due to the deposition of small amounts of ethylene glycol on the materials which would raise hygroscopicity and act as an activator of conidia settled thereafter.

More recently, Michaelsen et al. (2013), compared the sterilizing effect of EtO with gamma irradiation and freezing. EtO proved to be the most efficient method, however, no fungal DNA or RNA could be recovered from treated materials. The destruction of these molecules invalidates future molecular analysis to identify and characterize old microbial contaminations, therefore misleading diagnostics may be obtained in cultural heritage materials (Michaelsen et al., 2013).

Sterflinger and Engel (2013) evaluated the persistence of EtO on fumigated books 20 years after treatment and no EtO degassing from the materials was detected.

EtO is listed in Annex III to the Rotterdam Convention, which includes pesticides and industrial chemicals that have been banned or severely restricted for health or environmental reasons, and is also included in Annex I to Regulation (EC) No 689/2008 of the European Parliament and of the Council concerning the export and import of dangerous chemicals (EC, 2008). Although already banned in several countries, the use of EtO is still permitted in some, with restrictions, namely on the occupational exposure levels. For instance, the PEL value for the European Union countries varies between 0.56 and 5 ppm and in the USA this value is 1 ppm (EU-OSHA, 2009).

2.2.2.2. Formaldehyde

Formaldehyde (CH_2O) was first synthesized in 1859 but it was only in 1867 that it was conclusively identified. This compound has been produced commercially since 1889 (IARC, 2006). Besides being used for synthesis of several more complex organic compounds and materials, formaldehyde is also used as a microbicide. It is an electrophilically active microbicide with the ability to react with several different amino acids present in the microbial cell, including purine and pyrimidine groups of both DNA and RNA, an unique characteristic of this compound (Paulus, 2004:164). It is highly reactive and also considered sporicidal, by attacking the spore both at the surface and internally (Paulus, 2004:20; Fraise et al., 2013:145)

Formaldehyde is a gas at room temperature, and was frequently applied as a gaseous fumigant, and also used in solution as a preservative in the preparation of glues, like starch paste (Seibert, 1992:9).

As a fumigant, formaldehyde could be applied in a hermetically closed oven, at a temperature of 30°C, in a proportion of 250 g m^{-3}, with a similar quantity of water also being vaporized in order to prevent dehydration and cracking of sensible materials, like parchment or leather. The objects would remain in the oven for 24 to 48 hours, depending on the extension of fungal attack (Heim et al., 1968). Gallo (1963) suggested a 12 hours fumigation treatment with relative humidity above 60% and temperature above 18°C. Also, the treated materials should be afterwards exposed to air for several hours or days, since formaldehyde would polymerize and settle as a superficial film on materials and so its elimination was rather difficult (Gallo, 1963).

According to Valentín and Garcia (1999), this compound, when applied by fumigation, has a low power of penetration and a limited fungicide effect. Rather than to prevent dehydration, these authors indicate that the application of this treatment at high RH levels is made in order to keep formaldehyde from undergoing polymerization and precipitation on treated materials, which would result in the formation of a white deposit.

Formaldehyde has been reported to cause adverse effects on archival and library materials, like: cross-linking of cellulose, loss of flexibility on paper and protein containing materials, as parchment, leathers and silk, and also enhancing the corrosion of iron gall ink (Gallo, 1963; Valentín and Garcia, 1999).

Regarding the toxicity of formaldehyde to humans, the International Agency for Research on Cancer (IARC), classifies formaldehyde as carcinogenic (Group 1), besides causing several other health effects like irritation of the eyes, nose and throat, and contact dermatitis (IARC, 2006). The occupational exposure limits for this compound vary with country and can be found in the literature (IARC, 2006). According to the European Council regulations, formaldehyde should no longer be placed in the European Union market as a preservative for fibre, leather, rubber and polymerized materials since 01/07/2012 (EC, 2012).

2.2.3. Azole antifungals

Azole antifungals are membrane-active microbicides that can be classified as imidazoles or triazoles depending on whether they contain two or three nitrogen atoms, respectively, in the azole ring (Pérez-Rivera et al., 2009). The first azole with antifungal activity was reported in 1944, but it was not until 1958 that it begun to be marketed (Pérez-Rivera et al., 2009). In the last decades, intensive research has led to the establishment of azole compounds as antimycotics for human and veterinary uses and also as fungicides for agrochemical purposes (Paulus, 2004:26).

These compounds act by inhibiting the ergosterol biosynthetic pathway, which leads to changes of sterol composition in the plasma membranes of fungi (Gollapudy et al., 2004). This results in alterations in the membrane fluidity or in membrane disruption, causing a delay, or ultimately an arrest of fungal growth (Lamb et al., 2000; Paulus, 2004:26).

Fabbri et al. (1997) tested several azole antifungals compounds, chitin synthase inhibitors and antimicrobials for the control of fungal growth in different kinds of paper. The obtained results show significant variations on the efficacy of these compounds depending on the fungal strain

and on the kind of paper used. Overall, the best inhibiting effect on fungal growth was obtained with two of the azole antifungals - miconazole and econazole at concentrations of 1 mol/m^3. In this study the secondary effects on paper were not evaluated.

Rakotonirainy et al. (1999) studied the aerial disinfection of emptied storerooms in libraries and archives after a fungal contamination using different aerosolized compounds: three azole antifungals – econazole, thiabendazole, and imazalil (enilconazole) and also orthophenylphenol. The used aerosol method (thermal fogging) revealed to have a very good penetration and homogeneity power, having also the advantage of not causing any change in relative humidity and temperature values in the room. Regarding the antifungal compounds, none of them exhibited a fungicidal action, only a fungistatic one. Even tough, thiabendazole revealed the best aerial disinfection and surface decontamination properties. Also, this compound does not cause the deposition of a fatty film on surfaces unlike the other tested compounds but a powdery film that can be removed afterwards. Regarding the effects of thiabendazole on paper, slight changes in the brightness values have occurred, although the level of oxidation and fibre strength were practically unaltered after artificial ageing (Rakotonirainy et al., 1999). Nevertheless, the authors recommend removing all documents from the room during the treatment with thiabendazole since it has not been yet tested on parchment, leather or other materials.

Azole antifungals have showed in numerous studies to possess an embryotoxic potential, including teratogenic effects in laboratory animals and therefore should be avoided for use by pregnant women (Giavini and Menegola, 2010).

The legal restrictions of use of these antifungals depend on the specific azole compound and vary with country (US-EPA, 2002, 2005; EC, 2012).

2.2.4. Essential oils

In the last few decades, the increasing resistance of microbes to the available antifungal compounds has led to the search of alternative natural products derived from plants commonly used for their empirical antifungal properties in traditional medicine. Natural products can be used either as pure compounds or as standardized plant extracts, like essential oils (Abad et al., 2007).

Essential oils are mainly composed of terpenoid compounds (Karpouhtsis et al., 1998). Aromatic terpenes are cyclic hydrocarbons, which due to their hydrophobic character accumulate in the lipid bilayer of the microbial cell membrane according to a partition coefficient that is specific for each compound. This accumulation leads to alteration of the membrane structure and function, namely exerting negative effects on the proton motive force (Sikkema et al., 1994).

Although thymol is also a component of essential oils, it will be more thoroughly discussed below in section 3.2.5 Phenol Derivatives.

Rakotonirainy et al. (2005; 2007) have explored the use of alternative essential oils as antifungals for heritage objects. The antifungal activity of essential oils of armoise, clove, boldo, eucalyptus, ravensare, lavender, tea tree, thuya, wormseed and their main components linalool, linalyl acetate, eugenol, α+β thujone and cineole were tested in the vapour phase. The vapours of linalool showed the highest antifungal efficacy against the fungal strains tested, although its action was fungistatic rather than fungicidal at the tested concentration (415 ppm) (Rakotonirainy and Lavedrine, 2005). The assessment of the potential damaging effects of

linalool vapours on two types of paper showed that this compound did not affect the papers' brightness and degree of polymerization, but it did reduce the pH values (Rakotonirainy and Lavedrine, 2005).

Linalool applied by vapours and spraying was also tested on silver-gelatine photographs and bookbinding leathers (Rakotonirainy, Juchauld, et al., 2007). This compound caused oxidation of the photographs, which was revealed by changes in their optical density. On the leather test pieces, linalool caused a decrease on the temperature and enthalpy of denaturation, which was more pronounced after artificial ageing tests. These results may be explained by the fact that linalool undergoes autoxidation on air exposure, forming mainly a hydroperoxide (7-hydroperoxy-3,7-dimethyl-octa-1,5-diene-3-ol) (Sköld et al., 2002). This reaction is enhanced by temperature, and the hydroperoxides formed can oxidize the silver on the photographs (Rakotonirainy, Juchauld, et al., 2007). The possible reactions between this oxidation product and leather need further research.

Tests on linalool allergenic potential revealed that oxidized linalool caused sensitization to allergic contact dermatitis, while the pure compound gave no reactions (Sköld et al., 2002). Since linalool undergoes autoxidation just by being exposed to air, it can pose an allergenic risk to sensitive people.

Florian (1998) in a review regarding the use of natural products for insect and fungi control, states that some medicinal plants, like coriander, although may inhibit some fungal species, can also enhance the growth of other ones. Also, other varieties of plants that inhibited the growth of selected fungal species are at the same time classified as insect attractants (Florian, 1998).

2.2.5. Phenol derivatives

There are several phenol derivatives used for their disinfectant and preservative properties. These compounds are membrane-active antifungals, causing mainly damages in the plasma membrane of fungi (Russell, 2003).

Like acids, phenol derivatives dissociate hydrogen ions in solution and are able to form salts. Their antimicrobial effect only occurs in the undissociated state. When the compound is dissociated, its negative charged anions will be repelled by the negatively charged surface of the microbial cell, and therefore cannot exert antimicrobial effects. This means that in order to keep the antimicrobial properties of these compounds to a maximum, their pKa (the pH at which 50% of the phenol is in the dissociated state) has to be taken into account (Paulus, 2004:274). At a pH below its pKa, the compound exists mainly as a neutral species, while above that value it will predominantly have a negative charge. In this way, for example, if the pKa of a phenol is 5.0, it will be more effective at pH values below 5.0.

2.2.5.1. Dichlorophen

Dichlorophen (4-chloro-2-[(5-chloro-2-hydroxyphenyl)methyl]phenol), also known by the trade names Preventol GD or Panacide, is a chlorinated bisphenol that was first prepared in 1929 (Block, 2001:261). Besides being used as a fungicide for paper, cardboard, textiles, adhesives, and as a slimicide in paper manufacture, it is also used for treating skin fungal infections, as a germicide in soaps and cosmetics, and as an anthelmintic (Block, 2001:261; Cox et al., 2004). This compound is a weak acid with pKa values equal to 7.66 and 11.60 for the two hydroxyl groups, and can affect a multitude of microbial intracellular targets (McBain and Gilbert, 2001; Escalada et al., 2011). Its inhibitory processes are concentration-dependant and vary from

action as potassium proton antiporter and respiration uncoupler, to competitive inhibition of NADH-binding by malate dehydrogenase (McBain and Gilbert, 2001). Accordingly to Paulus (2004:570), dichlorophen is strongly effective against yeast and filamentous fungi. Being poorly soluble in water, dichlorophen is usually applied in organic solvents or in the solid state.

Triolo et al. (1968) studied the stability of paper samples treated with dichlorophen in ethanol (15 mol m^{-3}). With artificial ageing dichlorophen caused a decrease in the in polymerization degree and whiteness of paper samples. On the other hand, the pH values remained practically the same.

Kowalic (1980) refers as well that dichlorophen does not change the paper's pH, although it may cause harmful effects when applied on documents written with ink (those effects are not specified), and represents a potential danger to paper due to chlorine release. Thus, dichlorophen was suggested only for unwritten paper, or for impregnating blotting paper to interleave damp books (Kowalik, 1980).

Dichlorophen can cause mild skin irritation and severe eye irritation (Paulus, 2004:569). According to Yamarik et al. (2004) no reproductive or developmental toxicity data were available for dichlorophen and the overall available data were insufficient to support safety of this compound. In the European union, dichlorophen should no longer be placed in the market as a biocidal product (since 2009), or as a preservative for film, fibre, leather, and other materials (since 2011) (EC, 2012).

2.2.5.2. Ortho-phenylphenol

Ortho-phenylphenol ($C_{12}H_{10}O$), also known by the trade names Preventol O, Topane or Dowicide, is a membrane-active microbicide with a broad spectrum of activity, covering bacteria, yeasts and fungi (Valentín and Garcia, 1999; Paulus, 2004:556). This compound exhibits antimicrobial action even at low concentrations (between 10 and 50 ppm, pH 5–8) and performs best in an acidic, neutral or weakly basic environment (pKa=11.6) (Paulus, 2004:556).

For being less toxic than thymol, ortho-phenylphenol (OPP) started to be recommended in substitution of that compound in fumigation chambers (Haines and Kohler, 1986). It was also applied directly in infested documents, by interleaving or wrapping with paper soaked in a 10% aqueous solution. Due to the low volatility of OPP, its fumigation activity would last for six months or more (Strassberg, 1978). OPP was also used as a preservative in restoration products, synthetic adhesives and animal glues (Valentín and Garcia, 1999).

Considering OPP's antifungal activity, Haines and Kohler (1986) report that this compound applied by fumigation is unable to render unviable all the tested fungal colonies, and describe it as a non-effective method for treating mould infected books and papers. According to Rakotonirainy et al. (1999), OPP applied by thermal fogging, had a fungistatic but not fungicidal effect on spores, germ tubes and mycelium of all tested strains. Besides, it can leave a very irritating and suffocating odour in the air persisting for at least 15 days (Rakotonirainy et al., 1999).

OPP has been reported to cause alterations on paper materials, like changes in colour, depolymerization of adhesives and accelerated ageing (Triolo et al., 1968; Valentín and Garcia, 1999).

OPP's sodium salt (SOPP), with the trade names Preventol ON or Dowicide A, was also seldom used as an antifungal compound for paper items, but its marked colouration could produce severe stains on paper (Valentín and Garcia, 1999).

According to the International Agency for Research on Cancer (IARC, 1999b), OPP is not classifiable as to its carcinogenicity to humans (Group 3), although SOPP is considered as possibly carcinogenic to humans (Group 2B).

2.2.5.3. Pentachlorophenol

Pentachlorophenol (C_6HCl_5O), with the trade names Dowicide 7, G, EC-7 or Preventol P, is a chlorinated phenol that was first synthesized in 1841 but was not manufactured in large quantities until 1936 (Miller and Aboul-Ela, 1969). It is a highly lipophilic weak acid with a pKa of c. 4.75, and is considered a broad-spectrum microbicide (Fernández Freire et al., 2005).

According to Valentin and Garcia (1999), this compound was widely used as a fungicide for books, textiles and wood, although it attacks metals and pigments, and degrades paper and wood. Strassberg (1978) also states that by being an acid, pentachlorophenol may adversely affect archival materials.

Pentachlorophenol is considered carcinogenic by the International Agency for Research on Cancer (IARC, 1999c) and is listed as a priority pollutant due to its slow and incomplete biodegradation (Fernández Freire et al., 2005). For these reasons, the use of pentachlorophenol is banned as plant protection product and is severely restricted as a biocide in the European Union and in several other countries (Valentín and Garcia, 1999; Paulus, 2004; Fernández Freire et al., 2005; EC, 2008; US-EPA, 2008b).

2.2.5.4. Thymol

Thymol (2-Isopropyl-5-methylphenol) is a natural monoterpene phenol. Already used in the ancient Egypt for the preservation of mummies (Napoli et al., 2010), this compound is present in the essential oils of aromatic plants like thyme, oregano or savory (Numpaque et al., 2011). As a membrane-active microbicide, it alters the permeability of the microbial cell and causes alterations in the hyphal morphology, resulting in reduced hyphal diameters and lysis of hyphal wall, permitting the loss of macromolecules (Klarić et al., 2007; Numpaque et al., 2011).

Thymol has poor solubility in water and a 10.6 pKa (Coimbra et al., 2011). It is used mainly in its crystalline form as a sublimable solid, in fumigation chambers (Figure 2.1). Other methods of treatment include placing thymol impregnated paper interleaving books or inside picture frames, providing a continuous contact of the objects with the antifungal vapours, thus prolonging its effect (Strassberg, 1978; Daniels and Boyd, 1986). It can also be applied directly in paper in a 10% ethanolic solution (Strassberg, 1978).

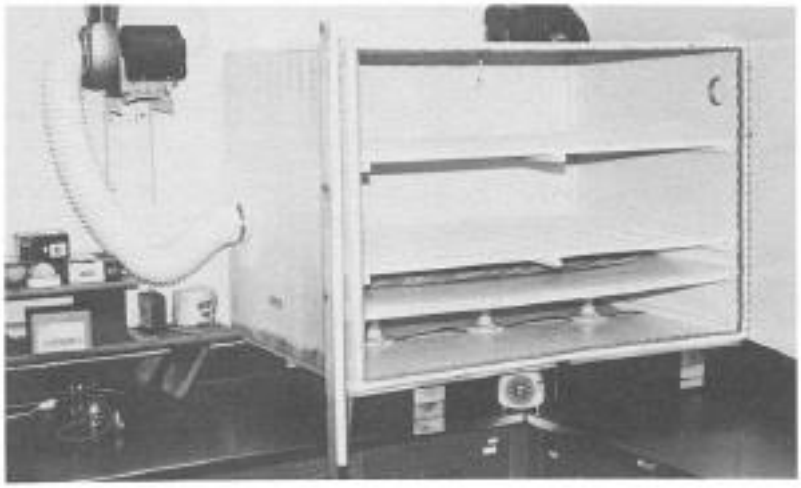


Figure 2.1: Thymol cabinet, from Gustafson et al. (1990).

In 1983 thymol was nearly the exclusive fumigant used in archives in the United Kingdom (Craig, 1986). The conditions by which thymol was applied, varied almost according to each institution. The concentration used ranged from 1 to 90 g m^{-3}, and the time of exposure from 24 hours to 3 weeks (Craig, 1986).

The efficacy of thymol as an antifungal agent for paper has been tested by several authors. According to Craig (1986), filter paper samples inoculated with four cellulolytic fungal species, and treated with thymol by fumigation (20 g m^{-3}), with a constant temperature (73°C) for 72 hours, showed no fungal growth, in comparison with control untreated samples that exhibited a strong fungal development. Nevertheless, following the treatment, after other 3 days at room temperature, all the treated samples exhibited fungal regrowth, which indicates that thymol did not kill the spores (Craig, 1986). Flieder (1965), reported that for inoculated filter paper samples treated in a room with 33 g m^{-3} of evaporated thymol for 14 days, with a temperature of 26.7°C, the antifungal treatment was totally ineffective.

Haines and Kohler (1986) and also Gustafson et al. (1990) stated that thymol was not totally fungicidal for the tested fungal species, since there was not a total elimination of the fungal spores.

According to Flieder (1965), the same thymol treatment described above, caused changes in the physical-chemical characteristics of filter paper samples. Namely, the folding endurance decreased considerably (27-63%), and the mechanical resistance slightly dropped (c. 10%), although the chemical properties (copper number, polymerization degree and pH) remained alike and there was practically no yellowing (Flieder, 1965).

Daniels and Boyd (1986) reported the yellowing of prints sealed in frames containing thymol vapours. His research revealed that the yellow colour was probably due to the formation of a polymeric form of thymol, through a photochemical reaction.

The experiments conducted by Isbell (1997) where different paper samples with different mediums were exposed to thymol vapours (three-tenths of an ounce of thymol, to four cubic feet) during one week, revealed an apparent degradation of paper, binders and iron gall ink. Even tough, the mechanism of degradation was not clarified.

Strassberg (1978) states that thymol may attack glues in books, dissolve inks, leave residues on parchment and on paper, and by direct application or interleaving it can cause discolouration on documents.

Concerning its toxicity to humans, thymol has proven to have a genotoxic effect (Karpouhtsis et al., 1998; Buyukleyla and Rencuzogullari, 2009), meaning that it is capable of damaging DNA, which may lead to cell mutations and possibly cancer.

US-EPA (2010) reports that additional mutagenicity data is required in order to review the registration of thymol, besides adding that this compound is extremely irritating to eyes and skin.

2.2.6. Photocatalysts

Photocatalysis is the type of reaction that takes place on the surface of a semiconductor in the presence of a very specific range of radiation (Pyrgiotakis and Sigmund, 2008). This chemical process has been used to decompose several pollutants and biological contaminations (Pyrgiotakis and Sigmund, 2008). Titanium dioxide has been the most used and studied compound for these purposes, namely for heritage materials protection and therefore it is further described below.

2.2.6.1. Titanium Dioxide

Titanium dioxide (TiO_2) is a natural occurring compound that exists more commonly as tetragonal crystallites of anatase or rutile, which are represented as octahedral structures of (TiO_2^{6-}) (Gavriliu et al., 2009). Crude TiO_2 or titanium white has been used as a pigment from the beginning of the 20th century and is still applied in contemporary art nowadays. Soon it gained other functions, like providing whiteness and opacity in food, medicines or toothpastes. It is an excellent semiconductor photocatalyst, which effectively transforms light energy into chemical energy (Markowska-Szczupak et al., 2011). TiO_2 has been used as a self-disinfecting compound on surface coatings and in purification of water and air (Huang et al., 2000; Chawengkijwanich and Hayata, 2008; Afsharpour et al., 2011). The knowledge about the microbiocidal effect of TiO_2 is rather recent, since the first published studies on this subject date from 1985 (Matsunaga et al., 1988).

The microbiocidal activity of titanium dioxide is due to its photocatalytic properties. When illuminated with UV light of less than 385 nm, TiO_2 generates strong oxidizing power, producing reactive species like hydroxyl radicals and superoxide ions, which can decompose and mineralize organic compounds, causing fatal damage to microorganisms (Blake et al., 1999; Huang et al., 2000). Nevertheless, the sensitivity of fungi to this kind of treatment is significantly weaker than that of bacteria (Gavriliu et al., 2009; Markowska-Szczupak et al., 2011). According to Markowska-Szczupak et al. (2011), this occurs due to the chemical composition, structure and thickness of the fungal cell walls, when compared to bacteria, namely the presence of chitin rather than peptidoglycan on cell walls. The studies performed so far show that TiO_2 irradiated with UV can only act as a fungistatic and do not affect conidia (Gavriliu et al., 2009; Markowska-Szczupak et al., 2011).

Recently, TiO_2 has been studied as a layered nanocomposite for protection of paper documents and works of art from UV radiation, pollutant gases, mould and bacteria (Afsharpour et al., 2011). In that study, TiO_2 nanoparticles dispersed in ethanol (0.2% w/v) were applied by spraying Whatman paper samples coated with a 0.7% Klucel G (hydroxypropylcellulose)

ethanolic solution. The antifungal properties of TiO_2 were tested on *Aspergillus niger* and *Penicillium sp*. Although the experimental procedure and results of the antifungal assay are not described in this article, the authors present their conclusions, stating that the TiO_2 coating may prevent the formation of biofilms, rather than killing the microorganisms (Afsharpour et al., 2011). A biofilm is a community of microbial cells that becomes attached to a surface and/or to other cells within an extracellular polymeric matrix. One of the main features of cells growing in biofilms is their increased resistance to antimicrobial agents, besides their significant increased tolerance to chemical, biological or physical stresses (Harding et al., 2009).

In the study by Afsharpour (2011), the effects of the titanium oxide nanocomposite on the physical and chemical properties of paper samples were evaluated before and after artificial ageing. The results showed that the TiO_2 nanoparticles alone caused a minor chemical degradation of cellulose and accelerated the fading of organic colourants. This effect was not observed with the nanocomposite with the Klucel G layer. As follows, hydroxypropylcellulose may be protecting the paper from deterioration catalysed by TiO_2 (Afsharpour et al., 2011). According to Chawengkijwanich and Hayata (2008), a TiO_2 surface when illuminated, forms hydroxyl radicals and reactive oxygen species. These unstable chemical species can react with cellulose, which can justify the results obtained. On the other hand, both the nanoparticles alone and on top of the Klucel G layer protected the paper samples from losing tensile strength (Afsharpour et al., 2011).

Concerning the health hazards, the International Agency for Research on Cancer, considers titanium dioxide as possibly carcinogenic to humans - Group 2B (IARC, 2010). Research on the application of ultrafine titanium dioxide particles on healthy skin revealed that the compound only penetrates the outermost layers of skin. Nevertheless, there were no studies available about the penetration of TiO_2 on compromised skin (IARC, 2010).

2.2.7. Quaternary ammonium Compounds

Quaternary ammonium compounds (Quats) were discovered by August Hofmann in 1851 (Paulus, 2004:306). These membrane-active microbicides are cationic surfactants that are attracted by the negatively charged surface of the microbial cell, forming an electrostatic bond with the negatively charged sites on the cell wall (Paulus, 2004:306; Fraise et al., 2013:22). The permeability of the cell wall is altered causing leakage of chemical species, and enabling the active ingredients of the microbicide to penetrate into the cell membrane, ultimately leading to cellular death (Paulus, 2004:130; Fraise et al., 2013:22).

Quats are fungistatic rather than fungicidal compounds and are more effective at alkaline and neutral pH (Fraise et al., 2013:22).

Some Quats are considered to be highly toxic with a detrimental effect on the immune system (Nittérus, 2000b).

2.2.7.1. Dimethyl-lauryl-benzyl ammonium bromide

Dimethyl-lauryl-benzyl ammonium bromide is a Quat used in medicine as a low-level disinfectant with no sporicidal activity (Bello-Gonzalez et al., 2008).

Strzelczyk and Rozanski (1986) have tested this compound, with the trade name Sterinol, for the disinfection of paper affected by mould. They have reported that a 15 minutes warm bath containing 0.75 – 1.0% of a commercial 10% aqueous solution of dimethyl-lauryl-benzyl

ammonium bromide (Sterinol) was effective on the tested objects. Nevertheless, they recommend that after the disinfection treatment, the objects should be thoroughly washed with tap water and distilled water to remove the remaining Quat, since this compound has the ability to accumulate in the paper. If Dimethyl-lauryl-benzyl ammonium bromide remains in the paper, it can negatively affect the physical properties, pH, whiteness, and alpha cellulose content of the treated material (Strzelczyk and Rozanski, 1986).

2.2.7.2. Lauryl-dimethyl-Carbethoxymethyl ammonium bromide

Lauryl-dimethyl-carbethoxymethyl ammonium bromide is a Quat used in the past in archives and libraries for elimination of mould. A 5% concentration solution in denatured alcohol at 70° could be applied by nebulisation using an air compressor and a spray gun or an atomizer in a proportion of 5ml of solution per cubic metre of air (Heim et al., 1968; Strassberg, 1978).

Triolo et al. (1968) studied the effects of lauryl-dimethyl-carbethoxymethyl ammonium bromide on paper. This compound, commercialized by the trade name Cequartyl BE in a 50% aqueous solution, was diluted to 3%, 1.5% and 0.5% aqueous solutions in which the paper samples were immersed without subsequent washing. Artificial ageing tests revealed a minor darkening and acidification of paper, with depolymerization of cellulose. A strong acidification of the treatment solutions occurred with time. Not even neutralization with NaOH could stabilize the pH, which continued to slowly decrease, probably due to the scission of a covalent bonding on the molecule (Triolo et al., 1968). The negative effects of lauryl-dimethyl-carbethoxymethyl ammonium bromide on cellulose were also pointed out by Strassberg (1978) and Kowalik (1980).

Due to its surfactant properties, Quats can also affect the adhesion of inks used on paper (Triolo et al., 1968; Strassberg, 1978).

Heim et al. (1968) adviced for the use of masks during the fumigation with lauryl-dimethyl-carbethoxymethyl ammonium bromide, since it irritates the mucous membrane.

In the European Union, products containing quaternary ammonium compounds as active substances (benzylalkyldimethyl (alkyl from C8-C22, saturated and unsaturated, tallow alkyl, coco alkyl, and soya alkyl) chlorides, bromides, or hydroxides)/ BKC, are no longer to be placed in market for film, fibre, leather, rubber, and polymerized materials preservatives since 2011 (EC, 2012).

2.2.8. Salts and Esters of acids

As described for the phenol derivatives, salts and esters of acids only exert their antimicrobial effects in the undissociated state, and so, the pKa values must be taken into account when using these compounds.

2.2.8.1. Calcium Propionate

Calcium propionate [$Ca(C_2H_5COO)_2$], a salt of propionic acid, is an antimicrobial substance that has been commonly used as a food preservative since the late 1930's (Paulus, 2004:290). Its ability to inhibit moulds but not yeasts to the same extent, have made it a common option for bread preservation since it does not impede fermentation. This salt is more often used than the acid itself due to its higher solubility in water (Suhr and Nielsen, 2004).

The pH of the substrate is of major importance in the use of these kind of preservatives (weak acids), since their antimicrobial effectiveness is much stronger in the undissociated form than in

the dissociated one. The pKa of propionic acid is 4.87 and the maximum pH for propionate activity is 5.0-5.5 (Suhr and Nielsen, 2004).

The use of calcium propionate in conservation of heritage objects was first studied, to the best of our knowledge, by Dersarkissian and Goodberry (1980), who tested its antifungal efficacy on leather and paper. Latter, Zappalà (1990) tested also the calcium propionate's ability to deacidify paper, and considered it a good cellulose deacidifier and stabilizer.

The study performed by Dersarkissian and Goodberry (1980), consisted on testing calcium propionate for its antifungal properties on paper and leather against *Aspergillus niger*. Since the authors could not achieve any fungal growth on the paper samples, the antifungal properties of calcium propionate were only tested on leather samples. A 5% calcium propionate aqueous solution was applied using a cotton swab on samples with fungal development. Fungal growth was totally stopped only after a fourth antifungal application, and no fungus was visible 10 days after the treatment. However under ideal growing conditions, the fungus slowly restarted its development. The authors suggest that a modest increase in concentration should greatly enhance the effectiveness of this compound, and highlight its low cost in comparison with the other tested compounds (Dersarkissian and Goodberry, 1980).

Zotti et al. (2007) also evaluated the antifungal properties of calcium propionate on paper, both in aqueous and in ethanolic solutions, and compared it with a commercial solution of *p*-hydrobenzoates (parabens). Their results show that a saturated ethanolic solution of calcium propionate (0.35% w/v), albeit having a lower concentration that the tested aqueous solutions (3% and 5% w/v) exhibits a much higher efficacy against the fungal species tested. The authors point out that this superior effectiveness is due to the ability of ethanol to act as a vehicle for the calcium propionate molecule through the fungal cell wall (Zotti et al., 2007).

Neves (2006) studied the antifungal and deacidification potential of calcium propionate and also its effects on Whatman #1 filter paper. Calcium propionate was used at a 5% concentration dispersed in an 85% (w/w) ethanol/water solution. The fungal species tested were *Cladosporium sp*. and *Penicillium corylophilum*. Two days after inoculation all tested paper samples showed fungal growth, though on the untreated ones the growth was wider. However, eight days after inoculation, paper samples inoculated with *Penicillium corylophilum* and treated with calcium propionate revealed a higher fungal development than the control samples (Neves, 2006).

In the above mentioned studies, the efficacy of calcium propionate was never related to the pH at which it was applied. Since propionic acid has a pKa of 4.87, its efficacy will be much higher in the acidic range. In the study reported by Neves (2006), e.g., it can be assumed that the compound was applied in an alkaline solution, since it raised the pH of filter paper which had already a nearly neutral pH. This factor could justify, at least in part, the poor antimicrobial results achieved. Although, adding an acidic compound to paper would not be reasonable in a paper conservation practice.

Regarding the effects of the calcium propionate solution on Whatman #1 filter paper, Neves (2006) reported a minor increase on the lightness and yellowness after treatment ($\Delta L^* = 0.43$ and $\Delta b^* = 0.37$, respectively), an increase on the pH values, a maintenance of the tensile strength, and an increase on the polymerization degree (probably due to the ability of the

calcium ions to form gels with polysaccharides improving the viscosity of solutions) (Neves, 2006).

Concerning the health hazards of calcium propionate, Paulus (2004:291) reports that even when administrated at large doses in a diet, propionic acid is excreted in the urine and there is no risk of accumulation in the human body. The United States Environmental Protection Office states that the tests for the teratogenicity (ability to cause birth defects) and reproductive toxicity for calcium propionate are negative (US-EPA, 1991). Nevertheless, the research performed by Şifa (2008) indicates chromotoxic effects (toxic action on haemoglobin) caused by calcium propionate, and advises caution on its use as a food preservative.

2.2.8.2. Esters of *p*-hydroxybenzoic acid

The esters of *p*-hydroxybenzoic acid, commonly known as parabens, are among the most common antimicrobial agents in pharmacy and cosmetics' industries, due to their low toxicity, pH range of activity, good stability and minimum secondary effects (Nguyen et al., 2005; Zhang et al., 2005).

These compounds were firstly synthesized in the early 1920's in order to replace salicylic and benzoic acids which were only effective at low pH values (Paulus, 2004:294).

Parabens are mainly fungistatic and bacteriostatic, being more effective against yeast and moulds than bacteria (Russell, 2003). The most widely used esters of *p*-hydroxybenzoic acid are methyl, ethyl and propyl esters (Russell, 2003). pKa values of methyl-*p*-hydroxybenzoate (methylparaben) and propyl-*p*-hydroxybenzoate (propylparaben) are 8.2 and 8.4, respectively, and so they are still active in slight alkaline media (Soni et al., 2005).

The use of parabens as antifungal agents on the conservation of heritage objects was published for the first time in 1990, on a study about alternatives to thymol on fumigation chambers (Gustafson et al., 1990). On that research, butyl-paraben and propylparaben were tested separately, applied in the gaseous form, and compared with other antifungal compounds. The results showed that parabens, as well as the majority of chemicals tested, did not exhibit a positive antifungal effect, and so did not provide an alternative for use in thymol cabinets.

The direct application of parabens in solution on paper materials started to be studied by Neves (2006) in her graduation thesis, and was published later in 2009 (Neves et al., 2009). In this study, several concentrations and proportions of methylparaben and propylparaben were tested against two fungal species - *Cladosporium sp*. and *Penicillium corylophilum*. The mixture of 0.5% methylparaben and 1% propylparaben in an 85% ethanolic solution totally inhibited those fungal species. The addition of 5% calcium propionate improved the characteristics of this mixture as calcium propionate, besides being a fungistatic compound, it has also deacidification properties. The effects of this parabens/calcium propionate mixture on paper were tested on Whatman #1 filter paper. The obtained results revealed a minor increase on yellowing and a slight decrease on the tensile strength after treatment application, while considerably raising the pH and moderately increasing the percentage of deformation (Neves et al., 2009). Zotti et al. (2007) tested the antifungal properties of parabens on fungi isolated from foxing spots (name commonly given to brownish-red spots on paper), and compared them with aqueous and ethanolic solutions of calcium propionate. The tested parabens were methylparaben and propylparaben as part of a commercial spray formulation, whose concentrations and brand name are not revealed. The tested fungi strains were *Penicillium spinulosum*, *Trichoderma*

oseudikoningii, *Geomyces pannorum* and *Aureobasidium pullulans*. The commercial parabens spray showed an intermediate fungistatic activity, between the water based and the alcohol based calcium propionate solutions. Since there is no indication of the quantity of parabens applied on the paper samples, these results can hardly be compared with the ones obtained in other studies.

Parabens are absorbed and metabolized through the the skin or gastrointestinnal tract, hidrolized into *p*-hydroxybenzoic acid (the nonoestrogenic metabolite) and rapidly excreted into the urine (Soni et al., 2005; SCCS, 2013). In the past few years, the detection of parabens in some breast tumour tissue samples has engaged a controversy on the possible oestrogenic hazards of these compounds. Some studies conducted thereafter claim that the previous ones fail to consider the metabolism and elimination rates of parabens, which are dose, route, and species dependent (Soni et al., 2005). The Scientific Committee on Consumer Products e European Commission, reports that "viewing the current knowledge, there is no evidence of demonstrable risk for the development of breast cancer caused by the use of underarm cosmetics" (SCCP, 2005). According to the same institution, several studies have proven parabens to be practically non-toxic, not carcinogenic, not genotoxic and not teratogenic (SCCP, 2005).

2.3. Physical Methods

Physical antifungal methods are the ones that do not require the application of chemical compounds. They leave no residues *pers se*, which can be a positive characteristic, but for the same reason they only exert a momentary action. The physical antifungal methods are described below and summarized in Table 2.2.

2.3.1. Dehydration

Water is one of the main limiting factors to fungal growth, and dehydration consists in lowering or even ceasing the availability of water to microorganisms. Already in early civilizations, drying of meat and fish was used as a food preservation method (Hugo, 1995).

In heritage conservation, partial dehydration (complete dehydration would cause paper materials to become brittle), or drying, is considered by many as the best way to stop fungal growth on wet materials. It should be performed quickly to prevent microbial development, but on the other hand, slow drying is recommended to prevent dimensional changes on heritage objects.

Dehydration can be performed using dehumidifying equipment (fans can be used to increase air circulation) and/or wrapping or interleaving wet objects with dry absorbing materials.

Florian (Florian, 1997:149, 2002:88) recommends drying objects under constraint to prevent distortions and dimensional changes, for example, drying water-soaked books without removal from the shelves. However, large volumes of wet absorbing materials, like shelves full of books will respond slowly to a dry environment due to its self-buffering effect (Garside and Knight, 2011), and will remain wet for several days, which can allow the development of fungi.

Air drying is considered as the least expensive of all recovery methods in terms of immediate cost (CCAHA, 2000). Nonetheless, it is labour-intensive and limited by available air-drying space and availability of trained professionals to monitor the drying materials (CCAHA, 2000).

Table 2.2: Summary of the antifungal physical methods reviewed, in alphabetical order.

Antifungal method	Advantages	Disadvantages	Effects on paper	First report of use	References
Dehydration	Does not leave toxic residues Economical	Fungistatic rather than fungicidal High volumes of wet paper respond slowly to a dry environment and meanwhile fungi development can occur	Physical deformations	Since early civilizations	(Hugo, 1995; Florian, 1997, 2002; CCAHA, 2000; Garside and Knight, 2011)
Freezing	Does not leave toxic residues Can prevent soluble compounds from bleeding and migrating	Dry conidia can resist freezing The concentration of solutes in the freezing process can increase deteriorating chemical reactions (ex. pH dependent and lipid oxidation) Fungicidal effect is time and temperature dependent	Ice crystals increase the porosity and thickness of paper materials, which can alter its absorbance and mechanical properties	1000 BC	(Flink and Hoyer, 1971; Takenaka et al., 1996; Florian, 1997, 2002; Adamo et al., 1998; Schwerdt, 1998; CCAHA, 2000; Gunde-Cimerman et al., 2003; Archer, 2004; Basset and Draïs, 2011)
Gamma Irradiation	Does not leave toxic residues. Can be used for mass treatment Can sterilize materials, depending on the radiation dosage	Can induce fungi to produce more coloured metabolic products Can turn cellulose more susceptible to further infestations	Diminishment of mechanical resistance, pH and polymerization degree, raise of copper number and yellowing	1896 (1960's on heritage materials)	(Ben-Arie and Barkai-Golan, 1969; Pavon Flores, 1976; Hanus, 1985; Butterfield, 1987; Hugo, 1995; Tomazello et al., 1995; Adamo et al., 1998, 2001, 2003; Florian, 2002; Magaudda, 2004; da Silva et al., 2006; Bank et al., 2008; Havermans, 2011; Mesquita, 2013)
High frequency current	Also dries the materials, which is an advantage in a case of wet documents. Does not leave toxic residues. Can be used for mass treatments	Mediocre antifungal activity Uses high temperature and physical pressure Soiled paper may ignite	Increasing of carbonyl groups on cellulose Diminishment of tear resistance.	1947	(Flieder, 1965; Strassberg, 1978)

Antifungal method	Advantages	Disadvantages	Effects on paper	First report of use	References
High temperature	Can lower the moisture content on materials	Can act as a conidia activator Temperatures above 100°C need to be reached in order to kill spores	Accelerates the rates of chemical deterioration		(Thevelein et al., 1979; Strang, 1992, 2001; Rajasekaran and Maheshwari, 1993; Brokerhof, 2002; Florian, 2002; Pinniger, 2003; Ackery et al., 2004)
Low-oxygen environments	Does not leave toxic residues Can prevent deteriorating chemical reactions that are oxygen dependent Can also kill insect pests	Only causes a decrease on fungal growth, does not stop it	Atmospheres high on CO_2 can change the acid/base balance on the materials	1990: on heritage materials	(Hocking, 1990; Valentin et al., 1990; Florian, 1997)
Refrigeration, 4°C	Does not leave toxic residues Slows down chemical deterioration rates	Only slows down but does not stop fungal growth Some fungal species can produce heavy coloured pigments, more conidia and polyols under this kind of stress Can activate conidia	Solubilisation and migration of water soluble compounds		(Florian, 1997, 2002)
Ultraviolet Radiation	Does not leave toxic residues Can act as a fungicidal, depending on the radiation dosage	Poor penetration power Has cumulative degrading effects on the treated materials	Causes oxidation of cellulose, leading to yellowing, bleaching and brittleness of paper	1903	(Gallo, 1963; Nyberg, 1987; Hugo, 1995; Florian, 2002; Bukovsky and Trnkova, 2003a, 2003b; Paulus, 2004; Belloni et al., 2006)

2.3.2. Gamma Irradiation

Gamma radiation (γ-radiation) is the electromagnetic radiation of highest energy and shortest wavelength. Among other functionalities, this kind of radiation is often used as a sterilizer in several fields like medicine, pharmacy or agriculture. It is usually obtained from Cobalt 60, a radioisotope that emits gamma rays continuously and therefore the irradiation cabinet must be correctly shielded from the outside to prevent health hazards. The antimicrobial effect of γ-radiation was shown by Mink already in 1896 (Hugo, 1995).

The microbiocidal activity of this highly penetrating ionization radiation is due to its ability to generate radicals capable of cleaving carbon-carbon bonds thus destroying cellular DNA and turning microbial cells unviable (Bank et al., 2008).

The disinfection of archival materials with γ-radiation was firstly studied in Russia in the early 1960s (Tomazello et al., 1995; Magaudda, 2004). Since then, several studies have been performed in order to determine the minimum radiation level required to eliminate fungal species identified in graphic documents, and also evaluate the effects γ-radiation has on paper materials in the short and long term.

On the reviewed literature, several minimum lethal radiation dosages are reported, which is not surprising since different fungal species were evaluated and the susceptibility to γ-radiation varies with species (Pavon Flores, 1976; Butterfield, 1987; Tomazello et al., 1995; Adamo et al., 2001, 2003; da Silva et al., 2006).

The minimum lethal radiation dose for all the fungal species tested by Pavon Flores (1976) was 18 kiloGray (kGy) at a rate of 5 kGy h^{-1}, although lower dosages were already lethal for many of those species. A remarkable increase in the pigmentation intensity on some fungal species after irradiation was observed, which could represent a problem when cultural heritage documents are treated by this method (Pavon Flores, 1976). The effects of γ-irradiation on paper samples were tested under normal and nitrogen atmospheres in order to clarify the role of oxygen in the deterioration reactions. According to Pavon Flores (1976), the mechanical resistance of paper did not suffer considerable modifications after the irradiation. However, after artificial ageing some changes were noticed, mainly in the folding endurance. Copper number and polymerization degree were severely affected both before and after artificial ageing. This chemical deterioration was mainly observed on papers with high cellulose content, by comparison with papers with high lignin content, like newspaper, revealing a protective effect of lignin. Moreover, the nitrogen atmosphere did not have any protective effect on the deterioration of the papers (Pavon Flores, 1976).

These results on paper deterioration can be justified by the effect γ-radiation has on organic materials. By the same mechanism it works on microorganisms - through the formation of radicals that deteriorate organic matter - it can also act on other organic materials, like cellulose.

Some authors indicate a synergistic effect between γ-radiation and heat, referring that this effect could allow a twenty-fold decrease in minimum effective radiation dosage (Ben-Arie and Barkai-Golan, 1969; Hanus, 1985). Nevertheless, no studies were performed in order to verify the effects of this combination on paper materials, since the deterioration reactions could also be synergistic.

Butterfield (1987), assuming 10 kGy gamma irradiation as the lethal dose for most fungi, and using a 156 Gy h^{-1} rate, tested its effects on paper samples. The results point out that irradiation causes similar deterioration as artificial ageing. Also, the combined treatment of irradiation followed by artificial ageing acts synergistically, since the deteriorating effects of this combination are more severe than the sum of the effects of the two separate treatments (Butterfield, 1987). Therefore, heat may be acting as a catalyser on the deteriorating reactions caused by radiation.

Tomazello et al. (1995) evaluated the lethal activity of γ-radiation on fungal spores existent on naturally contaminated papers and also the effect of pre-treatments with variable temperature and humidity levels on the minimum lethal dose of radiation. By increasing the doses of radiation (2-20 kGy at 4.103 kGy h^{-1}), the number of fungi decreased, although at higher doses between 17.5 and 20 kGy there were still viable fungi on the paper samples. Dried conidia were more resistant to radiation, as fungi on contaminated samples subjected to a drying pre-treatment at 50°C showed a higher survival rate than fungi in samples pre-treated with moist heat or at room conditions (Tomazello et al., 1995).

While fungal conidia were more susceptible to radiation after moist heat pre-treatment, bacteria on the other hand tremendously improved their resistance to radiation after this treatment (Tomazello et al., 1995). Since bacteria and fungi coexist in natural contaminated objects, this kind of pre-treatment would not be advisable and besides, moist heat is known to have deteriorating effects on paper.

Adamo et al. (1998) evaluated the effects of gamma irradiation treatment on pure cellulose paper samples, at room temperature, soaked with water, and soaked and frozen. Before analysis, the soaked and frozen samples were either dried in a ventilated oven or dried in vacuum by sublimation (freeze-dried). Irradiation was performed at doses between 0 and 10 kGy, using a dose rate of 2.8 kGy h^{-1}. The results achieved are similar to the ones obtained by Butterfield (1987), concluding that the alterations caused by radiation are similar to those caused by artificial ageing and that the combination of both treatments has a synergistic effect on cellulose deterioration, revealed by a drastic fall in the degree of polymerization. Besides, a very slight decrease in pH and a significant yellowing were observed on the irradiated samples, especially after ageing (Adamo et al., 1998). Regarding the variations between sample sets, the freeze-dried ones show significantly lower mechanical resistance than the other ones (Adamo et al., 1998). A possible explanation to this result is described in section 3.2.6.1 Freezing.

Different environmental conditions during irradiation, like nitrogen and vacuum atmospheres, and saturation of samples with water, were tested by Adamo et al. (2001). The administered radiation doses varied between 2 and 5 kGy at a dose rate of 14.7 kGy h^{-1}. The different environmental conditions tested did not reduce the deleterious effects caused by γ-radiation on paper. Regarding the antifungal essay, according to the authors, only the water saturated samples showed an additional antifungal activity by comparison with the altered atmosphere variants, maybe due to the radicals also formed in the water which could provide an extra germicidal power to the treatment (Adamo et al., 2001).

The exposure to γ-radiation has proven to turn cellulose more susceptible to further infestations (Adamo et al., 2003). The depolymerization caused by irradiation breaks the cellulose polymer into smaller units which can be more easily available to biodeteriogens.

Da Silva et al. (2006), determined 16 kGy (70 Gy min^{-1}) as the minimum dose to kill *Aspergillus versicolor* and *Eurotium chevalieri,* and two months after treatment, the materials remained sterile.

As the homogeneity of γ-radiation within high volumes of paper is important to achieve successful sterilization results, Havermans (2011) studied the effect of materials density on the homogeneity of a gamma radiation treatment. In the same study, the minimum lethal γ-radiation dose for four fungal species was 4 kGy under laboratory conditions. However, taking into account different fungal resistance and heterogeneities within paper volumes, a minimal dose of 8 ±2 kGy was recommended (Havermans, 2011).

Mesquita (2013:79), assessed the effects of γ-radiation on *Cladosporium cladosporioides*, and concluded that a 15.4 kGy radiation dose (21 kGy h^{-1}) completely sterilized the treated paper.

Summarizing the results obtained by the several reviewed studies, it can be concluded that the antifungal activity of γ-radiation is dose dependent and varies with fungal species and fungal development stage. Exposure to γ-radiation can induce fungi to produce more coloured metabolic products and after irradiation the treated paper will become more susceptible to further biodeterioration. Gamma radiation can cause severe depolymerization of cellulose and significant yellowing, which is highly enhanced by artificial ageing. On the other hand, the mechanical resistance and pH of irradiated papers remain practically unaffected. This antifungal treatment does not leave any toxic residues but has cumulative degrading effects on the treated materials.

2.3.3. High frequency current

The high frequency current method started to be used by Russians in 1947 for drying wet documents. During this practice, it was noticed that the method was also destroying fungal spores on documents, and so, high frequency current started to be used as an antifungal treatment as well (Flieder, 1965). This method acted through an extremely fast increase in temperature (a few seconds) in a homogeneous way, from the centre to the periphery of documents.

One of the high frequency current method procedures, as described by Flieder (1965), consists in placing the documents in a cabinet, pressing them strongly between two capacitor plates, where a current of 1.5–1.6 A and a grid current of 0.30–0.32 A passes through them. The temperature was kept between 90–100°C, for 12–15 minutes.

Strassberg, in 1978 (Strassberg, 1978), considered high frequency current as part of a group of newly developed fumigation techniques involving sophisticated electronic apparatus, which at the time were passed over in favour of chemical treatments.

The high frequency current method had also an insecticidal effect, and no chemicals remained in the treated materials. Nevertheless, its antifungal effect is described as mediocre. Leather, parchment, seals and documents with elements in relief could not be treated due to the pressure and temperature. Also, the documents have to be thoroughly cleaned previously, because the dust may ignite. Analysis on paper samples reveal an increase on the copper number (c. 40%) and a diminishment of tear resistance (c. 20%) (Flieder, 1965).

2.3.4. Low-oxygen environments

The use of modified atmospheres, i.e., low oxygen (O_2), carbon dioxide (CO_2) or nitrogen is common in the food industry. According to Hocking (1990), atmospheres with high percentage of CO_2 are more effective in controlling fungal growth than those which exclude oxygen by replacement with nitrogen. Also, the production of mycotoxins can be inhibited in modified atmospheres, and again high concentrations of CO_2 are more effective than reduced O_2 content (Hocking, 1990).

Valentin et al. (1990) have studied the microbial control potential of low oxygen and low relative humidity environments. Parchment samples were inoculated with *Aspergillus flavus* or exposed to environmental contamination. Low O_2 environments (5%, 1% and 0.1%) were achieved by addition of nitrogen. The results showed that even though the reduction of O_2 caused a decrease on fungal growth; it was the reduction of RH that highly diminished microbial development (Valentin et al., 1990).

Fungi need oxygen to growth, but some fungal species are efficient scavengers capable of near normal growth in oxygen concentrations below 1% (Hocking, 1990).

Low oxygen atmospheres besides reducing biodeterioration caused by fungi, can also eliminate insect pests, and in long term storage can prevent chemical reactions that are oxygen dependent like photooxidation or combustion in case of fire hazard. Nevertheless, atmospheres high in CO_2 can change the acid/base balance on treated materials (Selwitz and Maekawa, 1998:67).

2.3.5. Ultraviolet Radiation

Ultraviolet (UV) radiation, electromagnetic radiation with a wavelength between 10 and 400 nm, is used in many scientific and industrial fields according to the wavelength range. For sterilization purposes, UV radiation is used in the range of about 260 nm, since this is the wavelength that causes the highest damage in DNA molecules (Paulus, 2004:152). UV radiation is commonly used to sterilize air, as in inoculation chambers in mycological laboratories, or water, as a sanitizing treatment for recreational water.

The microbiocidal effect of UV radiation was discovered in 1903, by Barnard and Morgan, which by using a continuous arc current, found that the maximum bacterial effect occurred in the 226-328nm range (Hugo, 1995).

UV radiation causes degradation of microbial genetic material by forming dimmers between adjacent thymine nucleotides in DNA chains, which will inhibit the correct replication and transcription of this nucleic acid (Paulus, 2004:152). Due to its low energy content, this kind of radiation has poor penetration power (Hugo, 1995).

The exposure of mouldy books to sunlight due to the disinfecting properties of UV radiation has been performed as common sense and suggested for treating small, localized outbreaks (Nyberg, 1987), although, according to Florian (2002:61), the main process behind the inhibition of fungal growth by this method is probably dehydration.

Gallo (1963), considers UV radiation rather inefficient in disinfecting textiles and books due to its low penetration power.

Belloni (2006) studied UV radiation as a sterilizer for cellulose-based cultural heritage, using an excimer laser operating at 308 nm as UV source. The sterilizing properties of the laser were

tested against a mycelial actinomycete and the results show that after 75 seconds of irradiation, the survival rate decreased three orders of magnitude.

However, it is well known that UV radiation due to its high energy can cause damage to most heritage materials. Cellulose can be damaged significantly by high energy UV radiation (about 280 nm), which is within the range used to kill microorganisms (Florian, 2002:61; Bukovsky and Trnkova, 2003b). The oxidation of cellulose caused by UV radiation , leads to yellowing, bleaching and brittleness of paper (Bukovsky and Trnkova, 2003a, 2003b).

2.3.6. Temperature extremes

Temperature influences the rate of metabolic activity of microorganisms and thus their rate of growth. Each species of fungi has its own optimum growth temperature, and also its minimum and maximum limiting temperatures for development.

Temperature is related to the equilibrium moisture content (EMC) of microbial cells, and also the EMC of the substrate, which are limiting factors for fungal development.

Most fungi grow at temperatures between 5 and 35°C, with optimum temperatures for growth between 25 and 30°C (mesophiles) (Neville and Webster, 1995:322). However, there are species able to grow near or below 0°C (thermotolerants or thermophiles) or above 40°C (psychrotolerants or psycophiles) (Neville and Webster, 1995:322). Moreover, before activation, conidial cells are much more resistant to temperature extremes than on the subsequent developmental stages (Florian, 2002:57).

By exceeding the maximum or minimum development temperatures for each fungal species, fungal growth can be arrested and depending on the temperatures achieved, cellular damages can occur, leading to cell death.

2.3.6.1. Freezing

Freezing has been used in food preservation since as early as 1000 BC in ice cellars in China (Archer, 2004).

The lethal effects on microorganisms caused by freezing are time and temperature dependent and can originate from physical and/or chemical damages. The physical damages are related to the formation of extracellular or intracellular ice formation, causing the rupture of membranes and organelles (Florian, 2002:58; Archer, 2004). The chemical damages occur as water freezes and solutes become concentrated in the unfrozen fraction of water in which the microorganisms tend to concentrate. This process leads to a concentration of extracellular and intracellular solutes, causing lethal pH and ionic changes (Florian, 2002:58; Archer, 2004).

The formation of ice also lowers water activity on the substrate, thus limiting microbial development (Gunde-Cimerman et al., 2003).

In the field of heritage conservation, freezing can be used for long term preservation of photographic materials, or to kill insect pests. Regarding fungal development control, refrigeration (3.2.6.3) and freezing are used as an intermediate temporary methodology to prevent fungal growth in wet materials until they can be properly dried. Even though, low enough freezing temperatures can actually kill microorganisms, rather than just slowing down their growth as in refrigeration. Also, freezing can prevent soluble compounds in the materials from migrating and bleeding.

Schwerdt (1998) advises packaging the paper materials before freezing as packages with a thickness of 5 to 15 cm. Also, the materials should be separated from each other with plastic bags, freezer paper or silicone paper, in order to prevent adhesion between them.

Generally, the organisms are killed more quickly when held at or near their freezing temperatures, where freezing/thawing cycles occur, and when the rates of freezing and thawing are slow (Florian, 2002:58). At temperatures between 0 and -4°C, only a small quantity of water in materials is in fact frozen. In the fine capillaries of organic materials, water may not freeze even at much lower temperatures, and can still support microbial activity (Florian, 1997:148). Microbial growth can be stopped in storage at -20°C but at this temperature the formed ice may also cause damage on water-soaked materials (Florian, 2002:58).

It is important to take into account that dry conidia are much more resistant to freezing than hydrated spores and vegetative hyphae. This is due to their low water content which prevents the formation of internal ice crystals (Florian, 2002:58). These cells can still be viable after thawing and become activated when conductive conditions arise.

The increasing concentration of solutes in the materials during the freezing process, although contributing to the inactivation of microbes, can also lead to an increase in the deteriorating chemical reactions on the substrate, like pH dependent reactions and lipid oxidation which produces high energy radicals (Takenaka et al., 1996; Florian, 1997:147).

The formation of ice crystals increases the volume of water in about 8.5% (Florian, 1997:146). In water-soaked materials, the formation of ice crystals during freezing will also cause a volume expansion. The spaces created by the crystals will remain unaltered after the material is dried, and on materials such as paper the thickness and porosity will become higher (Florian, 1990). The porosity increase will change some of the paper's characteristics, like absorbance and mechanical properties, and dimensional changes can become a problem, for instance on bound volumes.

On the thawing process, moisture in materials will become high, which can activate dry conidia and also cause migration and bleeding of soluble components on the material. Moreover, the water evaporation forces on the drying process can cause dimensional changes and distortions.

In order to prevent the deteriorating processes caused by thawing, frozen heritage materials can be dried by sublimation, a method where the water passes directly from the solid to the gaseous state. This process of freezing and subsequently drying by sublimation is called *freeze-drying*. It is reported to be more lethal to fungi than freezing followed by thawing, due to its ability to remove also bound water from the microbial cells, however, more resistant cells may still survive (Florian, 1997:148).

According to Florian (1997:147) the process of sublimation can be carried out in a vacuum chamber maintained at freezing temperature. The frozen material is associated with a heat source for sublimation. In the system, a condensing surface is maintained at a lower temperature than the frozen material and so the sublimed water vapour will diffuse from the material to the condenser and then thawed. This is a slow process that can be accelerated by using vacuum. Flink and Hoyer (1971) tested this procedure on documents affected by a flood and subsequently frozen, using a heat source at 45°C and a 26.7 Pa vacuum pressure. According to their results, in none of the objects did ink running occur. The average drying time for

documents 2 to 3 cm thick was 1.5 to 2 days, while a photographic album 10 cm thick took 4.5 days to dry (Flink and Hoyer, 1971).

The freeze-drying method, besides requiring technology generally not available in cultural heritage institutions, such as museums, archives or historical houses, also presents some risks to paper based objects. Since the sublimation front moves from the outside to the inside of the object, the outer layers are continuously being dried and may lose some of their bound water and become impermeable and case-hardened (Florian, 1997:147). It is the bound water that provides plasticity to organic polymers like cellulose or collagen, and so, its loss will mean embrittlement of paper, parchment, leather and some adhesives (Florian, 1990). One possible example of this effect is presented on an article about gamma-irradiation (Adamo et al., 1998), in which a set of paper samples is irradiated while frozen and then freeze-dried. In comparison with other set of samples irradiated while frozen and dried in a ventilated oven at 35°C, the freeze-dried one exhibits a much lower mechanical resistance.

According to the Conservation Center for Art and Historic Artifacts Disaster Recovery bulletins (CCAHA, 2000), freeze-drying is limited to artworks with thinly applied media and no surface coatings. Also, it has the disadvantage of not allowing the interruption of the process for evaluation of the materials.

Basset and Draïs (2011) have developed an alternative to lyophilisation in drying frozen graphic documents. Firstly the excess ice on the surface of the object is removed with a hair dryer at 35°C and a spatula. Afterwards the object is wrapped on blotting paper, sealed in a hermetic bag and immersed in 70°C water. The blotters are replaced every 20 minutes. After thawing, the object is dried using fans and interleaving the book's pages with blotting paper. According to the authors, a book 15 cm thick can be thawed in 3 hours and dried in 48 hours with no additional damage caused, although the amount of handling that the book is subjected to when changing the blotting is an inconvenience. In this study it is not revealed if soluble media migrates with thawing and if any pressure is applied to prevent physical deformations during the drying process.

2.3.6.2. High temperature

Heat has been used for ages as a method of sterilization. Already Aristotle has recommended to Alexander the Great that his troops should boil the water before drinking in order to avoid illness (Hugo, 1995).

The use of high temperatures to eliminate insect pests from heritage objects has been studied by several authors (Strang, 2001; Brokerhof, 2002; Ackery et al., 2004). According to the literature, insect pests are killed by being exposed to 55 - 60°C for one hour (Strang, 1992; Pinniger, 2003). Fungi, on the other hand can resist higher temperatures. For instance, thermophilic fungi can still grow at 50–60°C (Rajasekaran and Maheshwari, 1993). The methods used to sterilize media and equipment in laboratory practice use temperatures above 100°C (autoclaving is performed at 121 °C, 100 kPa) in order to kill both vegetative forms and spores of microorganisms.

According to Florian (2002:57), herbarium samples are usually dried at temperatures around 50°C, which kills germinating conidia. On the other hand, dry conidia have longer viability and are more resistant to heat than hydrated conidia or vegetative cells of fungi, and so, they can survive a heat treatment that apparently has stopped fungal growth (Florian, 2002:57). Besides,

heat can also act as a conidia activator, allowing an immediate development of the fungus once the conductive conditions arise (Thevelein et al., 1979).

Heat can be an auxiliary method for drying wet heritage materials, since it helps to reduce moisture content, but the exposure to high temperatures can accelerate deterioration rates and cause dimensional changes on heritage materials, namely on paper.

2.3.6.3. Refrigeration

Refrigeration (c. 4°C) is universally used in domestic short term food preservation. In heritage conservation field, refrigeration is mainly used for temporary storage of water soaked materials, in order to slow down fungal growth, while gaining time for preparing dehydration or other antifungal procedures.

Long term storage at this range of temperatures is not advisable, since moisture in materials remains high and the fungal growth, although slowed down, still continues. The fungi being under this kind of stress can produce more conidia, melanin pigmented hyphae staining the substrate sometimes irreversibly, or polyols, which can easily undergo autoxidation and form free radicals and peroxides (Florian, 2002:57). Moreover, some conidia can become activated by this range of temperatures, and as soon as they are exposed to a proper growth temperature, they will immediately germinate (Florian, 2002:58).
Paper materials by remaining wet under refrigeration, can also suffer dissolution and migration of water soluble media, adhesives, sizing and grounds. On the other hand, chemical deterioration rates will remain lower at this range of temperatures.

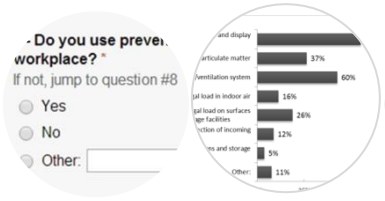
2.4. Conclusions

In this literature review it was found that the efficacy of an antifungal compound or method may vary with several factors, like mechanism of action, concentration/intensity used, or time of contact/action. Also, the susceptibility of fungi to a particular antifungal compound or method can depend on the fungal strain, on its stage of development, and if it is or not part of a biofilm.

According to these studies, regarding the physical methods, dehydration seems to present the best conjugation between antifungal effect and minor alterations on the properties of paper materials. Concerning the chemical antifungal compounds (fungistatic or fungicide), calcium propionate, esters of *p*-hydroxybenzoic acid (parabens) and ethanol, are the ones that combine minor health effects with less negative impact in paper properties. Among these three, parabens are the ones with best antifungal properties. However, these compounds still deserve additional testing to attest their efficacy and safeness for use in cultural heritage materials, and therefore they were selected for further analyses in this thesis (Chapters 6 and 7A).

All antifungal methods reviewed in this chapter have strengths and weaknesses. They present different degrees of efficacy, toxicity and some of them are safer to use in heritage materials than others. Development and evaluation of innovative antifungal methods in order to achieve and recognize better approaches than the ones already existent is still an active research field.

Knowing the pros and cons of the available methods to antifungal treatment of paper allows conscious decisions adapted to different situations. The information compiled in this chapter is intended to be an aid to this decision-making effort. This collection of data can be of great value when a fast intervention is needed, as in an emergency situation.



3

What is the panorama of fungal biodeterioration of paper worldwide? - Results of an international survey

Published with minor alterations as: Sequeira, S. O., Cabrita, E. J. and Macedo, M. F. (2014) 'Fungal Biodeterioration of Paper: How are Paper and Book Conservators Dealing with it? An International Survey.' Restaurator, 35(2) pp. 181–199.

Paper biodeterioration by fungi has always been a concern in archives, libraries and museums. Several guidelines have been published regarding the prevention of fungal development in paper collections and recovery of affected objects, but what is actually being implemented from the literature by worldwide paper and book conservators? How common is this type of biodeterioration? What needs to be further studied?

In order to access this information an online international questionnaire was conducted with participants from 20 different countries.

The results show that fungal biodeterioration is highly common in paper collections. All of the respondents already had to deal with paper deteriorated by fungi, and although the vast majority uses active measures to prevent fungal development, most of them have already experienced active fungal infestations. The mainly used preventive measures are the ones concerned with control of environmental conditions in storage and display rooms. Drying the affected paper objects and applying 70% ethanol are the most preferred options to stop active fungal growth. The study of non-toxic and safer antifungals is considered as the most relevant research topic in the area of paper biodeterioration by fungi, meaning that the currently available options are not totally satisfactory.

3.1. Introduction

Paper biodeterioration by fungi has always been a common problem in archives, libraries and museums. The way conservators have dealt with this occurrence has changed throughout the years. Precautionary and curative disinfection procedures were performed routinely along the last century (Flieder, 1965; Hengemihle et al., 1995). However, the acknowledgement of the risks that most of the used antifungal compounds, like ethylene oxide or formaldehyde, pose to health and to the treated objects has led to an increasing implementation of alternative less interventive and less toxic options.

Following this tendency, there are currently several publications available on what to do to prevent and treat this kind of paper deterioration (Florian et al., 1994; Florian, 1997, 2000, 2002; Brokerhof et al., 2007; Guild and MacDonald, 2007; National Park Service, 2007; Child, 2011; Price, 2013). In this literature, the recommendations to prevent fungal infestations consist primarily in the protection of heritage objects from dust, in keeping the objects and facilities

clean, controlling relative humidity (RH) and temperature (T), preventing the formation of microenvironments, implementing air ventilation systems, monitoring surface contaminations and fungal load in the environment, and regular inspection of collections for signs of visible fungal growth.

To recover already infested collections, the literature generally advises to isolate the contaminated artefacts, inactivate the fungi (stop fungal development) by air drying, freezing, freeze-drying, and cleaning with dry surface-cleaning methods. Only a few authors advise the application of ethanol directly on the artefacts (Florian et al., 1994; Brokerhof et al., 2007; Child, 2011).

This study aimed to disclose what is actually being adopted from the published literature by worldwide paper and book conservators, if there is a general agreement among conservators, how often does this type of biodeterioration occur, and what topics are in need of further research according to their working experience.

This kind of information is difficult to obtain due to several issues like institution and company's policies, concerns with anonymity and telling something incorrect or compromising, and the difficulty to reach and contact a broad spectrum of paper conservators globally scattered. To bypass such obstacles, a survey that permitted to collect information while maintaining the anonymity of the respondents was used.

Surveys are a research method used in many and diverse fields to collect information about people's features and ideas (Richardson, 2005; Kalantari D. et al., 2011; Kazi and Khalid, 2012). There are several methods to collect data on surveys, like interviews (in person or by telephone), or postal, e-mail and online questionnaires. Nowadays, electronic and internet surveys are becoming more common than the traditional methods due to their ability to reach larger groups of people, thus achieving more valid results, and also due to their speed of extraction and analysis of results, which improves scientific development (Kalantari D. et al., 2011).

For the first time, to our knowledge, a questionnaire was made to study the attitudes and experiences of conservators towards paper biodeterioration by fungi. The gathered information will help us to have a real panorama on the prevention and treatment of fungal biodeterioration in archives, libraries and museums. It will also aid conservation researchers aiming their research towards the needs of conservation-restoration practitioners, as an appropriately targeted research can reduce costs and improve the impact and practical use of science in conservation practice (Bradley, 2002).

3.2. Materials and methods

This survey was based on an electronic questionnaire designed for Google Drive®. This type of questionnaire is completed online, is totally anonymous and the gathered data are automatically compiled in a database.

The questionnaire was composed mainly of close-ended questions - questions where the respondents are asked to choose among a set of answers (Kazi and Khalid, 2012). These are easier and quicker to answer, require no writing, and its quantification and analysis is simpler

(Oppenheim, 2000:114). However, this type of questions can result in loss of spontaneity and expressiveness, and the choice of answers may fail to correspond to the respondents own ideas, so in all of the questions an "Other (please specify)" option was included, which was a free response answer that enabled the respondents to add their own preference. A part of the questionnaire is given as example in Figure 3.1, where it is shown that the used software allowed marking the main questions as mandatory, meaning that one could not submit the questionnaire without answering those.

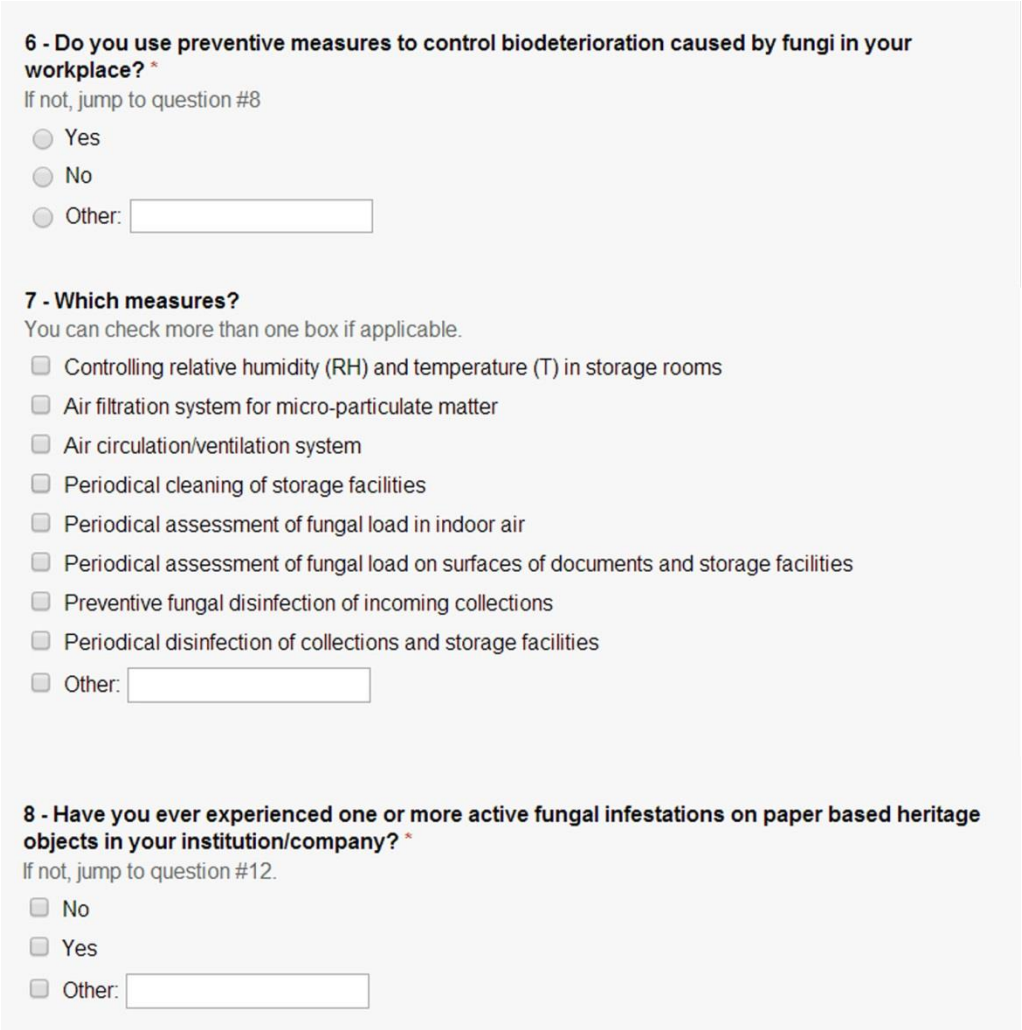


Figure 3.1: Part of the questionnaire as it appeared online. The asterisk indicates questions that were mandatory to respond in order to be possible to submit the filled questionnaire.

With a total of 14 questions, including the ones to determine the profile of respondents, the survey focused on the experiences paper and book conservator have had with fungal biodeterioration of paper. These experiences encompassed the prevention, causes and management of fungal infestations, and conservation-restoration treatments of paper biodeteriorated by fungi. Also, one of the questions was aimed at the respondents' opinion on which topics need further research in the field of paper biodeterioration by fungi.

With the exception of Yes/No answers, Country and Year of experience, the participants were allowed to select more than one option from the set of answers, and so, the total number of responses in those ones will add up to more than 100%.

The first version of the finalized questionnaire was administered to a small sample of the population, which allowed the detection of unclear questions or options. These were corrected before distributing the final version.

The questionnaire was available in English and Spanish languages. The links to the two versions of the online questionnaire were distributed by Conservation DistList in September 2012 and remained active for two months.

Conservation DistList is an e-mail distribution list created in 1987 operated nowadays by the Foundation of the American Institute for Conservation. With 9559 registered users from 95 different nationalities by September 2012, this list is considered "the most efficient means of worldwide communication between conservators"(Bordalo, 2009).

The questionnaire was directed only to paper and book conservators, starting with a screening question that excluded everyone that was not working or studying in the paper and/or book conservation field. From the totality of registered users on Conservation DistList, only 1034 stated to be conservators with paper or book as their field of interest (data provided by the Conservation DistList manager). Although these numbers can be accessed, it is not possible to calculate an exact response rate due to the impossibility of quantifying the exact number of people who did indeed receive the questionnaire email. According to the Conservation DistList manager it is unfeasible to have an exact figure, since there is no way of knowing how many of the messages have failed to be delivered, and also, many institutions forward these emails to other people.

The use of an e-mailing list as the distribution mean for a questionnaire has several advantages, like its anonymous character, its low cost of data collection and processing, and ability to reach respondents who are dispersed all around the globe. Still, every distribution method has limitations and by choosing this mean, the survey is being limited to people who have access to the internet, and who receive Conservation DistList. This has to be taken into account when interpreting the gathered data, as they will only be representative of this population.

In the treatment of the collected data, for each question of the questionnaire the respondents were divided by all profile segments (years of experience; working activity; geographic distribution and type of workplace) and the results examined in cross-tabulations to see if there was any relationship between the profile segments and the given responses. However, no patterns were observed and therefore none is presented.

3.3. Results and discussion

3.3.1. Respondents profile

Out of the 57 participants in this questionnaire, the great majority were Conservators-Restorers, followed by Preventive Conservators, Conservation Scientists, and Archivists/Librarians. Most of them worked in the public sector and had between 10-20 years of working experience in the conservation field (Table 3.1).

Table 3.1: Profile of questionnaire respondents.

	Segment	% of respondents
Professional activity	Archivist / Librarian	4
	Conservator-Restorer	79
	Conservation Scientist	7
	Preventive Conservator	19
Type of workplace (company/institute)	Public sector	68
	Private sector	21
	Both sectors	7
Years of experience	1-5	14
	5-10	25
	10-20	42
	>20	19

The responses came from people working in 20 different countries (Table 3.2), with the United States of America leading with the highest number of participants, followed by Brazil and the United Kingdom. This broad spectrum of geographic locations shows that the method of distribution of the questionnaire is efficient, although the percentage of respondents per country can be related to the limitations of this method, which are pointed out in the Methods section.

Table 3.2: Distribution of respondents per country.

Country	% of respondents
Afghanistan	2
Argentina	5
Australia	4
Bolivia	2
Brazil	16
Canada	5
Colombia	2
Chile	4
Denmark	2
Germany	5
Greece	2
Guatemala	2
Ireland	2
Malta	2
Netherlands	5
Norway	2
Pakistan	2
Portugal	4
United Kingdom	11
United States of America	25

3.3.2. Prevention of fungal infestations

Participants were firstly asked if they use preventive measures to control biodeterioration caused by fungi in their workplace. The vast majority who answered positively (Table 3.3) was then requested to specify which preventive measures they use (Figure 3.2).

Table 3.3: Frequency of responses to the questions that required a Yes/No response.

Question	Yes (%)	No (%)	Other (%)
Do you use preventive measures to control biodeterioration caused by fungi in your workplace?	90	5	4
Have you ever experienced one or more active fungal infestations on paper based heritage objects in your workplace?	79	19	2
Have you ever noticed a reactivation of the fungal growth after the returning of the affected paper items into the storage facilities?	20	76	23

Figure 3.2 shows that most people selected the control of RH and T in storage and display rooms. Following this option, the second most frequent was the use of an air circulation/ventilation system.

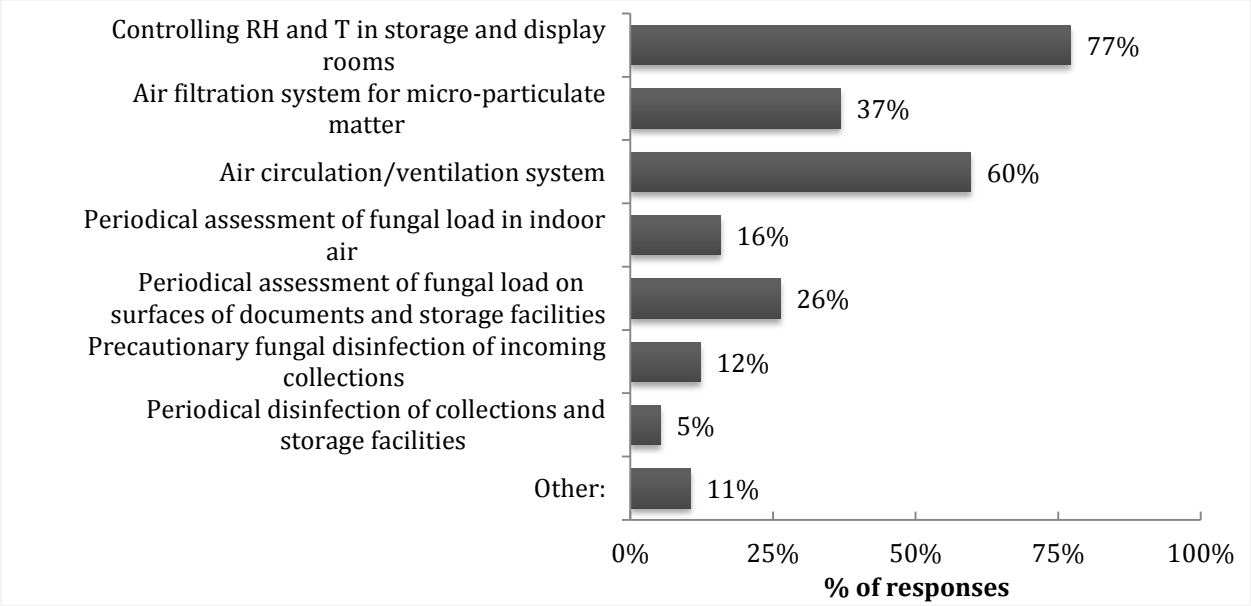


Figure 3.2: Frequency of responses to the question «Which preventive measures do you use to control biodeterioration caused by fungi in your workplace?».

These results reflect the fact that the control of the environmental RH and T is one of the best known and generalized preventive conservation methods, since it has influence in most types of deterioration processes, like chemical, physical and biological ones. Heating, ventilating, and air-conditioning (HVAC) systems, generally used to control environmental conditions, constitute a high economic burden due to energy consumption and maintenance, and therefore are not available to all institutions. Owing to the economic and environmental impact such energy waste represents, new more sustainable alternatives are being studied (Staniforth, 2011; Casanovas et al., 2013).

A suitable air circulation around the documents/objects (chosen by 60% of the respondents) is very important since it helps to homogenize T and RH in those areas, preventing the formation of local microclimates with higher RH prone to fungal development. Nonetheless, it is important to combine the air circulation system with a filtration system for micro-particulate matter, since

air circulation/ventilation can increase the spreading of spores in the environment (Valentin, 2007), and 37% of the respondents selected this option.

A periodical assessment of fungal load in air and surfaces, can act as an indicator of fungal contamination and development, enabling the monitoring of fungal presence along time. The percentage of respondents who selected this procedure was 16% and 26% for indoor air and surfaces, respectively. These percentages may be in part attributable to the fact that this kind of procedures normally have to be done by external companies or institutions and therefore compose an additional expense.

Precautionary disinfection of collections, either performed in incoming collections or as a periodical procedure, was the least selected option in this question (Figure 3.2), which is in accordance with the published guidelines.

In the "Other" option, which was a free-response one, most of the answers included visual inspection, and cleaning of collections and storage facilities.

The responses obtained in this question illustrate the tendency that is evidenced in the published guidelines, which is based on acting on the environment surrounding the objects instead of using antifungals systematically as a precautionary measure – a common practice in the past (Hengemihle et al., 1995).

3.3.3. Causes of fungal infestations

In spite of the high percentage of people using preventive measures shown in the previous question, 79% of them have already experienced one or more active fungal infestations on paper based collections (Table 3.3). This fact shows that the adoption of preventive conservation methods is not a 100% guarantee of fungal development prevention. When HVAC systems or electronic dehumidifiers fail, temperature and humidity levels can quickly raise allowing fungal development. Regular inspections for fungal growth and microclimates formation on all several linear kilometers of documentation in an archive or library can be impractical, and water related emergency situations can occur. The causes pointed by the respondents for the experienced fungal infestations are summarized in Figure 3.3.

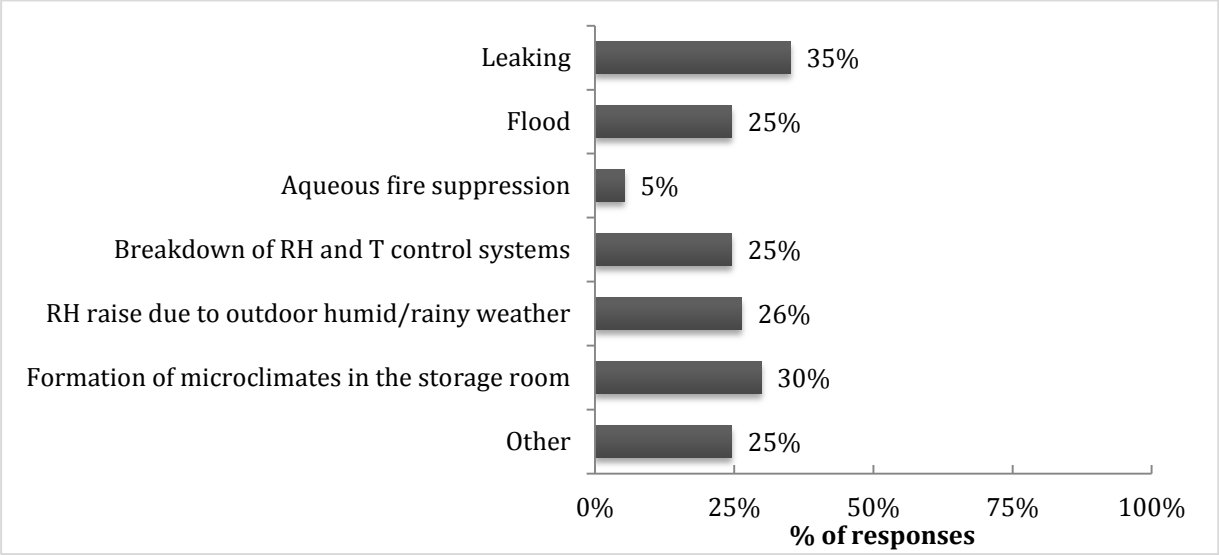


Figure 3.3: Frequency of responses to the question "Which were the causes of the active fungal infestations on paper based collections in your workplace?".

Leaking and formation of microclimates were the most selected options (Figure 3.3.), although there is an even distribution of the causes, where only the aqueous fire suppression shows a distinctive lower value. On the "Other" option, the most predominant causes for fungal infestations pointed out were the introduction of mouldy artefacts into clean environments, and poor hygiene/dust accumulation.

3.3.4. Deactivation of fungal infestations

Once faced with active fungal infestations in paper items, almost all of the surveyed conservators do take measures to stop further fungal development (Figure 3.4).

Drying the paper objects, either by interleaving them with dry absorbent paper, or by lowering the environmental RH, together with application of a 70% ethanol solution were distinctively the most selected options, followed by freezing.

On the contrary, sterilization procedures (fumigation with ethylene oxide or gamma irradiation), were the least chosen ones.

These results are in general accordance with the procedures recommended in the published literature. Though, the application of 70% ethanol directly on the paper artefacts - one of most frequently selected options in this question - is generally omitted in the referred literature.

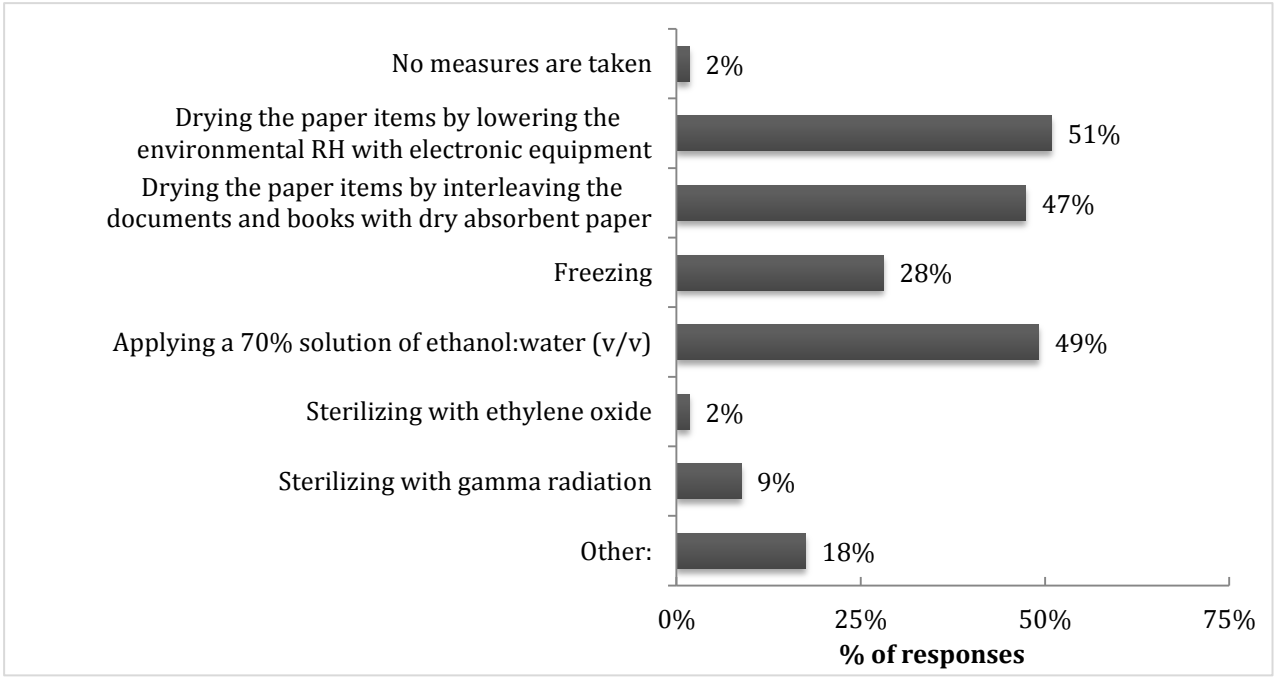


Figure 3.4: Frequency of responses to the question "Which measures do you usually apply to stop further fungal development on the paper items during an active fungal infestation?"

The respondents were then asked if after treatment of fungal infestations they have ever noticed a reactivation of fungal growth after the returning of the paper items to the storage facilities. 20% of the respondents did so (Table 3.3), meaning that the levels of humidity were still high, that what caused the fungal infestation in the first place was not corrected, and/or that the

performed interventions were not successful. However, the majority of the surveyed conservators responded negatively to this question.

3.3.5. Recovery of paper biodeteriorated by fungi

When biodeterioration by fungi has already taken place, the majority of the surveyed chose to isolate the affected paper objects, vacuum clean the entire page(s) and consolidate/reinforce the paper (Figure 3.5).

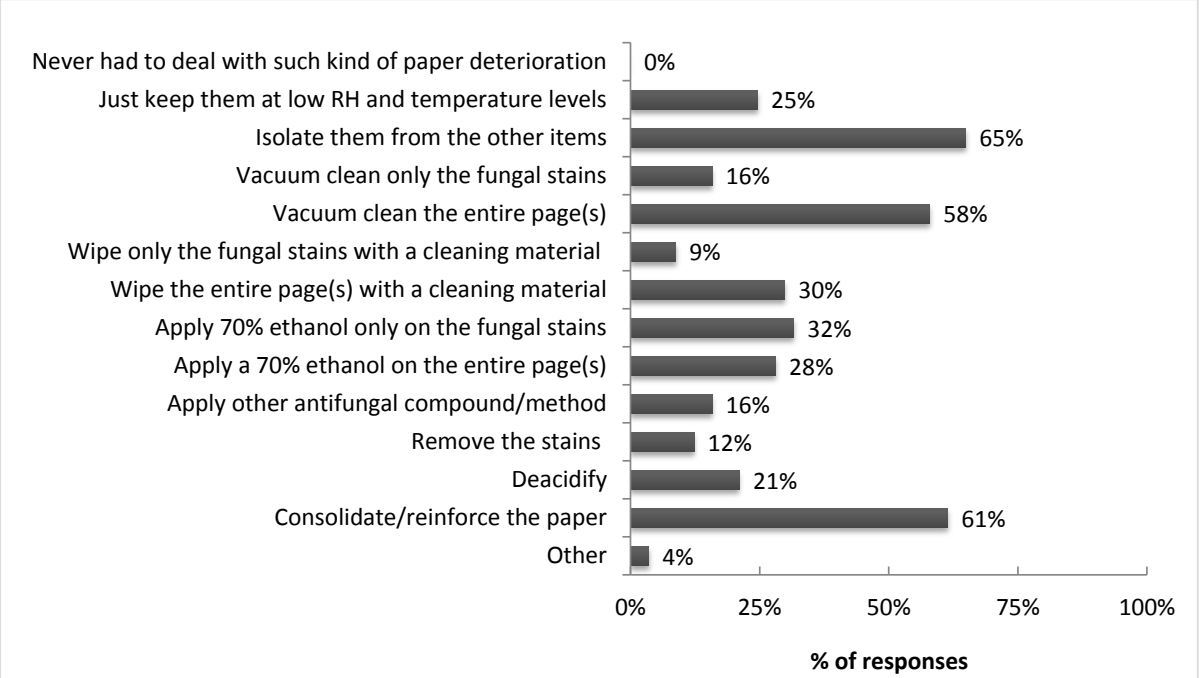


Figure 3.5: Frequency of responses to the question "How do you deal with paper based heritage objects that have already suffered biodeterioration by fungi (stained and with low mechanical resistance)?"

Regarding the given options of cleaning and applying 70% ethanol, there were two available variants: treating only the fungal stains or treating the entire page(s) (Figure 3.5).

In the cleaning options, there was a clear preference for treating the entire page(s), probably with the intention of doing a more thorough cleaning. However, the material used to clean (e.g. vacuum nozzle, brush, sponge, cloth) is going to retain at least some part of the fungal spores. If the same material is used to continue cleaning non-affected areas, a great amount of spores is spread on those areas, and in the case of high humidity conditions it can pose a serious risk of a worse new infection (Konkol et al., 2012).

On the other hand, when treating the objects with 70% ethanol, the localized application obtained a slight higher preference from the respondents. This may be due to the fact that this is a solvent and has a risk of dissolving paper or media components, and so the larger the areas where it is applied, the higher the risk of dissolution.

According to the gathered data, all the respondents of this questionnaire have already had to deal with paper based heritage objects deteriorated by fungi (Figure 3.5). These results can be

due to the high occurrence of this kind of paper damage, but can also indicate that whoever returned this questionnaire had a particular interest in this subject due to their past experience in dealing with fungal biodeterioration. According to the literature, there is a bias associated with mail surveys, since people who have a particular interest in the survey's topic are more likely to return the questionnaires than those who are less interested (Fowler, 2009:52).

In a free-response query, the respondents were asked to specify which materials/methods they typically use to treat paper biodeteriorated by fungi and the main results are presented in Table 3.4.

Table 3.4: List of the materials/methods specified by the respondents for wiping, deacidifying and consolidating/reinforcing of paper deteriorated by fungi. The percentages were calculated from the number of individuals who selected these treatments and not from the totality of the respondents.

	Methods/materials	Frequency of responses (%)
Wiping material (n=22)	Dry cleaning sponge (latex, rubber)	55
	Brush	27
	Eraser crumbs / gum powder	14
	Groom-stick	9
	Microfibre cloth	5
Deacidification (n=12)	Aqueous $CaOH_2$	42
	Bookkeeper deacidification spray	25
	Alkaline deionized water	8
	Magnesium ethoxide 0.05M in alcohol	8
Consolidation / reinforcement (n=35)	Japanese paper (JP) + starch paste	37
	JP + Methylcellulose (MC)	11
	JP + Klucel G in alcohol	11
	Resizing with gelatine or MC	11
	Heat-set tissue	6
	Encapsulation / isolation in polyethylene sleeves	6
	JP + carboxymethyl cellulose	6
	Lascaux acrylic adhesive[a]	3
	JP + mix of starch paste and MC	3
	Resizing with klucel G	3

[a] reactivation of tissue with this adhesive using acetone

Dry cleaning sponge is the most used wiping material and calcium hydroxide is the most used deacidification compound. From the respondents who had selected the option of paper consolidation/reinforcement, nearly 40% use Japanese paper/tissue with starch paste. Since most moulds can easily digest starch, and its nutrients are more easily available to fungi than cellulose itself (Nyberg, 1987; Florian et al., 1994), by adding this type of adhesive the susceptibility of treated paper to fungi may be enhanced.

The respondents who claimed to use other antifungal compounds besides the ones given as options (nine individuals), reported the use of isopropanol (by local treatment or immersion), sodium hypochlorite, quaternary ammonium compounds, azoles, immersion in ethanol 100%, or 0.35% calcium propionate in ethanol. Each of these methods was only mentioned by a single respondent, except the use of isopropanol, which was mentioned by two, and so its percentages

are not presented in Table 3.4. It is worth mentioning that some of these compounds can have deleterious effects on paper, like sodium hypochlorite that is a strong oxidizing agent of cellulose (Smith, 2012), and quaternary ammonium compounds that can cause depolymerization and acidification of paper (see Chapter 2).

Fungal stain removal was selected only by 12% of the totality of the respondents (seven individuals), and among them, the majority claimed that it was only done when the objects were to be displayed in an exhibition. Bleaching with hydrogen peroxide was chosen by two of the seven respondents, and the following methods were selected only once: bleaching with sodium hypochlorite; bleaching with chlorine dioxide (solution or gas); bleaching with sodium borohydride; washing with ethanol/water solutions; washing with calcium hydroxide solution in demineralized water.

3.3.6. The need for further research

In the end of the questionnaire it was enquired in which topics people consider there is a higher need for further research in the field of fungal biodeterioration of paper according to their working experience. Four options were given, plus an open response option "Other". The respondents had to classify each topic from 1 (lower need of research) to 5 (higher need of research).

Figure 3.6 shows that the topic "Non-toxic/safer antifungals" was considered the one with the higher need for further research by the majority of the respondents, and is clearly detached from the other topics.

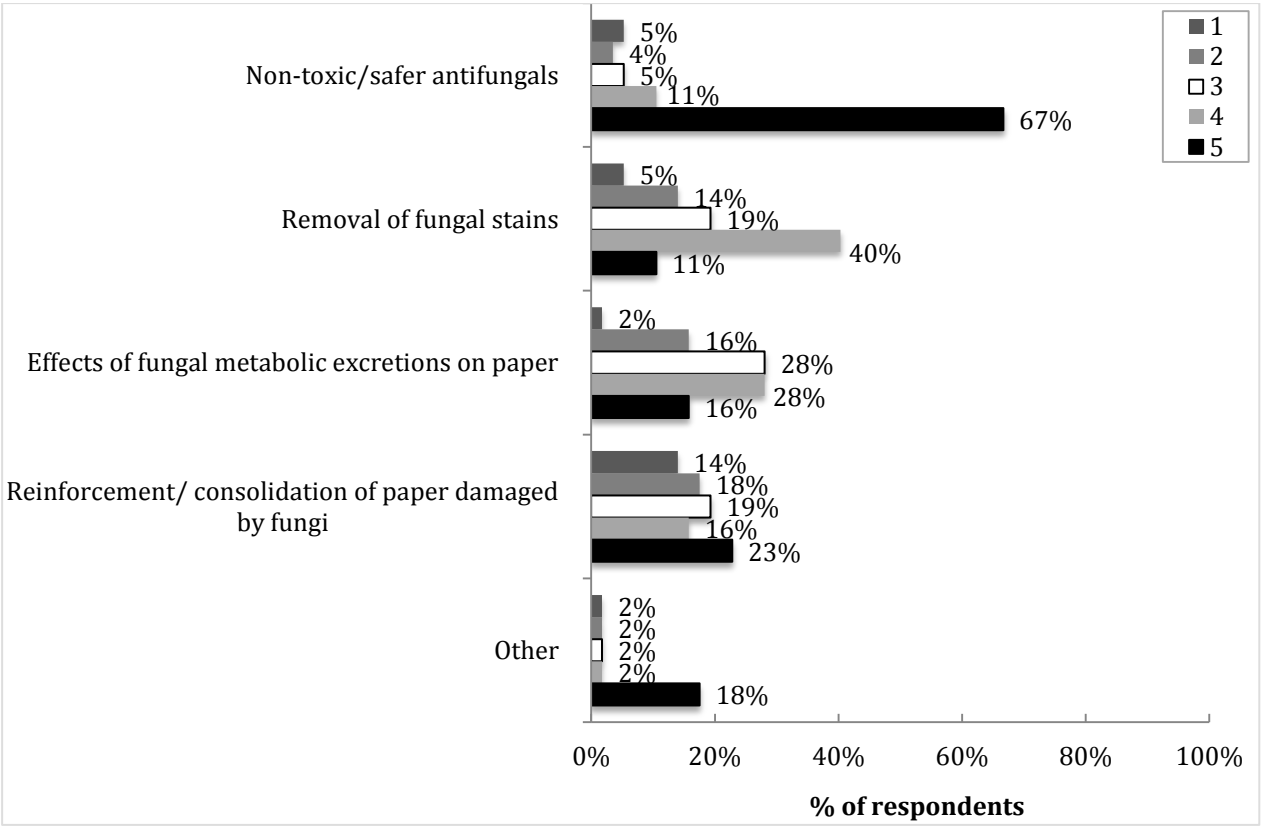


Figure 3.6: Results obtained for the question "In which areas do you think there is a higher need for research in the field of biodeterioration of paper by fungi?". The punctuations vary from 1 (lower need of research) to 5 (higher need of research).

The other three given topics obtained an average similar punctuation, although the "Removal of fungal stains" had a more pronounced score in level 4. This one could be considered the second topic in higher need for research.

The option "Other" was chosen by only a few respondents. Nevertheless, the respondents who selected this option mostly rated it with "5", the highest level. This indicates that in order to specify another research topic besides the given options, people had to consider it very important. Among these "Other" topics of research, the respondents referred mainly to safety measures for staff, and the effects and effectiveness of specific antifungal compounds and cleaning methods.

3.4. Conclusions

The main aim of this chapter was to have an insight into the real panorama of fungal biodeterioration of paper collections through the perspective of paper and book conservators distributed worldwide. It was intended to disclose how often does this type of biodeterioration occur, how do conservators deal with it, if there is any general agreement, if the published guidelines are followed, and what do conservators consider more important to be further studied according to their practical experience.

The obtained results show that fungal biodeterioration is very common in paper based collections. Even though almost all the respondents use preventive measures to control fungal biodeterioration, all of them have already had to deal with paper deteriorated by fungi, and the great majority has already experienced active fungal infestations in paper collections in their workplace. Preventive conservation should always be the first choice to control fungal biodeterioration, nonetheless the results obtained indicate that preventive conservation is not a 100% guarantee that no fungal biodeterioration will occur.

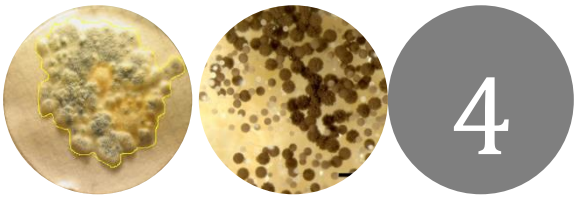
Among the population of conservators studied in this survey, the most frequently used methods to prevent fungal development on paper collections are the ones related with environmental control (T, RH and ventilation), which is in agreement with the current published literature.

When faced with active fungal infestations most of the respondents stop fungal development by drying the affected paper objects, applying a 70% ethanol solution, and/or by freezing them. The use of sterilization procedures are the least selected actions. There is a general agreement between conservators on this matter and it follows the recommendations existent in the literature, with exception of the use of ethanol directly on the objects which is scarcely mentioned in the published guidelines. Taking into account this result, and the fact that the efficacy of ethanol as an antifungal for paper collections is still an open discussion (see Chapter 2), ethanol was selected for further studying and the results are presented in Chapter 6.

To treat paper objects that have already suffered from fungal biodeterioration, most of the conservators choose to isolate the objects from other items, vacuum clean them and apply a consolidation /reinforcement treatment. In both these situations (stopping fungal development and treating paper deteriorated by fungi) a trend of using less interventive and toxic methods is observed: rarely any antifungals (besides ethanol) or removal of fungal stains are applied, contrarily to was usual in the past. Even so, the respondents considered the study of non-toxic/safer antifungals the topic that most needed further research. This information shows that the products and methods now available to prevent and suppress fungi are not fulfilling the

requirements of conservators according to their own experience. Therefore this topic was further developed on this thesis (Chapters 7B and 7C).

Surveys regarding the practical experiences and opinions of conservators are not common. With this study some of the potentials of surveys in the field of cultural heritage conservation have been shown. These studies can aid conservation practitioners to compare experiences with their peers and learn from them, and also help conservation researchers to target their research towards the needs of conservators. Moreover, the documentation and statistical treatment of this kind of data are very important not only for the present time, but also to aid future studies on the history of paper conservation.



4

Selection of fungal species for antifungal testing

Part of this chapter has been submitted for publication as: Pinheiro, A. C., Sequeira, S. O., Macedo, M.F., 'Fungi on paper collections in archives and libraries: a review on paper conservation and human health'.

This chapter is focused on the selection of the most frequent fungal species found on paper biodeterioration. The selected species will be used to test the efficacy of antifungal compounds in Chapters 6 and 7.

From the available literature dating from 1997 to 2014, 32 studies focused on the identification of fungal species from biodeteriorated paper materials were reviewed. *Cladosporium* sp. and *Penicillium* sp. were the most frequently identified fungi in the different studies. However, only fungi identified at the species level were considered for further selection. The most frequent fungal species were studied in greater depth, regarding their ideal growth conditions, pigmentation and enzymatic potential.

Aspergillus niger, *Chaetomium globosum*, *Cladosporium cladosporioides*, *Penicillium chrysogenum* and *Penicillium corylophilum* were the selected species for evaluation of antifungal compounds for paper conservation. This selection was done based on their frequency on paper materials and presence on reference studies regarding antifungal compounds used in paper. The characteristics of these five species are presented in greater detail.

4.1. Introduction

In Chapters 6 and 7, different chemical compounds are evaluated regarding their antifungal potential against filamentous fungi. Before initiating those studies, an insightful selection of representative fungal species to test was of utmost importance, since different species can react and resist differently to antifungal compounds (Vandeputte et al., 2012).

The selection of fungal species had as a starting point a review of the literature regarding the isolation and identification of fungi from paper or book materials.

The last review on fungi isolated from this type of materials was performed in 1997. In this work by Zyska (1997), data were collected from very diverse origins as it was not just focused on paper but on a panoply of materials one can find in museums, archives or libraries, such as parchment, wax seals, textiles, or photographs. Since 1997 numerous research works regarding fungi on paper collections were made and major methodological changes occurred in this field of knowledge, especially concerning new identification methods like molecular biology techniques. Now, a new literature review on that subject is requisite in order to systematise the fungi occurring on paper, its biodeterioration activity and go further by studying their role on human health.

The most frequently isolated fungal species were studied in greater depth, regarding their ideal growth conditions, pigmentation and enzymatic potential.

This chapter describes the selection process and presents the characteristics of the selected fungal species.

4.2. Materials and methods

From the available literature on isolation and identification of fungi, a selection was made considering only the studies where samples were collected from paper materials on areas with fungal biodeterioration signs. In this way, there was a higher probability that the identified species were paper biodeteriogens and not only an aerial deposition.

From that first screening a table was constructed where each bibliographic reference corresponds to a different study (Appendix I). Fungal species with the higher number of references were then presented in a subsequent table in greater detail.

For the five selected species for testing with antifungals a more thorough description was made, together with a photographic record. *Chaetomium globosum* Kunze, *Cladosporium cladosporioides* (Fresen.) G.A. de Vries and *Penicillium chrysogenum* Thom were obtained from the mycological collection of Universidade do Minho (Braga, Portugal). *Aspergillus niger* Tiegh. was obtained from the mycological collection of Instituto Nacional de Saúde Doutor Ricardo Jorge (Lisbon, Portugal), and *Penicillium corylophilum* Dierckx was isolated from library materials at Universidade de Coimbra (Coimbra, Portugal) and identified by DNA analysis.

Colour and visual characteristics of the fungal colonies growing on potato dextrose agar (PDA) were photographed on Petri dishes; the reproductive structures and other distinctive characteristics were registered *in situ*, with a stereo microscope (Leica MZ16) and digital camera (Leica ICD). The reproductive structures and fungal spores were also captured at a higher magnification with optical microscopy using a Zeiss Axioplan 2 Imaging system (HAL 100) coupled to Nikon DXM1200F digital camera and ACT-1 software.

4.3. Results and discussion

4.3.1. Fungi reported in paper materials – Literature review

All the fungal species isolated from paper materials in the reviewed literature, in a total of 32 studies between 1997 and 2014, are presented in Appendix I.

Cladosporium sp. and *Penicillium* sp. were the fungi with the highest number of references (Appendix I). However, only fungi identified at the species level were considered for further selection. The most frequently identified species, with four or more references are presented in greater detail in Table 4.1.

Five of the 12 species presented in Table 4.1 belong to the genus *Aspergillus* and four belong to the genus *Penicillium*. This illustrates the high frequency of such ubiquitous genera. The most frequently identified species was *Penicillium chrysogenum* (10 references), followed by *Aspergillus niger* and *Aspergillus versicolor* (9 references).

Table 4.1: The most frequently identified fungal species in paper/book materials - quantification and characteristics. Fungal identification using DNA analysis and classical morphological characterization are abbreviated as DNA and M, respectively. Enzymatic potential is assigned with (+++) high; (++) medium); (+) low; or (-) negative levels. Minimal and maximal water activity (aw) and temperature (T) values for growth are presented, with optimum values in brackets.

Fungal species	**Isolation from altered paper/book materials**		**Characteristics**				
			Colouration/ Pigments	**Enzymes**	**Growth conditions**		**References**
	Identification	**Reference**			**a_w**	**T (°C)**	
Aspergillus candidus Link	M	Das et al., 1997 Zotti et al., 2008 Bankole, 2010	White to off-white, yellow orange, or dull brown	Cellulases (+++) Amylases	>0.75 (>0.98)	3 - 55 (20 - 24)	(Samson et al., 2000; Milala et al., 2009; Pitt and Hocking, 2009; Panesar et al., 2010)
	M+DNA	Pinheiro, 2014					
Aspergillus fumigatus Fresen.	M	Das et al., 1997 Nol and Kenneth, 2001 Lourenço et al., 2005 Bankole, 2010	Turquoise-grey	Cellulases (+/+++) Gelatinases (+) Lipases (++) Proteases	> 0.83 >0.82	12 - 55 (37 - 42)	(Pasanen et al., 1991; Das et al., 1997; Tepsic et al., 1997; Samson et al., 2000; Chang et al., 2004; Gopinath et al., 2005; Janda et al., 2009; Pitt and Hocking, 2009; Oyeleke et al., 2010; Low et al., 2011)
	DNA	Di Bonaventura et al., 2003 Kraková et al., 2012					
	M+DNA	Mesquita et al., 2009					
Aspergillus niger Tiegh.	M	Das et al., 1997 Nol and Kenneth, 2001 Adelantado et al., 2005 Lourenço et al., 2005 da Silva et al., 2006 Michaelsen et al., 2009 Bankole, 2010 Borrego et al., 2012 Bergadi et al., 2014	Black/dark brown (melanin and hexahydroxyl pentacyclic quinoid pigments)	Cellulases (++/+++) Amylases (-/+) Gelatinases (++) Proteases (+)	>0.77 (0.97)	6 - 47 (30 - 37)	(Fogarty and Kelly, 1979; Das et al., 1997; Samson et al., 2000; Parra and Magan, 2004; Gopinath et al., 2005; Janda et al., 2009; Jorgensen et al., 2011; Borrego et al., 2012; Bergadi et al., 2014)
Aspergillus terreus Thom	M	Fabbri et al., 1997 Ricelli et al., 1999 Nol and Kenneth, 2001 Zerek, 2003	Hues of brown	Cellulases (+++) Amylases (-/+) Gelatinases (+)	>0.78	11-48 (35-40)	(Samson et al., 2000; Pitt and Hocking, 2009; Shahriarinour et al., 2011; *www.mycobank.com*, 2016)
	DNA	Michaelsen et al., 2010					
	M+DNA	Pinheiro, 2014					

Fungal species	Isolation from altered paper/book materials		Characteristics				
			Colouration/ Pigments	Enzymes	Growth conditions		References
	Identification	Reference			a_w	T (°C)	
Aspergillus versicolor (Vuill.) Tiraboschi	M	Hyvärinen et al., 2002 Zerek, 2003 Lourenço et al., 2005 da Silva et al., 2006	Mycelium: white to buff or orange; Conidia: greyish green	Cellulases (+/++) Amylases (+++) Gelatinases (+++) Lipases (+)	>0.78 (0.90 - 0.95)	5 -39 (25-27)	(Reiss, 1978; Samson et al., 2000; Pitt and Hocking, 2009; Adan and Samson, 2011; Borrego et al., 2012)
	DNA	Michaelsen et al., 2010					
	M+DNA	Mesquita et al., 2009 Michaelsen et al., 2009 Sato et al., 2014 Pinheiro, 2014					
Aureobasidium pullulans (de Bary) G. Arnaud	M	Zotti et al., 2007, 2008	Pink; grey to black	Cellulases (-) Amylases (+/+++) Gelatinases (-/+++) Lipases (+++)	>0.89	-5 - 35 (25)	(Federici, 1982; Gunde-Cimerman et al., 2004; Pitt and Hocking, 2009; Leathers et al., 2013)
	DNA	Michaelsen et al., 2010					
	M+DNA	Pinheiro, 2014					
Chaetomium globosum Kunze	M	Das et al., 1997 Szczepanowska and Cavaliere, 2000 Corte et al., 2003 Lourenço et al., 2005	Dark brown, chrome, slight yellowish or purplish tinge	Cellulases (++/+++) Amylases Proteases	>0.90	4 - 38 (25)	(Florenzano, 1949; Lakshmikant, 1990; Das et al., 1997; Sharma and Shukla, 2008; Pitt and Hocking, 2009; Abdel-Azeem et al., 2014)
	DNA	Rakotonirainy et al., 2007					
	M+DNA	Mesquita et al., 2009					
Cladosporium cladosporioides (Fresen.) G.A. de Vries	M	Silva et al., 2006 Zotti et al. 2008	Coloured olive, reverse bluish grey or black	Cellulases (-/+++) Lipases (-/+) Proteases (-/+)	> 0.86 (0.985)	-4 - 32 (25)	(Sautour et al., 2002; Gopinath et al., 2005; Pitt and Hocking, 2009; Borrego et al., 2012)
	M+DNA	Mesquita et al., 2009 Michaelsen et al., 2009					

Fungal species	Isolation from altered paper/book materials		Characteristics				
			Colouration/ Pigments	Enzymes	Growth conditions		References
	Identification	Reference			a_w	T (°C)	
Penicillium chrysogenum Thom	M	Fabbri et al., 1997 Ricelli et al., 1999 Corte et al., 2003 Borrego et al., 2012	Dull blue, greyish turquoise, dull green blue-green conidia, and yellow exudate, soluble pigment and reverse	Cellulases (+/+++) Amylases (+) Proteases (+)	>0.78-0.81 (0.985)	-4 - 37 (23-25)	(Samson et al., 2000; Sautour et al., 2002; Pitt and Hocking, 2009; Chinedu et al., 2011; Bergadi et al., 2014)
	DNA	Di Bonaventura et al., 2003 Bergadi et al., 2014 Michaelsen et al., 2010					
	M+DNA	Mesquita et al., 2009 Michaelsen et al., 2009 Sato et al., 2014					
Penicillium citrinum Thom	M	Corte et al., 2003 Lourenço et al., 2005 da Silva et al., 2006 Borrego et al., 2012	Greyish Orange, greyish turquoise; bright yellow	Cellulases (-/+++) Amylases (+/++) Proteases (+) Lipases (+)	>0.80 - 0.84	5 - 37 (26-30)	(Samson et al., 2000; Gopinath et al., 2005; Pitt and Hocking, 2009; Borrego et al., 2012)
	DNA	Rakotonirainy et al., 2007					
Penicillium commune Thom	M	Michaelsen et al., 2009 Borrego et al., 2012 Bergadi et al., 2014	Greyish turquoise to dull green conidia	Cellulases (+++) Amylases (+) Proteases (+)	>0.85	? - 30 (25)	(Samson et al., 2000; Pitt and Hocking, 2009; Borrego et al., 2012; Bergadi et al., 2014)
	DNA	Kraková et al., 2012					
	M+DNA	Sato et al., 2014					
Penicillium spinulosum Thom	M	Zerek, 2003 Lourenço et al., 2005 Zotti et al., 2007, 2008	Mycelium white; pinkish or pale Brown; Orange Brown or red brown	Cellulases (+)	>0.80	22-25	(Ruegger and Tauk-Tornisielo, 2004; Pitt and Hocking, 2009)

It is important to notice that some of the most recent studies make use of DNA analysis protocols for fungal identification, some of them in association with conventional culturing methods where identification is made through morphological characteristics (Table 4.1.). While conventional culturing methods enable the evaluation of the viability of the fungal flora, DNA analyses allow for the identification of non-culturable microorganisms (Pinheiro et al., 2011) and also cell debris which can be responsible for allergic reactions (Wlazło et al., 2008).

Almost all the fungal species presented in Table 4.1 possess cellulolytic activity. Although different studies can indicate different cellulolytic potential for a same species, this can be related to strain differences or test conditions. Besides cellulases, most species produce other enzymes that degrade paper composing or binding materials like amylases (starch decomposition) and proteases (gelatine, glues, or leather decomposition).

The great majority of fungal species presented in Table 4.1 are already able to develop at water activity levels near 0.80, which qualifies them as xerotolerants. Regarding temperature, the optimum levels for fungal growth are around the ones existent in ambient temperature, circa 20-25 °C.

Fungi can excrete acidic compounds to alter the pH of the substrate according to their development needs. Fungal growth of species such as *Aspergillus niger, A. terreus, A. ustus* and *A. versicolor, Cladosporium cladosporioides, Penicillium chrysogenum, P. citrinum* and *P. commune* have shown the ability to greatly decrease (up to 4 pH units) the pH of saline solutions initially buffered to pH 7 (Borrego et al., 2012). This reveals a significant production of acidic metabolites by the most common fungal species found on paper materials. As paper is highly susceptible to acidic hydrolysis, such acid production represents an important biodeterioration potential.

4.3.2. Selected fungal species

From Table 4.1, four fungal species were selected for subsequent antifungal testing. The criterion for selection was the highest frequency of a species within each of the most common genera, *Aspergillus*, *Chaetomium*, *Cladosporium* and *Penicillium*, for a higher representativeness. Therefore the selected species were: *Aspergillus niger* Tiegh. (*A. niger*), *Chaetomium globosum* Kunze (*Ch. globosum*), *Cladosporium cladosporioides* (Fresen.) G.A. de Vries (*Cl. cladosporioides*) and *Penicillium chrysogenum* Thom (*P. chrysogenum*).

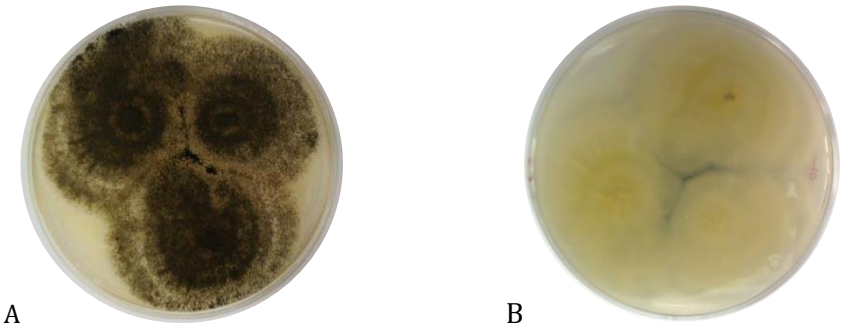
Although *A. niger* and *A. versicolour* had the same number of references, *A. niger* was the one selected since it is more often used as a test microorganism for antifungal evaluation on paper materials (Dersarkissian and Goodberry, 1980; Nittérus, 2000a; Ponce-Jimenez et al., 2002; Rakotonirainy and Lavedrine, 2005; Bacílková, 2006; Rakotonirainy et al., 2008; Afsharpour et al., 2011), which would allow for a comparison with other studies.

The fifth species selected was *Penicillium corylophilum* Dierckx (*P. corylophilum*), which although was the most frequent species in the study by Lourenço et al. (2005) regarding archival repositories in Portugal, was not common in the remaining consulted literature. This species was designated to allow for a closer comparison with the study by Neves et al. (2009) in Chapter 7A, as the same antifungal compounds were tested against *P. corylophilum*. Since a transversal comparison within the tested antifungals in this thesis was desired, *P. corylophilum* was also included in the other antifungal tests in Chapter 6, 7B and 7C.

The selected fungal species are described in greater detail below, accompanied by a photographic record at different magnification scales.

4.3.2.1. Aspergillus niger

Aspergillus niger is a filamentous ascomycete, belonging to one of the most ubiquitous genera, *Aspergillus* (Sharma, 2012). Colonies of *A. niger* are generally composed by white mycelium covered by a layer of dark brown to black conidial heads (Figure 4.1 A). The reverse is usually pale yellow (Figure 4.1 B) (Pitt and Hocking, 2009:313).



A B

Figure 4.1: *Aspergillus niger* colonies on PDA, 14 days, 22°C, (A) front and (B) reverse. Petri dish = 90mm.

Conidial heads (Figure 4.2 A) are composed of closely packed metulae, phialides and conidia over the surface of spherical vesicles (Pitt and Hocking, 2009:313). The conidia are spherical (4-5 µm) with roughened walls, as shown in Figure 4.2 B.

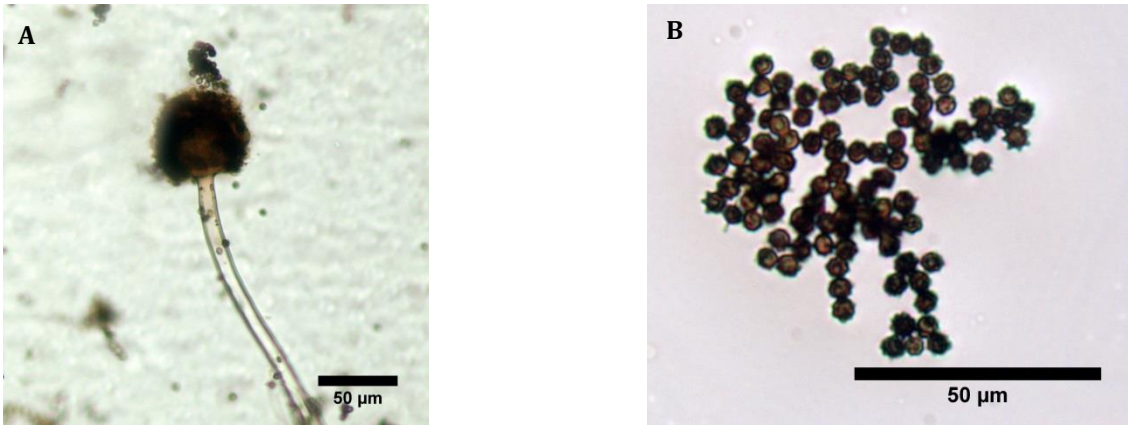


Figure 4.2: *A. niger* (A) conidiophore, (B) conidia, bars = 50 µm.

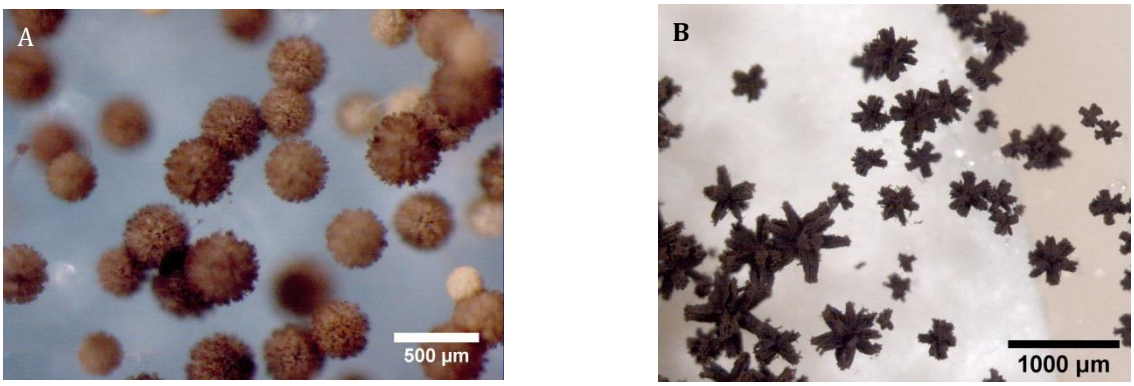


Figure 4.3: *A. niger* growing on paper over PDA (conidial heads detail), at (A) 7 and (B) 14 days of incubation, 22°C. Bars= 500 µm and 1000 µm, respectively.

With age, the initially globose conidial heads of *A. niger*, become radiate and tend to split into several loose columns (Figure 4.3) (Sharma, 2012).

A. niger is reported as a xerotolerant, being able to germinate at 0.77 a_W at 35°C and is also classified as a thermotolerant, being able to grow at temperatures up to 47°C (Table 4.1). Having an optimum growth temperature around 30-37°C, this species is not able to develop below 6°C, at refrigerator temperatures (Table 4.1).

One of the main characteristics of *A. niger* is the production of black or dark brown conidia resulting from combination of dark brown melanins and hexahydroxyl pentacyclic quinoid green pigments (Jorgensen et al., 2011). Melanins are known for their chemical and physical endurance (Butler and Day, 1998), rendering the safe removal of melanised fungal stains from paper very problematic (Nieto-Fernandez et al., 2003). Several biological functions have been proposed for these macromolecules on fungal structures, like protection against radiation (e.g. UV, gamma radiation), enzymatic lysis, high temperatures, or oxidizing agents; and also for binding of metals (preventing the entry of toxic metals or concentrating essential metals); as a virulence factor; or increasing resistance to fungicides (Butler and Day, 1998).

A. niger is an exceptionally efficient producer of organic acids like oxalic acid, citric acid, or gluconic acid, and is actually used in biotechnology industries for acid production (Schuster et al., 2002). If grown on an unbuffered medium, this species can acidify the medium to a pH below 2 (Andersen et al., 2009). *A. niger* is capable of reducing a pH 8 buffered iron gall ink growth medium to pH 1.7 (Messner et al., 1988). Numerous hypotheses have been raised on the evolutionary strategy behind this trait, like being a contribution to the degradation of cellulose by acid hydrolysis; slowing down or inhibiting the growth of competitors (a majority of rapidly growing bacteria and many fungi cannot grow below pH 3); chelation of sparse metals for assimilation on metabolic functions; iron solubilisation/translocation; or precipitation of ions that may interfere with the normal metabolic function of the fungus, like precipitation of calcium oxalate trough oxalic acid production (Jellison et al., 1997; Magnuson and Lasure, 2004; Hastrup et al., 2011). *A. niger* also produces gluconic acid from glucose as a way to store this nutrient and turn it unavailable to other competing organisms (Andersen et al., 2009).

Besides deteriorating paper by acid hydrolysis, *A. niger* is an efficient producer of paper degrading enzymes, like cellulases, amylases, gelatinases, proteases, which are able to decompose cellulose and paper additives like starch, gelatine or animal glues (Janda et al., 2009; Borrego et al., 2012; Bergadi et al., 2014).

This species is one of the three recommended for Standard Test Methods for Mildew (Fungus) Resistance of Paper and Paperboard (ASTM, 2003) and has also been used in several studies for testing the efficacy of antifungals on paper (Dersarkissian and Goodberry, 1980; Nittérus, 2000a; Ponce-Jimenez et al., 2002; Rakotonirainy and Lavedrine, 2005; Bacílková, 2006; Rakotonirainy et al., 2008; Afsharpour et al., 2011).

A. niger is not particularly problematic regarding allergies or mycopathologies when compared with other filamentous fungi (Schuster et al., 2002). Only a few strains are able to produce the mycotoxin ochratoxin A under favourable conditions (Schuster et al., 2002). This species also produces the slightly toxic naphta-γ-pyrones and more recently it was found that *A. niger* can produce the mycotoxins fumonisins (Adan and Samson, 2011:257).

4.3.2.2. Chaetomium globosum

Chaetomium is a black ascocarps producing genus, belonging to the phylum Ascomycota (Pitt and Hocking, 2009:70). These ascocarps are perithecia, which have an opening (ostiole) through which ascospores are released. Ascospores are meiospores, which contrarily to conidia (mitospores) result from sexual reproduction.

Figure 4.4: *Chaetomium globosum* colonies on PDA, 14 days, 22°C, (A) front and (B) reverse. Petri dish = 90mm. On the left: detail of perithecia *in situ*, bar= 500 µm.

Colonies of *Ch. globosum* are generally composed of white mycelium and sparse black perithecia (Figure 4.4 A). On PDA the reverse is usually orange-brown (Figure 4.4 B). Perithecia (ca. 150-200 µm) are black, with numerous dark hairs (setae) attached (Figure 4.5 A). The ascospores are smooth walled, with spheroidal, ellipsoidal or apiculate shapes, ca. 8-10 µm (Figure 4.5 B) (Pitt and Hocking, 2009:73).

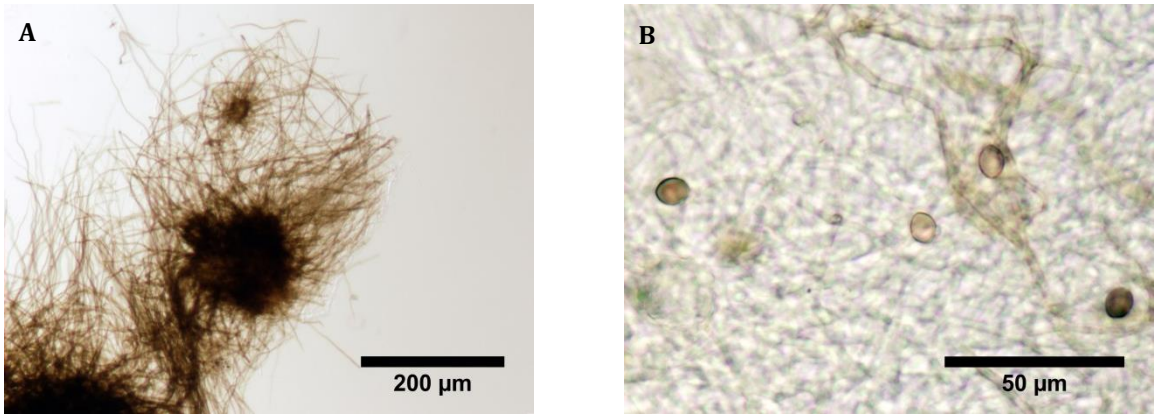


Figure 4.5: *Ch. globosum* (A) perithecia (bar = 200 µm) and (B) ascospores (bar = 50 µm).

Ch. globosum requires water activity levels above 0.90 to develop (Table 4.1), and therefore is considered a tertiary colonizer or water damage mould (Adan and Samson, 2011:246). Its heat resistance is low, with zero rate of survival of ascospores at 57°C, and its optimum growth temperature is 25°C (Pitt and Hocking, 2009:73).

This species is a remarkable producer of cellulases and is commonly found on paper and wood (Lakshmikant, 1990; Das et al., 1997; Pitt and Hocking, 2009:70). It also produces amylases and

proteases (Sharma and Shukla, 2008; Abdel-Azeem et al., 2014), capable of degrading paper additives, like starch, gelatine, or other proteinaceous glues.

Ch. globosum can grow from pH 4 to 9, although optimal growth occurs at neutral to alkaline pH (Pinzari et al., 2006; Fogle et al., 2008). Interestingly, the range of pH values at which *Ch. globosum* grows complies with the pH of paper collections (Trafela et al., 2007), and the optimal pH for growth is the one considered ideal for cellulose in terms of slowing down chemical degradation (Strlic and Kolar, 2005:6).

According to Fogle et al. (2008), sporulation of *Ch. globosum* if favoured in acidic environments, but Pinzari et al. (2010) observed that neutral to alkaline pH promoted the maturation of perithecia and production of ascospores.

Ch. globosum causes severe loss of mechanical strength on paper and produces yellow and greyish brown stains on paper, which are not possible to remove by laser treatment (Szczepanowska and Lovett Jr, 1992; Szczepanowska and Moomaw, 1994).

Like *A. niger*, *Ch. globosum* inclusion is also recommended in the Standard Test Methods for Mildew (Fungus) Resistance of Paper and Paperboard (ASTM, 2003). This species is also commonly used for testing antifungals on paper conservation studies (Pavon Flores, 1976; Rakotonirainy et al., 1999; Nittérus, 2000a; Ponce-Jimenez et al., 2002; Rakotonirainy and Lavedrine, 2005; Michaelsen et al., 2013).

Ch. globosum has been associated with sick building syndrome and is known to produce chaetoglobosins, which are highly cytotoxic metabolites inhibiting cytoplasmic division and glucose transport in tissue (Adan and Samson, 2011:254). According to Fogle et al. (2008) the optimal conditions for chaetoglobosin C production occur at a neutral pH. Colonies of this fungus should be handled with caution, as when they are dry, fungal fragments containing large quantities of chaetoglobosin could be aerosolized (Nielsen, 2003).

4.3.2.3. Cladosporium cladosporioides

Cladosporium cladosporioides is a dematiaceous (pigmented) filamentous fungus (Bensch et al., 2010) and is one of the most common airborne fungi (Zielińska-Jankiewicz et al., 2008; Flannigan et al., 2011:30).

Colonies of *Cl. cladosporioides* on PDA are grey-olivaceous to dull green or olivaceous-grey (Figure 4.6 A). The reverse is iron-grey, leaden-grey or olivaceous-black (Figure 4.6 B) (Bensch et al., 2010). The conidiophores *in situ* are dendritic (tree-like) and closely packed (Figure 4.6 A) (Pitt and Hocking, 2009:75).

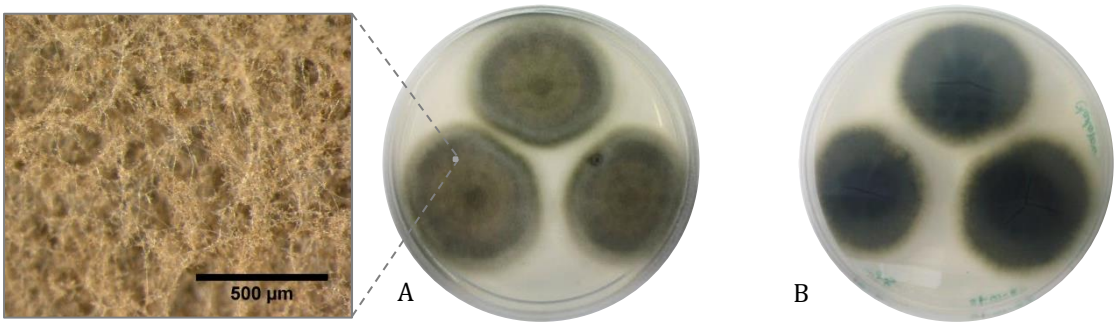


Figure 4.6: *Cladosporium cladosporioides* colonies on PDA, 14 days, 22°C, (A) front and (B) reverse. Petri dish = 90mm. On the left: detail of conidiophores *in situ*, bar= 500 µm.

Under the microscope, conidiophores bearing branched conidial chains can be observed (Figure 4.7 A). Conidia are mostly one-celled, ellipsoidal to lemon-shaped (3-7 x 2-4 µm), and mostly smooth-walled (Figure 4.7 B)(Samson et al., 2000:108).

Figure 4.7: *Cl. cladosporioides* (A) conidiophore (bar = 50 µm) and (B) conidia (bar = 50 µm).

This species is considered a secondary colonizer for growing at a minimal a_w between 0.8 and 0.9 (Table 4.1) (Adan and Samson, 2011:246).

Cl. cladosporioides is a psychrotolerant species, being able to grow at negative temperatures, and having a maximum temperature for growth at 32 °C (Table 4.1). It can produce several enzymes for paper digestion, namely cellulases, lipases and proteases (Table 4.1). It can also effectively produce acids, being able to reduce the pH of a saline solution initially buffered to pH7 to nearly 4 (Borrego et al., 2012).

This species has been used for testing antifungals in the field of paper conservation, mostly gamma irradiation treatments (da Silva et al., 2006; Mesquita, 2013:3; Michaelsen et al., 2013).

No mycotoxins have been identified and no allergenic potential has been detected from this fungus (Adan and Samson, 2011:262; Low et al., 2011). From the several metabolites excreted by *Cl. cladosporioides*, some have antifungal and/or plant inhibition properties (Nielsen, 2002:96; Adan and Samson, 2011:262; Yanjuan et al., 2013).

4.3.2.4. Penicillium chrysogenum

Penicillium chrysogenum is one of the most common indoor moulds and has been extensively studied in the last decades due to its use in the production of the antibiotic penicillin (Nielsen, 2003; Houbraken et al., 2011).

Colonies of *P. chrysogenum* are generally composed by white to yellowish mycelium with yellow green or pale green-blue conidia (Figure 4.8 A), becoming darker with age (Samson et al., 2000:194; Pitt and Hocking, 2009:235). The reverse is usually bright yellow or yellow brown (Pitt and Hocking, 2009:235).

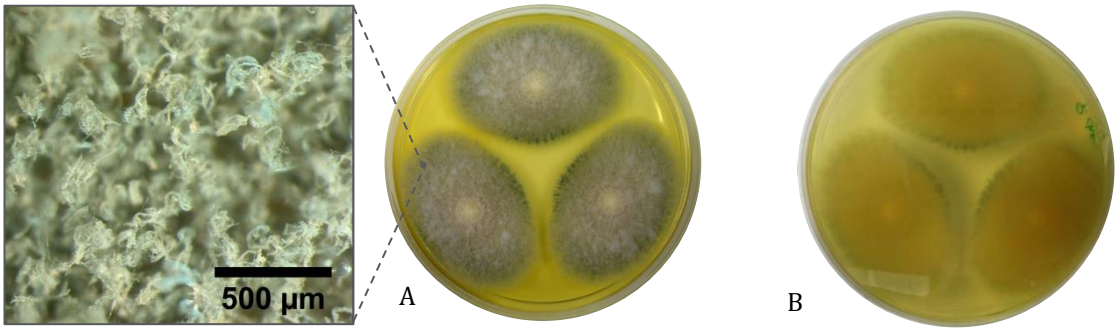


Figure 4.8: *Penicillium chrysogenum* colonies on PDA, 14 days, 22°C, (A) front and (B) reverse. Petri dish = 90mm. On the left: detail of conidiophores *in situ*, bar= 500 µm.

One of the distinctive features of this species is the production of a yellow exudate (Figure 4.9). The formation of these droplets varies with the growing stage of the colony and is more frequent at the optimum growth temperature of the fungus and at a high relative humidity (Adan and Samson, 2011:92).

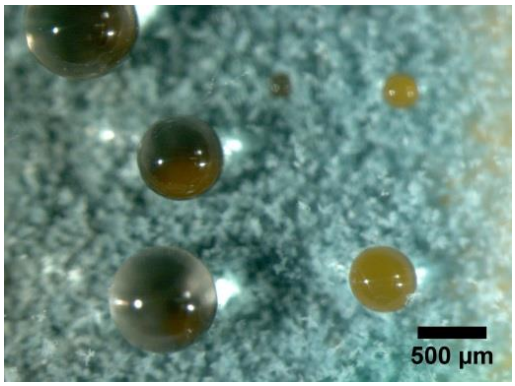


Figure 4.9: Yellow exudate on *P. chrysogenum* colony growing on paper over PDA.

In situ it is possible to observe the conidiophores' heads composed of loose columns of conidia, similar to brushes (Figure 4.8 A). The conidiophores of *P. chrysogenum* are commonly ter- to quaterverticilate (Figure 4.10 A) and the conidia (2.5 – 4 µm) are ellipsoidal to subspheroidal, smooth walled (Figure 4.10 B), produced in long irregular columns (Samson et al., 2000:194; Pitt and Hocking, 2009:235).

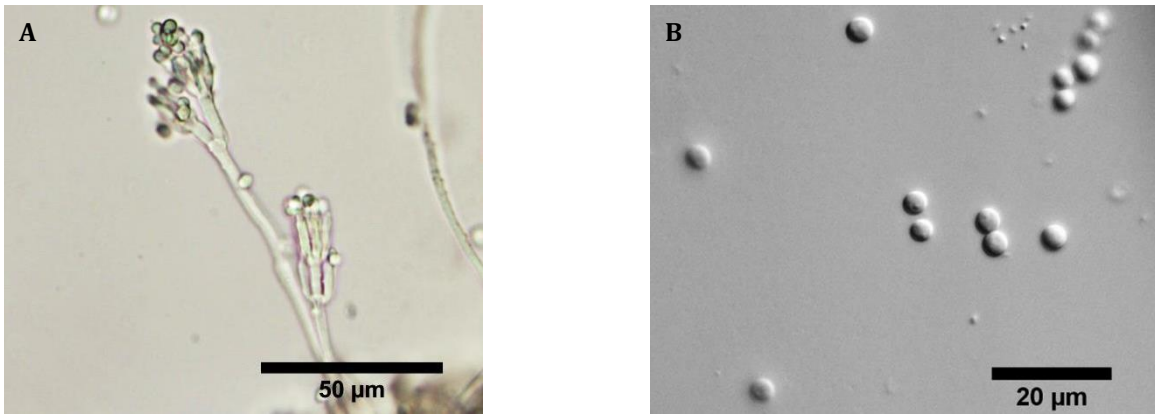


Figure 4.10: *P. chrysogenum* (A) conidiophore (bar = 50 µm) and (B) conidia (bar = 20 µm).

P. chrysogenum is considered a xerotolerant fungus and a primary colonizer or storage mould (Adan and Samson, 2011:246), as it is able to grow at a_W > 0.78 (Table 4.1). Like *Cl. cladosporioides*, this is a psychrotolerant species, being able to grow at negative temperatures, and having a maximum temperature for growth at 37 °C (Table 4.1).

Having cellulolytic, amylolytic and proteolytic activities, and being able to effectively acidify the substrate (Borrego et al., 2012), *P. chrysogenum* has a high biodeterioration potential for paper materials.

This species has been widely used in antifungal testing in the field of paper conservation (Fabbri et al., 1997; Ricelli et al., 1999; Adamo et al., 2003, 2007; Rakotonirainy et al., 2003).

P. chrysogenum has been reported to have allergenic potential by producing several antigenic proteins (Adan and Samson, 2011:196). This fungus also produces mycotoxins, namely roquefortine C, secalonic acid D, and PR toxin (Nielsen, 2003; Hocking et al., 2006:20; Adan and Samson, 2011:259). Contaminated materials should, therefore, be carefully handled.

4.3.2.5. Penicillium corylophilum

Penicillium corylophilum was the most commonly isolated species from altered paper samples in four Portuguese archives by Lourenço et al. (2005), making up 20% of a total of 260 isolates. Although this species is not common in other studies regarding isolation of filamentous fungi from fungal stains on paper materials, it has been isolated from colonies growing over cinematographic films (Rakotonirainy et al., 2016), from areas with signs of biological activity on parchment (Carvalho et al., 2016), from book bindings and document surfaces (Maggi et al., 2000; Zielińska-Jankiewicz et al., 2008) and from the air in paper collections' depositories (Lugauskas and Krikstaponis, 2004; Zielińska-Jankiewicz et al., 2008).

Colonies of *P. corylophilum* are generally coloured in blue-green, grey-green or dull green, with green, dark-green to blackish or brownish pale reverse (Figure 4.11)(Samson et al., 2000:200; Pitt and Hocking, 2009:210). Mycelium is white or light yellow (Pitt and Hocking, 2009:210).

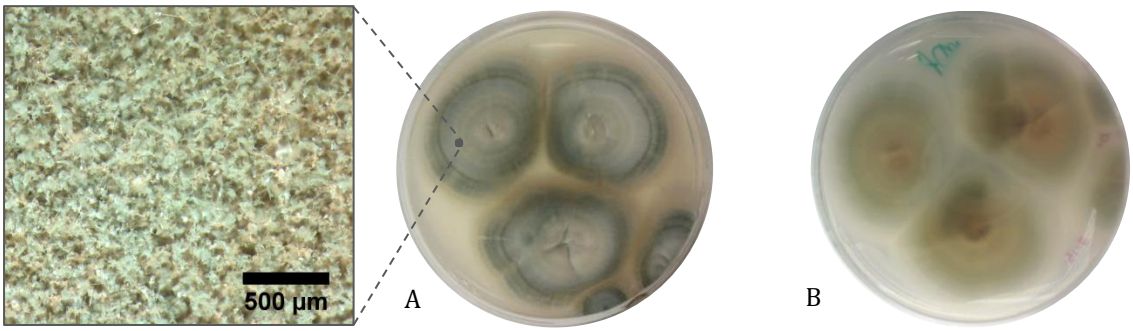


Figure 4.11: *Penicillium corylophilum* colonies on PDA, 14 days, 22°C, (A) front and (B) reverse. Petri dish = 90mm. On the left: detail of conidiophores *in situ*, bar= 500 µm.

Penicilli are mostly biverticilate or monoverticilate, with 2-5 metulae (Figure 4.12 A) and conidia are spherical to subspheroidal (2.5-3 µm) (Figure 4.12 B) (Samson et al., 2000:200; Pitt and Hocking, 2009:210).

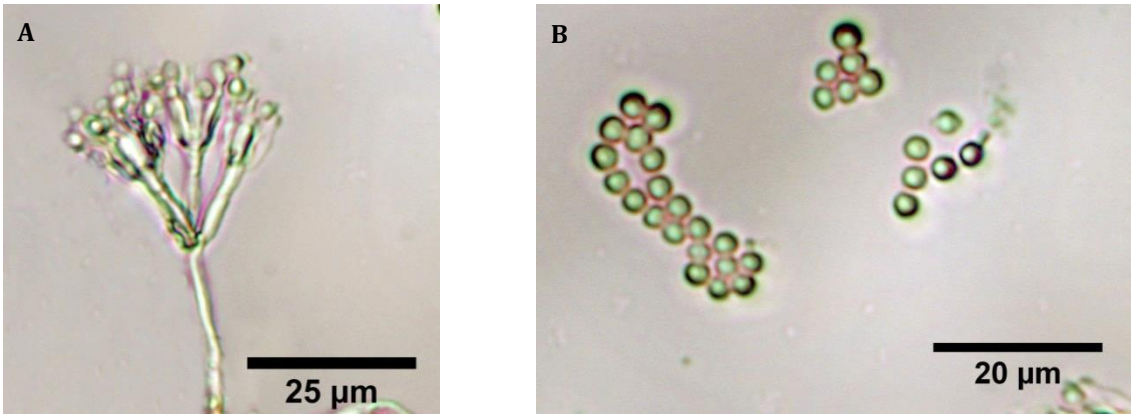


Figure 4.12: *P. corylophilum* (A) conidiophore (bar = 50 µm) and (B) conidia (bar = 20 µm).

This species is considered a xerotolerant, being able to germinate at 0.80 a_w and growing at 0.85 a_w (Pitt and Hocking, 2009:210). The minimum and maximum growth temperature for this fungus are, respectively, -2°C and 37°C (Lowry and Gill, 1984; Pitt and Hocking, 2009:210).

P. corylophilum is able to grow in acidic and alkaline substrates (pH 3 to 9), although its growth and conidiation are reduced below pH 6 (Sacks et al., 1986; Marín et al., 2002).

Having cellulolytic and proteolytic activity (Sharma et al., 1995; Ruegger and Tauk-Tornisielo, 2004; Silva et al., 2013), *P. corylophilum* has the ability to decompose paper substrates. This fungus has been used for testing antifungals in the field of paper conservation by Neves et al. (2009).

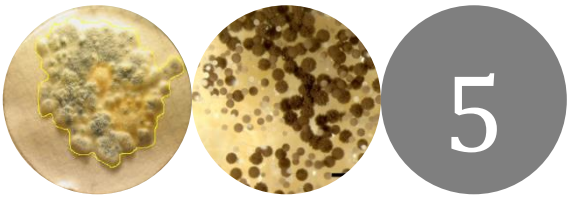
P. corylophilum has shown to produce metabolites with antibacterial activity (Garcia Silva et al., 2004) and no mycotoxins are known to be produced by this species (Pitt and Hocking, 2009:211).

4.1. Conclusions

The main objective of this chapter was a proper selection of fungal species to test in the subsequent experiments with antifungal compounds on paper samples (Chapters 6 and 7).

A detailed review of several studies concerning the identification of fungi from paper materials allowed for a more informed choice of the most common and representative fungal species. From the four genera with higher representativeness, *Aspergillus*, *Cladosporium*, *Chaetomium* and *Penicillium*, the four species more frequently identified were selected - *Aspergillus niger*, *Chaetomium globosum*, *Cladosporium cladosporioides* and *Penicillium chrysogenum.* The fifth selected species, *Penicillium corylophilum* was designated taking into account its high ocurrence in Portuguese Archives and also because it was used in one of the main antifungal studies with which a future comparison was desired.

A more profound insight into the characteristics of the selected species revealed that all of them possess paper deterioration abilities, like cellulolytic, amylolytic or proteolytic activities, or production of acidic compounds. Also, most of the selected species are xerotolerants, being able to germinate at water activity values near 0.80. All of the selected fungi have already been used in antifungal testing in the field of paper conservation, which allows for future comparisons.



Quantifying fungal growth on paper

Part of this chapter has been accepted for publication as: Sequeira, S. O., Coutinho, M. L., Lima, J. C., Phillips, A. J. L., Macedo, M.F., Cabrita, E. J., '4-MUF-NAG for fungal biomass determination: scope and limitations in the context of biodeterioration studies'. Jounral of Cultural Heritage (in press). DOI: 10.1016/j.culher.2016.06.002.

A reliable fungal growth quantification method is essential for a proper evaluation of antifungal treatments. Quantification of filamentous fungi on paper is a rather complex task, due to the multicellular character of these microorganisms and opacity of the substrate, among other aspects.

From the existing fungal quantification methods, three of the most promising ones were evaluated: a fluorometric method consisting in the detection of β-N-acetylglucosaminidase (NAGase) activity; measurement of colonized area using digital image analysis; and biomass dry weight determination.

The NAGase detection method revealed several limitations which compromised its use for the intended purposes of antifungals evaluation. The correlation between fluorescence and biomass differs according to fungal species and stage of growth. Therefore, a measurement of fluorescence may not be indicative of a specific value of biomass. Also, false positive and negative results for fungal growth can be obtained.

The measurement of colonized area demonstrated to be a simple, non-destructive method, with high sensitivity and high correlation with biomass dry weight. The optimization of the illumination technique improved the detection and quantification of minute fungal colonies.

Biomass dry weight determination although being a destructive method and having less sensitivity than the previous method, is more appropriate for quantification of aged cultures and takes into account the density of fungal colonies, rather than just their extent.

5.1. Introduction

The study of fungal biodeterioration of cultural heritage often requires the quantification of fungal growth to assess contamination levels or bio-susceptibility/resistance of a certain material or conservation treatment (Valentin, 1986). In this thesis it was necessary to determine the antifungal efficacy of various formulations intended to treat biodeterioration on paper artefacts by filamentous fungi. To do so, the adoption of a proper method for measurement of fungal growth was crucial.

The growth of filamentous fungi is not easy to quantify since, unlike yeasts and bacteria, these are multicellular organisms and therefore cannot be quantified using the common cell

enumeration techniques. Filamentous fungi can differentiate to produce unicellular spores, however the quantification of colony forming units (CFU) is rather an indication of the degree of sporulation than of actual biomass production (Marín et al., 2005).

In solid opaque substrates, like paper, and in most organic materials from which cultural heritage objects are made, fungal mycelium penetrating the substrate is not visible to the naked eyed, thus restricting the use of colony diameter quantification techniques. Also, since the mycelium penetrates solid substrates, its extraction is difficult or even impossible, when performing biomass dry weight quantification.

Taking into account these difficulties, several chemical and biochemical techniques have been developed to estimate the extent of fungal growth in solid substrates. The developed techniques usually rely on the quantification of chemical markers found in fungi, like in cell membranes (ergosterol, 18:2ω-phospholipid fatty acids) or cell walls (chitin, β-glucans), intracellular biochemical components (Adenosine triphosphate - ATP), metabolic compounds (fungal volatiles, fungal antigens, β-N-acetylglucosaminidase), or on molecular biology techniques (qRT-PCR) (Nielsen and Madsen, 2000; Pitt and Hocking, 2009). The most prominent methods are briefly described below.

Ergosterol (ergosta-5,7,22-trien-3β-ol) is a predominant cell membrane component of most filamentous fungi and yeasts that is absent from bacteria, plant or animal cells, although it can also exist in green algae (Miller et al., 2012) and amoeba (Smith and Korn, 1968). Ergosterol quantification, mainly used for fungal quantification in the food industry, has also been used in the cultural heritage field (Fabbri et al., 1997; Adamo et al., 2003). Ergosterol extraction from fungal cells can be made by different methods (Padgett and Posey, 1993) and analysis is usually performed by chromatographic techniques, mainly High Performance Liquid Chromatography (HPLC) (Dambolena et al., 2008; Porep et al., 2014).

Positive correlations between amount of ergosterol and fungal biomass have been obtained by several authors (Reeslev et al., 2003; Marín et al., 2005; Porep et al., 2014). Also, the relative chemical instability of ergosterol after cell death may provide a good quantitative measure of viable cells when combined with total biomass quantification methods (Ekblad et al., 1998). However, the amount of ergosterol in fungi can vary within fungal species, isolates of the same species, and even among the same strain depending on the growth medium, incubation time and physiological state (Charcosset and Chauvet, 2001; Gessner and Newell, 2002:396; Taniwaki et al., 2006), which limits the direct use of ergosterol as a means of evaluation and comparison of fungal growth.

Chitin, a polymer of N-acetyl-D-glucosamine, is the second main cell wall component of fungi (Borkovich and Ebbole, 2011). Although it also occurs in the exoskeleton of insects, it is absent from bacteria. Quantification of chitin can be used for estimation of fungal contamination. Still, the extraction and analysis of chitin is a complex and slow process, the relationship between dry weight and chitin content varies for different fungal species, and chitin does not increase proportionally with fungal growth (Pitt and Hocking, 2009:35).

ATP is a coenzyme that is responsible for intracellular energy transfer operations in living cells. After cell death, ATP stops being produced and is readily degraded by ATPases (Rakotonirainy et al., 2003). This characteristic makes ATP analysis a good indicator of viability in microorganisms and can be used to quantify viable biomass (Suberkropp et al., 1993). ATP analysis is not fungi

specific since ATP is present in all living cells. Therefore, the type of organisms to quantify has to be the only one present in the analysed sample. Rakotonirainy et al. (2003) have adapted ATP analysis protocols to cultural heritage as a non-destructive analysis of fungal spores viability. As a disadvantage of this method, the extraction of molecules from fungal cells is notoriously difficult, and only viable biomass rather than total biomass is detected (Pitt and Hocking, 2009:37).

Quantitative real-time Polymerase Chain Reaction (qRT-PCR) is a molecular biology technique that has proven to successfully quantify bacteria (Furet et al., 2004). In yeast or bacteria, which are unicellular, one molecule of DNA is representative of one cell, and conversion of DNA quantity to biomass is possible. However, in growing filamentous fungi, biomass is mostly composed of multicellular hyphae, which vary considerably in cell length and cell volume, and also in the number of nuclei and DNA amount per cell, making it impossible to determine a universal factor to convert DNA quantities into fungal biomass (Guidot et al., 2002). For these multicellular microorganisms, standard curves need firstly to be constructed for each fungal species and each growth model (Guidot et al., 2002; Furet et al., 2004). qRT-PCR has already been tested in cultural heritage by Martin-Sanchez et al. (2013), who were able to detect and quantify *Ochroconis lascauxensis* in the Lascaux Cave in France. The high assay costs involved in this kind of methodology and the need of a previous thorough calibration procedure may limit the use of this method.

Another recently used molecular biology technique for fungal detection is the analysis of ribosomal RNA (rRNA). This method can be used as a fungal viability indicator, based on the fact that metabolically active cells transcribe more rRNA for ribosome synthesis than inactive cells. Michaelsen et al. (2013) have applied the analysis of fungal rRNA as an indicator of long-term fungal viability in paper-based collections after treatment with different antifungal methods. Besides the high assay costs related to molecular biology techniques, rRNA analysis is rather an indication of fungal viability than on actual fungal biomass.

Among the present methods for fungal quantification based on fungal chemical markers, the one that seemed more suitable for the present study on antifungal compounds for paper was the one where the activity of β-N-acetylglucosaminidase (NAGase) was quantified. According to Konkol et al. (2012), this method permitted the detection of minute fungal growth, even before it could be detected by the naked eye. Also, this method combined high quantification sensitivity with speed and simplicity of analysis (Krause et al., 2003; Reeslev et al., 2003; Konkol et al., 2010). These characteristics would be very useful for the present study, and so, the application of this method was tested. The results are presented below in Section 5.2 – "Fungal growth measurement by NAGase activity quantification".

Additionally, the optimization of common fungal growth quantification techniques like diameter measurements and mycelium dry weight estimation was performed and the results are presented in Section 5.3 "Optimization of colony and dry biomass measurement techniques for fungal growth measurement on paper".

5.2. Fungal growth measurement by NAGase activity quantification

Measurement of fungal growth through quantification of NAGase activity was initially used for soil and building materials (Miller et al., 1998; Krause et al., 2003; Reeslev et al., 2003). More

recently it was tested for detection and estimation of *Aspergillus niger* biomass on cultural heritage materials (Konkol et al., 2010, 2012).

NAGase is an enzyme that catalyzes the hydrolysis of terminal non-reducing N-acetylglucosamine (NAG) residues in chitin oligomers (Díez et al., 2005).

The cell wall of fungi is composed mainly by chitin, a homopolymer of NAG residues. In filamentous fungi, NAGase is expected to be secreted at all stages of active growth, as chitin-degrading enzymes are thought to contribute to a number of morphogenic processes including spore germination, branch formation, and autolysis (Gooday et al., 1992). For instance, in the rigid cell wall of a hypha, the site of formation of a new branch must be weakened with chitinolytic enzymes to allow the apex to be formed (Gooday et al., 1992).

The measurement of NAGase activity involves the use of the fluorogenic substrate 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide (4-MUF-NAG), a molecule composed of a chitin monomer (NAG) and a 4-methylumbelliferone (4-MUF) molecule. At pH values around 5 (the optimum pH for enzymatic activity), the NAGase secreted by active fungi will assume the NAG part of 4-MUF-NAG as a terminal NAG residue in chitin, therefore hydrolyzing the synthetic substrate and liberating the fluorescent 4-MUF (Figure 5.1). The fluorescence intensity is in this way correlated with enzymatic activity, and consequently, to the quantity of active enzymes excreted by the fungi, which is expected to be proportional to fungal growth.

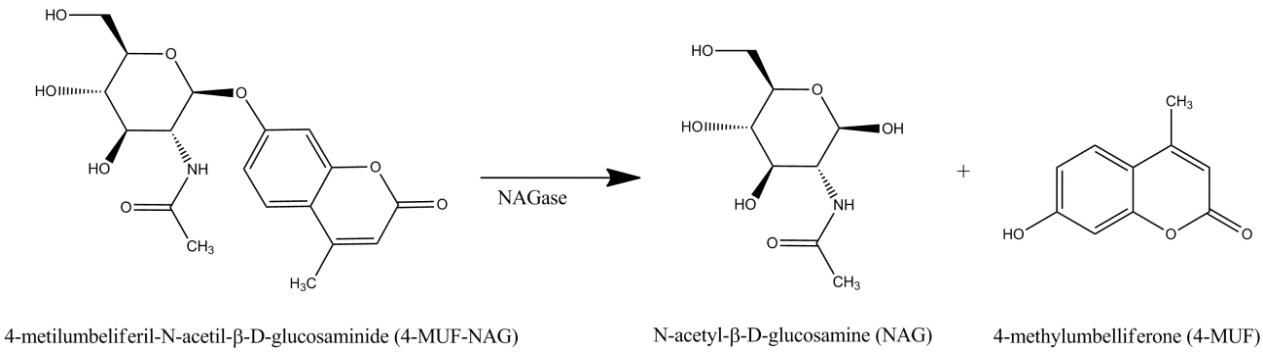


Figure 5.1: Scheme of 4-MUF-NAG substrate hydrolysis by NAGase.

To relate fluorescence with fungal biomass, a calibration curve must be developed first. Konkol et al. (2010, 2012) found a linear correlation between fungal dry biomass and NAGase activity for *Aspergillus niger*, concluding that a specific fluorescence intensity of 4-MUF would be indicative of a particular biomass value in paper samples. Reeslev et al. (2003) found a linear correlation between NAGase activity, ergosterol content and fungal biomass for two fungal species. Miller et al. (1998) on the other hand, found a weak correlation between NAGase activity and ergosterol content (R^2 = 0.36), a moderate correlation between NAGase activity and 18:2ω6 phospholipid fatty acid (a cellular component used as a fungal biomarker) content (R^2 = 0.61), and from the 21 fungal species tested, 14% did not reveal any fluorescent response signaling NAGase activity.

The technical aspects of the photochemistry behind this process, namely why using the 377 nm excitation wavelength, why 4-MUF-NAG does not fluoresce at 446nm while 4-MUF does, and why is there a need to raise the pH to 10 before analysis of fluorescence like described in Konkol et al. (2010, 2012) are presented in greater detail in Appendix II.

The main purpose of the present study was to evaluate the potential and the limitations of the 4-MUF-NAG method for quantification of fungal biomass in paper biodeterioration studies. Firstly it was necessary to understand if 4-MUF fluorescence values would always correspond to a specific biomass for diverse fungal species and at different stages of growth. If this first premise was not satisfied, it was important to understand why, and in what way it could influence the obtained results. To answer to these questions, three experiments were made:

- First experiment: the protocol for the standard curve development, described by Konkol et al. (Konkol et al., 2012), was reproduced, by testing only one fungal species (*Aspergillus niger*) and one incubation period. Different methods of preparation and separation of the fungal suspension were evaluated to select the best one and determine the presence of NAGase in the growth medium.
- Second experiment: The preparation method selected in the first experiment was used to develop standard curves for three different fungal species at shorter incubation periods (younger cultures).
- Third experiment: The preparation method was adapted to analyse younger cultures. Four fungal species were analysed at four different incubation periods (shorter and longer than the one used in the first experiment) to verify the correspondence between 4-MUF fluorescence and biomass for diverse fungal species at different stages of growth.

5.2.1. Materials and methods

5.2.1.1. Fungal cultures

Ch. globosum, *Cl. cladosporioides* and *P. chrysogenum* strains were obtained from the mycological collection of Universidade do Minho (Braga, Portugal) and *A. niger* strain was obtained from the collection of Instituto Nacional de Saúde Doutor Ricardo Jorge (Lisbon, Portugal). Fungal strains were plated on Potato Dextrose Agar (PDA) medium and incubated at 22°C for 5 (*A. niger* and *P. chrysogenum*) or 15 days (*Cl. cladosporioides* and *Ch. globosum*). Conidia were harvested in sterile 0.05% Tween 80 (Panreac) by pipetting 100 µl volumes of this solution onto the surface of colonies and collecting the spore suspension with a pipette. For *Ch. globosum*, an extra step to release the spores from the perithecia and asci was required, which consisted of a gentle grinding with a sterile glass rod. The concentration of spore suspension for each fungal species was determined with a haemocytometer and adjusted to $1x10^6$ spores/ml. For the following experiments 100 µl of spore suspensions were used as inoculum.

All fungal cultures were incubated in liquid 1.5% malt extract growth medium (Scharlau, Spain) at 22°C.

For the first experiment *A. niger* was chosen since this was the fungal species used by Konkol et al. (2010, 2012). Two separate cultures of *A. niger* were grown in 250 ml volumes of growth medium in Erlenmeyer flasks (500 ml) fitted with cotton wool plugs and incubated for 16 days.

For the second and third experiments with various fungal species (*A. niger, Ch. globosum, Cl. cladosporioides* and *P. chrysogenum*) and different incubation periods, individual fungal cultures were grown in 50 ml of growth medium in 100 ml flasks. Blank control samples were kept uninoculated. As the biomass determination used for calibration is a destructive technique, different samples had to be prepared for analysis at each incubation period. Samples of uninoculated growth medium were used as blank controls.

5.2.1.2. Preparation for enzymatic activity measurement

5.2.1.2.1. First experiment: One fungal species, one incubation period

For the first experiment testing one fungal species (*A. niger*) and one incubation period (16 days), the protocol described by Konkol et al. (2012) was reproduced with minor changes (Figure 5.2). After harvesting the fungal mycelium by filtration through nitrocellulose membrane filters (Whatman, 0.45 µm pore diameter) and re-suspending it in 50 mM Tris Maleate (pH=5), instead of using a homogenizer to homogenize the fungal pellets, different types of fungal suspensions preparation were tested to evaluate the influence of the presence of fungal cells and/or its disruption on the liberation of the enzyme. Also, samples from the culture medium were collected to evaluate if the enzyme would pass through the nitrocellulose filters.

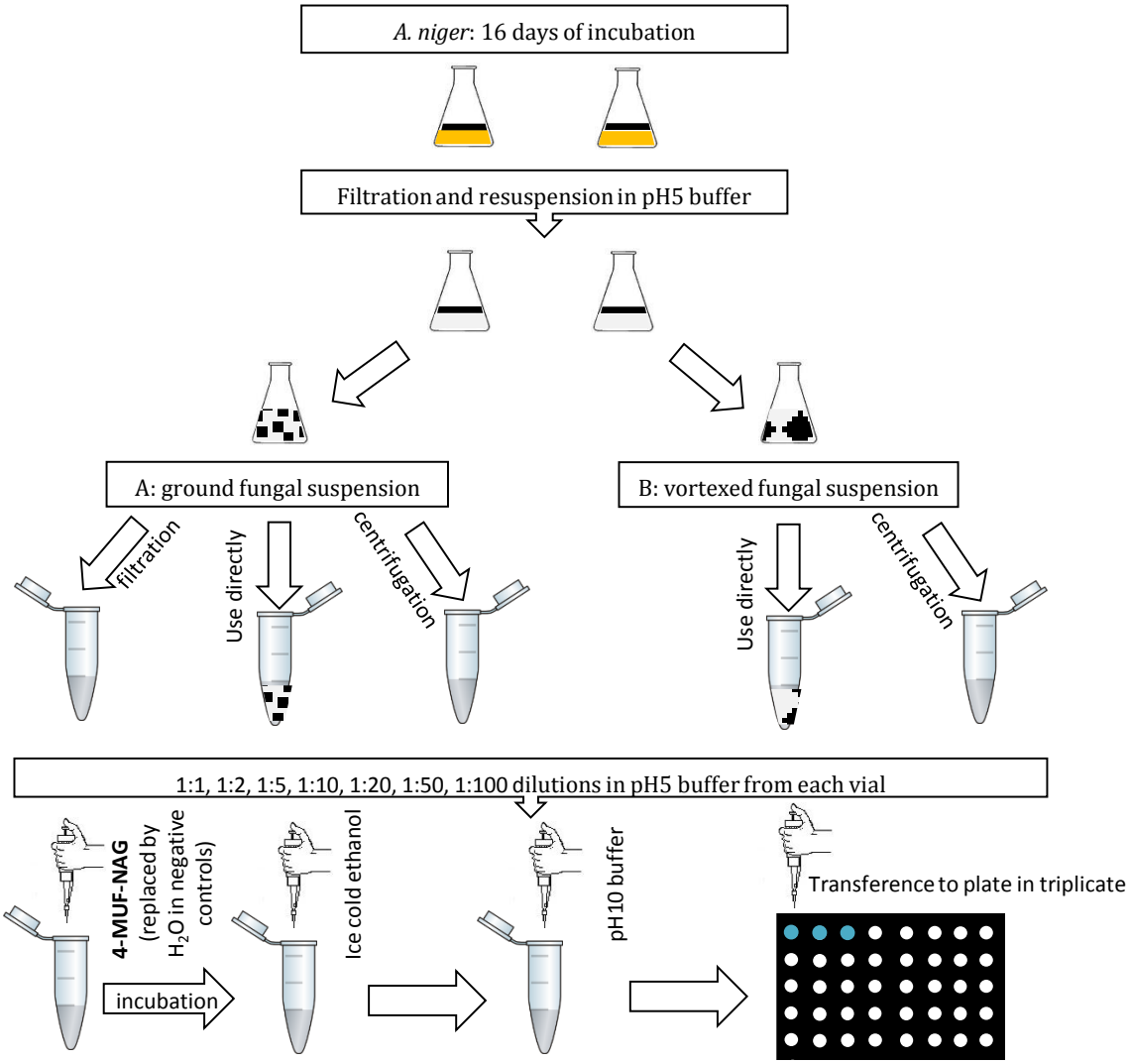


Figure 5.2: Diagram of experimental design used for the study of different preparation methods (first experiment).

Of the two prepared *A. niger* cultures, one (Culture A) was ground with a blender and the other (Culture B) was vortexed vigorously for 60 seconds. The homogenized Culture A was divided into three volumes (Figure 5.2). One volume was centrifuged and the supernatant collected. The second volume was filtered through Whatman #1 paper filter and the filtrate collected. The third volume was directly used without further purification.

For Culture B the same procedure was performed, except the filtration variant was omitted, since the difference between filtration and centrifugation would already be analyzed with Culture A (Figure 5.2). Several dilutions (1:1, 1:2, 1:5, 1:10, 1:20, 1:50, 1:100) were made (in triplicate) from each fungal/enzymatic suspension for correlation between fluorescence and different fungal biomass values. To raise the pH before analyzing the fluorescence, instead of using 30 µl of 2.5 M Tris-HCL (pH 10) like described by Konkol et al. (2012), 200µl were used, as this was the quantity needed to increase the pH of the solution to 10, the pH by which all the 4-MUF liberated in solution will be in the anionic form (Moriya, 1983; Seixas de Melo and MaCanita, 1993) (See Appendix II).

Negative control samples were prepared from the lowest dilution (1:1) where in replacement of 4-MUF-NAG, the correspondent volume (13 µl) of Millipore water was added, to verify the fluorescence from any other components (Figure 5.2).

5.2.1.2.2. Second experiment: three fungal species, three incubation periods

Flasks with single cultures of *A. niger*, *Ch. globosum* and *P. chrysogenum*, and also blank control samples (uninoculated) were prepared in triplicate. After 2, 4 and 8 days, one flask of each culture was removed from the batch and assayed for enzymatic activity (Figure 5.3). For enzymatic activity measurement, the same protocol described in 5.2.1.2.1 was used. For preparation of the fungal suspensions, the method giving the best results (see 5.2.2.1) was used.

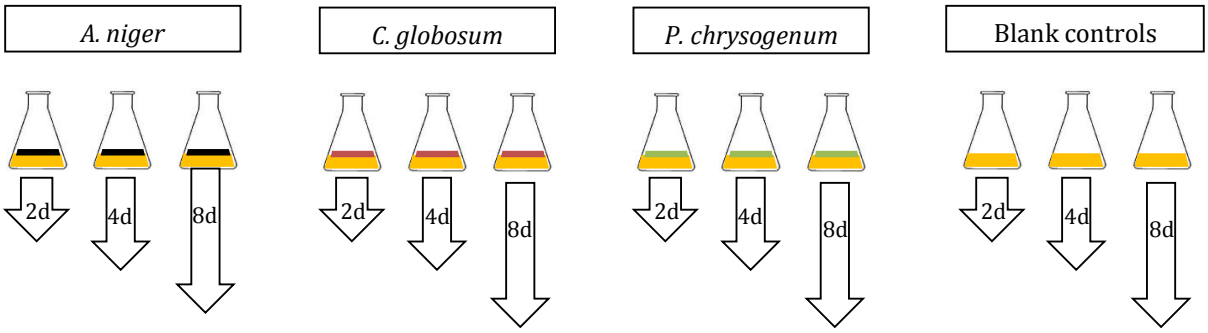


Figure 5.3: Diagram of experimental design used for the study of three different fungal species and three incubation periods (second experiment).

5.2.1.2.3. Third experiment: four fungal species, four incubation periods

Flasks with single cultures of *A. niger*, *Ch. globosum*, *Cl. cladosporioides* and *P. chrysogenum,* and blank control samples (uninoculated) were prepared in quadruplicate. After 3, 7, 14 and 42 days of incubation, one flask of each fungal species culture and a blank control flask were removed from the batch and assayed for enzyme activity (Figure 5.4). Instead of filtering the fungal material and re-suspending it in buffer solution, like in the previous experiments, the growth medium was directly analyzed (see section 5.2.2.2) as stated in the literature (Sigma-Aldrich, 2013).

After vortexing the culture for 60 seconds, 4 ml of growth medium were harvested and centrifuged at 10,000 x g for 2 minutes to separate cells and debris from the medium. The supernatant was collected and the pH adjusted to 5 (the optimal pH for NAGase enzyme activity) with the addition of 50 mM Tris Maleate (pH=5). Six 130 µl replicates of the pH=5 main solution and four dilutions (to a maximum of a 1:100 proportion of the supernatant) were distributed in Eppendorf tubes and 13 µl of 200 µM 4-MUF-NAG (Sigma-Aldrich) were added to three of the six replicates, which were then vortexed briefly, and incubated at 25°C for 30 min. For the other three replicates, 13 µl of Millipore water was added instead of 4-MUF-NAG to constitute a negative control. After incubation, 130 µl of ice cold ethanol was added to stop the reaction, followed by brief vortexing. The pH was then raised with the addition of 200 µl of 2.5 M Tris-HCL (pH 10) and the mixtures were briefly vortexed before analyzing. Figure 5.4 presents the experimental design of this experiment.

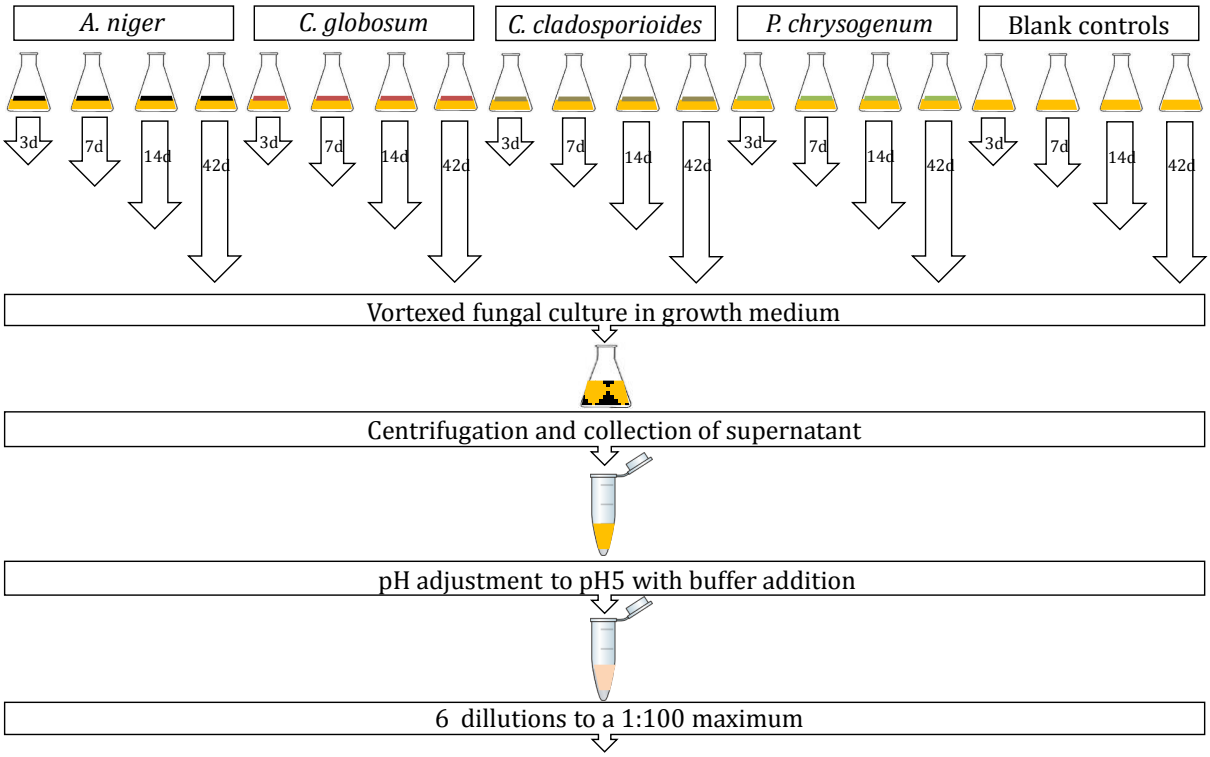


Figure 5.4: Diagram of experimental design used for the study of four fungal species and four incubation periods (third experiment).

5.2.1.3. Measurement of fluorescence

In all experiments, 100 µl of each reaction mixture were transferred to a 96-well microplate (U-bottom black immunoGrade™ BRANDplates®). Fluorescence was measured using fiber-optics with a Fluorolog® Horiba Jobin Yvon at 366 nm excitation (wavelength of maximum absorbance measured for the 4-MUF anion in the solvent mixture used in these assays) and 448 nm emission (wavelength of maximum fluorescence emission measured for the 4-MUF anion in the solvent mixture used in these assays, when excited at 366 nm) (see Appendix II).

The average fluorescence of the three replicates from each dilution was calculated and plotted against the negative control samples (without 4-MUF-NAG) to evaluate the contribution of any fluorescence originating from the growth medium itself and/or from any metabolic fungal excretions.

5.2.1.4. Measurement of biomass

To calculate the dry biomass, fungal cultures were vacuum filtered through pre-weighed nitrocellulose filters (Whatman, 0.45 μm pore diameter) and dried at 65°C for 24 hours, followed by 24 hours in a desiccator with silica gel before being weighed again.

5.2.1.5. Treatment of data

To compare fluorescence intensities obtained in the different assays at different days, which could vary with the spectrometer lamp intensity, a normalization procedure was done. The fluorescence of commercial 4-MUF (97%, Sigma-Aldrich, USA), in concentrations that could occur through the enzymatic decomposition of the added quantity of 4-MUF-NAG, was used as a calibration curve to normalize the readings in each assay. A normalization factor was calculated from the division of the slope from the linear regression equation of the 4-MUF calibration curves obtained in each assay by $1x10^6$ (as the obtained slopes generally had this order of magnitude). The values of fluorescence intensity obtained for the samples were then divided by that normalization factor.

Statistical analyses (one-way analysis of variance ANOVA and multiple regression analysis) were performed using a significance level of 0.05, with Statistica software v12 (StatSoft, Inc., 1984–2013, Tulsa, OK, USA).

5.2.2. Results and discussion

5.2.2.1. First experiment: Study of different fungal suspension preparation methods and presence of NAGase in the growth medium

The results of the first experiment concerning one fungal species, one incubation period and two methods of preparation of fungal suspension (grinding or vortexing) are shown in Figure 5.5.

The values of fluorescence in all preparation methods (Figure 5.5) tend to level out at higher biomass values. Since fluorescence is not leveling at the maximum intensities that can be obtained from the total hydrolysis of the amount of added 4-MUF-NAG (c. $5.82x10^6$ fluorescence, according to the developed calibration curves), this could be due to enzymatic inhibition or fluorescence quenching caused by fungal debris.

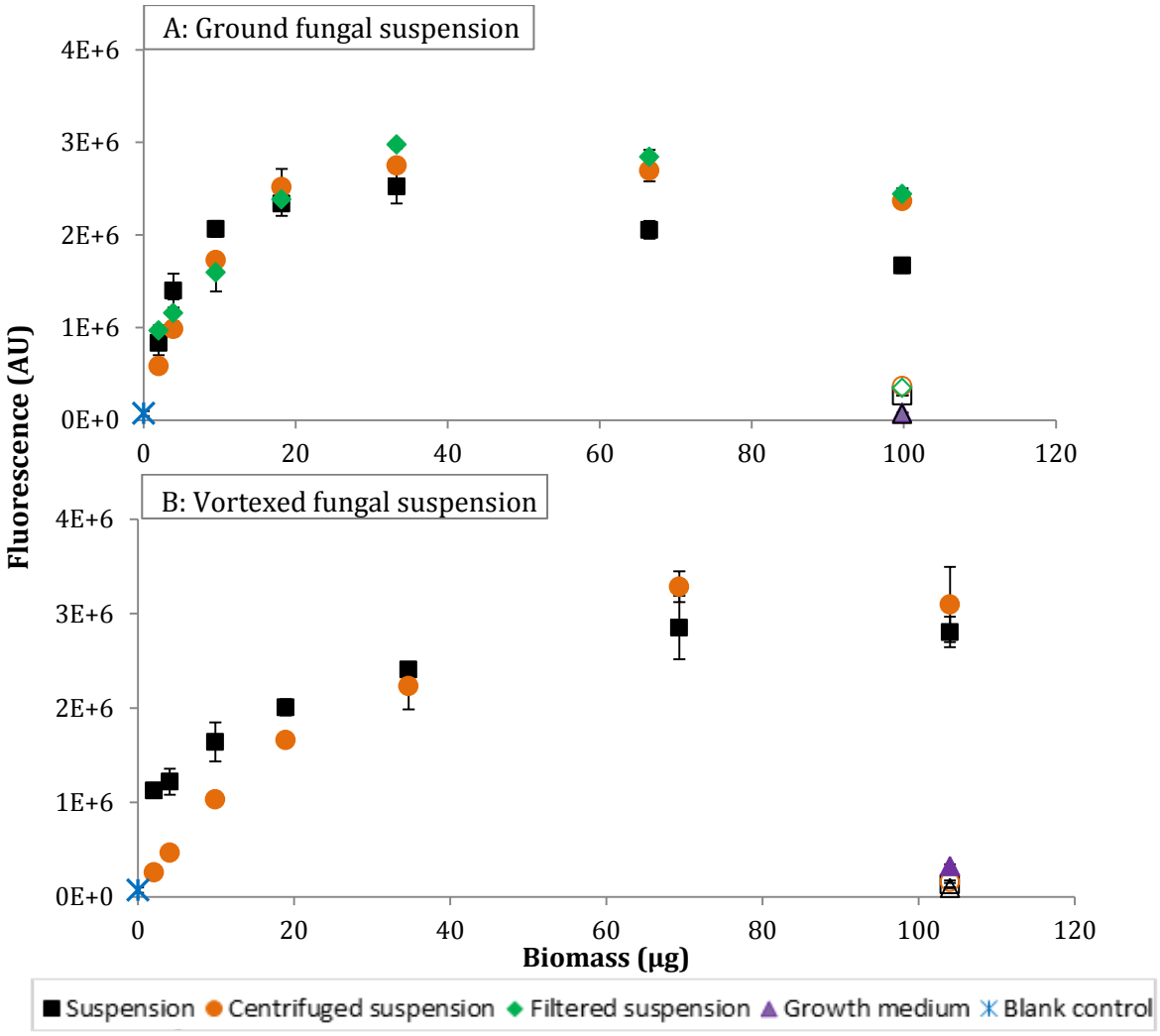


Figure 5.5: Biomass dilutions versus fluorescence for *A. niger* at 16 days of incubation. A: Ground suspension; B: Vortexed suspension. Hollow symbols indicate control samples without 4-MUF-NAG. Error bars = standard deviation of three replicates.

In Figure 5.5 A, where the fungal suspension was ground, the fluorescence decreases at the highest biomass values, for all three preparation methods. The preparation method that exhibits a higher gradient in the correlation between biomass and fluorescence is the one where the fungal culture was vortexed and centrifuged (Figure 5.5 B). These results indicate that NAGase is excreted into the growth medium and disruption of the fungal cells does not aid in the detection of its enzymatic activity. In fact, the presence of fungal debris actually diminished the obtained fluorescence at higher concentrations.
The preparation method exhibiting the best results - vortexing followed by centrifugation - was therefore used in the following experiments.

Figure 5.5 also shows that the fluorescence of the growth media from cultures A and B was approximately at the same low levels ($6x10^4$ – $4x10^5$) as the blank controls (without fungi) and as the negative controls (without 4-MUF-NAG). These results point out to the retention of NAGase by the membrane filter used in the fungal suspension preparation (see section 5.2.1.2.1).

5.2.2.2. Second experiment: testing three different fungal species at three incubation periods

In the second experiment, *A. niger* together with *Ch. globosum* and *P. chrysogenum* were tested at three shorter incubation periods than in the first experiment. This assay was aimed at developing calibration curves for diverse fungal species and relating them with the period of incubation.

Fungal growth was clearly visible in the test flasks and biomass values increased with incubation, however, very low fluorescence values were obtained for all fungal species in the tested periods. In the followed protocol, before analysis, the fungal cultures were collected from their growth media by filtration and resuspended in pH5 buffer. To verify if NAGase was being effectively collected on the filtration step, the growth medium left after filtration of the fungal suspensions was sampled and analysed. As shown in Figure 5.6, the fluorescence obtained from the growth medium was indeed higher than on the filtrated samples, especially for *Ch. globosum*.

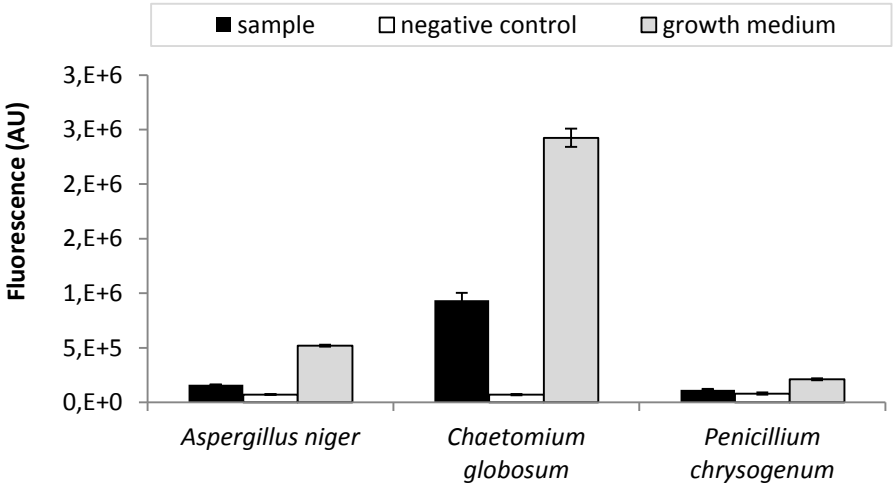


Figure 5.6: Fluorescence measured in filtered sample, negative controls (samples without 4-MUF-NAG) and growth medium, for *A. niger* (36µg biomass), *Ch. globosum* (19µg biomass) and *P. chrysogenum* (37µg biomass), at 8 days of incubation.

Since the same type of filters and filtration method were used in first and second experiments, it can be inferred that the 0.45 µm pore filters used to collect the fungal cultures do not retain NAGase and consequently only the enzyme retained in the fungal culture pellet is analyzed after re-suspension in the buffer. When fungal colonies develop a mycelial mat that occupies the entire surface of the liquid growth medium, like in the first experiment, the mat formed may itself function as a biological filter and the enzymes released are retained above that mat.

In the Sigma-Aldrich protocol for their "Chitinase assay kit" using 4-MUF-NAG, the culture medium is directly sampled from the growing culture, followed by centrifugation to remove the microorganisms and debris (Sigma-Aldrich, 2013). This protocol was therefore adapted in the following experiment to prevent the loss of NAGase in the initial filtration step.

5.2.2.3. Third experiment: testing four fungal species at different incubation periods

In the third experiment, the protocol for preparation of the fungal suspension used in the previous experiments, had to be adapted to prevent the loss of NAGase on the filtration process.

Besides *A. niger*, *Ch. globosum* and *P. chrysogenum,* an additional species, *Cl. cladosporioides*, was added in this assay to increase the range and representativeness of fungal species. Also, four periods of incubation, both shorter and longer than the one used in the first experiment were tested to analyse the differences occurring in younger and older cultures.

5.2.2.3.1. pH adjustment

Since the samples were directly collected from the culture medium, without previous filtration and resuspension in pH5 buffer, an extra step of pH adjustment had to be performed. The adjustment to pH=5 by adding Tris Maleate buffer was essential to enable the comparison between enzymatic activities from different fungal species, since enzymatic activities are pH dependent. This step required dilution of the mother (initial) solutions.

While for *Ch. globosum*, *Cl. cladosporioides* and *P. chrysogenum* a maximum of 1:5 dilutions were sufficient to attain pH5, *A. niger* cultures required much higher dilutions (up to 1:60 sample : buffer proportions), as shown in Table 5.1. After such high dilutions, the assayed biomass values for *A. niger* were much lower than the other species at all incubation periods.

Table 5.1: pH of mother solution (growth medium after incubation without further purification) and consequent dilution to obtain pH=5 for each fungal species after 14 and 42 days of incubation.

	14 days of incubation		42 days of incubation	
Sample	pH of mother solution	Dilution to obtain pH=5	pH of mother solution	Dilution to obtain pH=5
Blank control	5.27	1:3	5.06	1:1
A. niger	1.67	1:60	1.87	1:50
Ch. globosum	6.22	1:5	4.50	1:2
Cl. cladosporioides	4.78	1:3	6.23	1:10
P. chrysogenum	4.48	1:3	6.22	1:10

The mother solutions obtained from *A. niger*, a species known to be a powerful producer of organic acids (Andersen et al., 2009), always exhibited exceptionally low pH values (<pH2) and seemed to be acting like pH buffers themselves.

5.2.2.3.2. Relationship between biomass and fluorescence

Biomass dilutions from *A. niger*, *Cl. cladosporioides*, *Ch. globosum* and *P. chrysogenum* were plotted against the correspondent obtained fluorescence, as described in the literature (Konkol et al., 2010, 2012), at 3, 7, 14 and 42 days of incubation (longer and more spaced incubation periods to cover a higher time spectrum than in the previous experiment) (Figure 5.7). The regression results and linear fitting of the straight part of the obtained slopes are presented in Table 5.2.

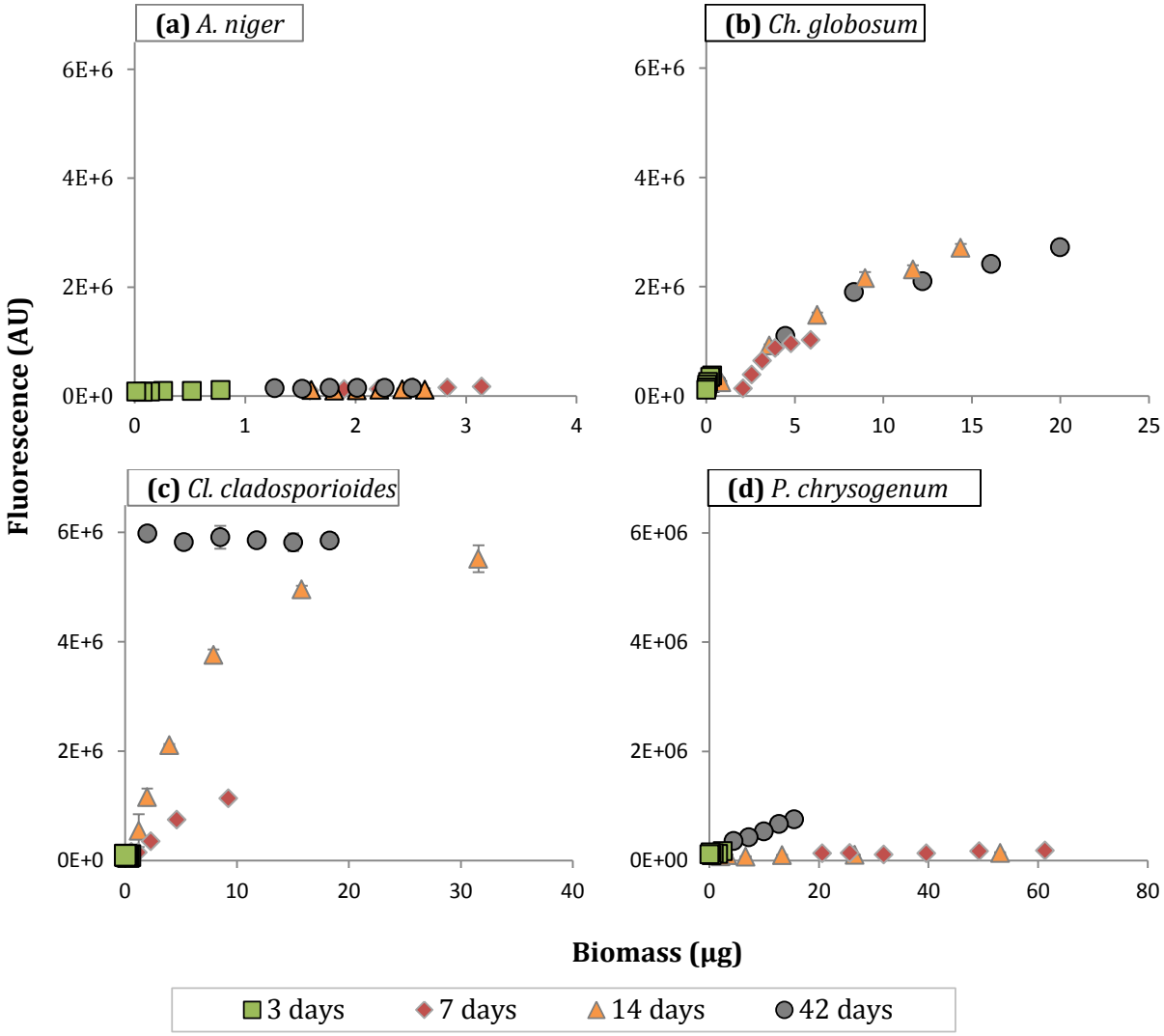


Figure 5.7: Relationship between fluorescence and biomass dilutions for *A. niger* (a), *Ch. globosum* (b), *Cl. cladosporioides* (c), and *P. chrysogenum* (d) at 3; 7; 14 and 42 days of incubation. Error bars =standard deviation of three replicates.

The difference between fluorescence values obtained from *A. niger* samples and the corresponding negative controls (without 4-MUF-NAG) were not statistically significant (p>0.05) at 3, 7 and 14 days of incubation. The results obtained at 42 days of incubation, although significantly different from the controls, do not show a significant correlation between biomass dilutions and fluorescence (p>0.05), as shown in Table 5.2.

Due to the experimental limitations occurred with *A. niger* it is not possible to conclude about its NAGase activity, since the lack of detectable fluorescence may be due to the excessive dilutions.

Table 5.2: Correlations between fluorescence and biomass dilutions obtained for *A. niger* (a), *Ch. globosum* (b), *Cl. cladosporioides* (c) and *P. chrysogenum* (d) at 3, 7, 14 and 42 days of incubation.

Fungal species	Incubation (days)	Linear fitting	R^2	*P* value
A. niger	3	-	-	-
	7	-	-	-
	14	-	-	-
	42	$y = 1.28E5 + 8973.62x$	0.533	0.100
Ch. globosum	3	$y = 1.43E5 + 7.80E5x$	0.930	0.00191
	7	$y = -6.74E5 + 4.06E5x$	0.987	0.00668
	14	$y = 2.56E5 + 1.81E5x$	0.975	0.00023
	42	$y = 4.92E5 + 1.22E5x$	0.925	0.00219
Cl. cladosporioides	3	-	-	-
	7	$y = 81523.21 + 1.19E5x$	0.981	0.00014
	14	$y = 1.41E5 + 4.67E5x$	0.990	0.00478
	42	$y = 5.92E6 - 2687.75x$	0.270	0.29042
P. chrysogenum	3	-	-	-
	7	-	-	-
	14	-	-	-
	42	$y = 1.24E5 + 42086.30x$	0.980	0.00015

-, fluorescence values not statistically different from control samples without 4-MUF-NAG.

For a more immediate comparison between fungal species regarding fluorescence at different incubation times, the estimated fluorescence for 5 µg (a biomass value that fell within the range of the tested biomass dilutions at 7, 14 and 42 days of incubation) was calculated from the curve fitting equations in Table 5.2, and is shown in Figure 5.8. The results from *A. niger* were not included in this graph due to the lack of statistical significance presented above.

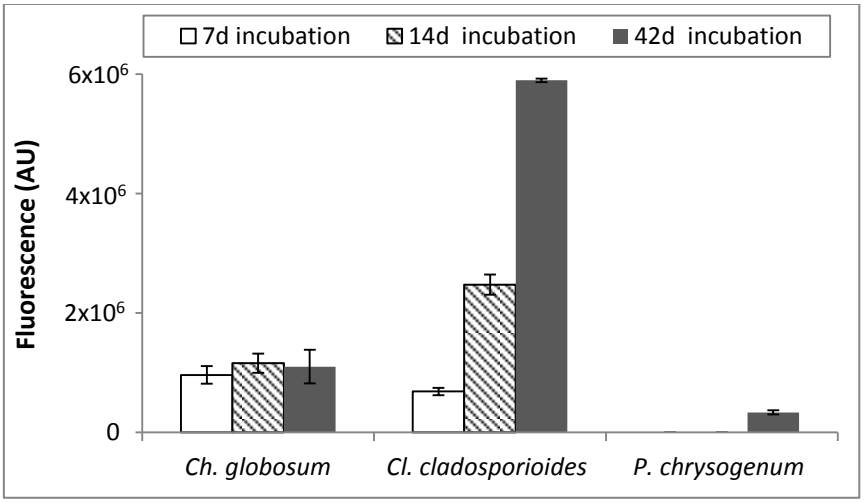


Figure 5.8: Estimated fluorescence values for 5µg of fungal biomass of *Cl. cladosporioides*, *Ch. globosum* and *P. chrysogenum*, after 7, 14 and 42 days of incubation. Error bars represent standard errors for estimated values.

As presented in Figure 5.8, very diverse results were obtained concerning the relationship between fluorescence and biomass for the three fungal species and periods of incubation.

Ch. globosum was the only species presenting detectable NAGase activity after 3 days of incubation (Table 5.2) despite having the lowest total biomass values of the tested group (Table

5.3). This denotes a higher excretion of active NAGase in the initial development phase of this species. *Ch. globosum* was also the fungus with the most constant relationship between biomass and fluorescence along time, where approximately the same fluorescence values were obtained at 7, 14 and 42 days of incubation (Figure 5.8).

For *Cl. cladosporioides*, fluorescence augmented as incubation time progressed for a same biomass (Figure 5.8). This points out to an accumulation of active enzyme in the growth medium, since these enzymes have shown to not significantly lose their activity for up to 40 days (Madsen, 2003).

Cl. cladosporioides was the fungus that produced the highest fluorescence intensities of the tested fungal group. After 42 days of incubation even at a 1:100 dilution of the supernatant, the fluorescence was on the maximum level that can be obtained from the total hydrolysis of the added 4-MUF-NAG (c. 5.82 x 10^6). To estimate the total enzymatic activity for this species, a much higher quantity of MUF-NAG would have to be used. The enormous quantity of active NAGase detected in *Cl. cladosporioides* in this last assay is not proportional to its total biomass, which is in fact lower than the maximum biomass obtained in the previous assay with 14 days of incubation (Table 5.3). Thus, autolysis could be occurring and enzymes produced for this process could be contributing to the intense values of fluorescence. Autolysis is the process of self-digestion that occurs locally in aged hyphae when active growth has ceased. During this process, biomass falls, although there may be renewed growth resulting from metabolism of autolysis products (Prosser, 1996). In this case NAGase activity is a poor marker for fungal growth.

Table 5.3: Total dry biomass of *A. niger*, *Cl. cladosporioides*, *Ch. globosum* and *P. chrysogenum*, at 3, 7, 14 and 42 days of incubation. The maximum biomass values obtained for each fungi are in bold.

	Total biomass (mg)			
	3 days	7 days	14 days	42 days
A. niger	45.80	**224.27**	184.25	179.37
Cl. cladosporioides	7.35	38.72	**176.79**	112.60
Ch. globosum	5.61	49.55	120.68	**167.76**
P. chrysogenum	20.38	171.56	**297.10**	86.67

P. chrysogenum produced the highest level of dry biomass of all tested species, and thus it would be expected to also have the highest levels of 4-MUF fluorescence. However, at 3, 7 and 14 days of incubation, the measured fluorescence levels were considered null, as the difference between the fluorescence obtained from samples and the corresponding negative controls (without 4-MUF-NAG) was not statistically significant (p>0.05). NAGase activity was only detectable for *P. chrysogenum* when its biomass started decreasing, at 42 days of incubation (Figure 5.7 and Table 5.3).

If a continuous increase in biomass of *P. chrysogenum* was observed in the first three assays (Table 5.3), detection of NAGase activity would be expected on all of them, and not only on the last assay, when biomass was decreasing.

According to the literature (Sámi et al., 2001), during hyphal growth in *P. chrysogenum*, microsomal chitinase(s) have demonstrated to be membrane-bound zymogen(s) (inactive enzyme precursors that require a biochemical change to become active enzymes). This means that they are activated only when associated with the fungal membrane. The fact that the NAGase produced by *P. chrysogenum* could be membrane-bound zymogens could justify the obtained results. In this way, when the growth medium was tested, the enzymes would not be associated with the membrane thus would be inactive and unable to break the 4-MUF-NAG substrate and generate fluorescence. Furthermore, the same authors (Sámi et al., 2001) observed that during autolysis, the chitinases of *P. chrysogenum* were already active, as no zymogen activation was observed. This could explain why NAGase activity was only detected on the last assay, when autolysis was already taking place. NAGase activity is therefore also a poor marker for the growth of *P. chrysogenum*.

5.2.3. Conclusions

The main aim of this study was to assess the potential and limitations of the 4-MUF-NAG fluorometric method to quantify fungal biomass, for subsequent use in the testing of the antifungal treatments presented in this thesis in Chapters 6 and 7.

Several experimental conditions were tested to improve the development of calibration curves which would enable the correlation between fluorescence and biomass.

The obtained results show that the 4-MUF-NAG method, while measuring NAGase activity, has several limitations as a technique for the quantification of fungal biomass. These limitations include the fact that even when analyzing a single fungal species, different stages of growth produce different quantities of NAGase, and therefore a measurement of fluorescence may not be indicative of a specific value of biomass. Even after preparing a calibration curve with known amounts of biomass, it is not possible to know in subsequent analysis in which exact stage(s) of growth the fungus is, and any minor changes in incubation conditions or substrate composition could generate different results.

The behavior of *A. niger* demonstrates that the developed calibration curve methodology, where the growth medium is directly analyzed, is not appropriate for fungi that are strong producers of acids. An alternative procedure could be an initial growth medium filtration using the 0.45 μm pore filters and a second filtration using lower pore filters, which could retain the enzyme.

Regarding the detection of fungal activity, as observed for *P. chrysogenum*, a fungus may be growing without excreting active NAGase, and thus give a false negative result. On the other hand, the released NAGase can remain active for several days (Madsen, 2003) and accumulate in the substrate (as inferred from the results obtained with *Cl. cladosporioides*). So, even if the fungus is inactive, by measuring enzyme activity, false positives may be obtained. Fungi also release active NAGase during autolysis and consequently the detection of increased fluorescence may not signify an increase in fungal biomass, but the opposite.

The intensity of fluorescence varied enormously between the fungal species tested, and so this method is not appropriate to quantify fungal biomass in natural samples, where a diversity of species is expected to be present.

The most constant correlation between biomass and fluorescence was obtained with *Ch. globosum*, which for a given biomass value, approximately the same fluorescence intensities were obtained throughout the different incubation periods.

This study intended to be a first screening to conclude if the 4-MUF-NAG fluorometric method could be used for fungal growth quantification in the following antifungal testing experiments presented in this thesis. If the results were promising, further optimization would have to be conducted, like having replicates for each fungal species in a same incubation period; and developing calibration curves with experimental conditions closer to the ones to be used in the test experiments, like growing fungi on paper samples instead of liquid cultures. However, given the obtained results, the method was considered inadequate for use in the following antifungal experiments and alternative methods were evaluated, as described hereinafter.

5.3. Optimization of colony and dry biomass measurement techniques for fungal growth measurement on paper

Given the limitations of the existing fungal biomass quantification methods and the need to analyse a large amount of samples for antifungal activity evaluation (Chapters 6 and 7), two of the most simple and most common methods for fungal quantification were evaluated and optimized: colony size and dry weight measurements. Both methods are frequently used as references for validation of new quantification methodologies (Taniwaki et al., 2006; Marín et al., 2008).

5.3.1. Colony size measurement

Hyphal growth in filamentous fungi proceeds in a radial pattern from the point of inoculation and as mycelium develops it soon forms a circular colony (Gow and Gadd, 1996:310; Bottone et al., 1998). As colonies develop circularly, the measurement of colonies diameter has been one of the most common methods to quantify fungal growth in the field of microbiology (Brancato and Golding, 1953; Gow and Gadd, 1996:303). It is also one of the simplest techniques, as it can be done in situ with no other instruments than a simple ruler. This technique is also more sensitive than dry weight, hyphal length or ergosterol quantification, since a colony as small as 2 mm can be successfully measured (Taniwaki et al., 2006). However, colony diameter measurement does not take into account the density of colonies or sporulation, and while in early growth stages the correlations between colony diameter and biomass are high, in more mature colonies the correlation decreases (Marín et al., 2005; Taniwaki et al., 2006).

On paper biodeterioration studies, the colony size measurement technique has been frequently applied to test the efficacy of antifungals (da Silva et al., 2006; Neves et al., 2009; Mesquita, 2013:87).

5.3.1.1. Diameter vs area measurement

Measuring the diameter of single circular fungal colonies can be a very simple task, but for multiple and irregular colonies, the measurement of colonization areas instead of diameters can be more precise. Calculating the areas of irregular shapes is not as straightforward as measuring a diameter. Though, current digital photography and imaging software techniques have made area measurements much easier and quicker.

This kind of analyses has already been performed before. Zotti et al. (2007) photographed paper samples with fungal growth and segmented the digitally captured images in black and white levels using an image-analysis program. The percentage of colonisation was then computed as the ratio between the black area (colonies) and the total area.

Saunders & Kohn (2008) used a technique where the colonies areas were traced onto paper, scanned, and the digital image measured using ImageJ, a freeware program designed mainly for microbiology image analysis.

Barry et al. (2009) also used ImageJ for fungal quantification on solid substrates, but with digital capture of image in a microscopic scale, for measuring microscopic development.

Miller et al. (2010) used digital image analysis to quantify the colonisation of stone by phototrophic microorganisms. The collected RGB images were decorrelated by Principal Component Analysis (PCA), using Hypercube software. The most appropriate PCA band, the one that improved the visualization of the biomass on the stone surfaces, was afterwards used for area quantification by ImageJ software.

5.3.1.2. Distinguishing fungi from paper

The problem with quantifying colonisation of paper by fungi using visual techniques lies on the difficulty in distinguishing white mycelium from white paper. The same technique (PCA decorrelation) used by Miller et al. (2010) was tested on our paper samples, but since the microorganisms, or at least part of them, have the same coloration as the substrate, as opposed to phototrophic green microorganisms on limestone, the results were not satisfactory.

There are colorants used in the microbiology field that can selectively stain fungal material, like lactophenol cotton blue or lactofuchsin, or the fluorescents calcofluor white or lectin (Santos et al., 2009). However, these methods are destructive and the compounds can also bind to cellulose (Herth and Sghnepf, 1980).

Alternatively, illumination techniques that are commonly used for paper artwork like ultraviolet (UV), transmitted and raking lighting, were tested and compared with direct or normal lighting, to evaluate their potential to differentiate between paper and fungi.

With direct lighting, the source light is incident to the surface of the object at a right angle, to give an even illumination. This allows for the observation of staining caused by the fungi and can give a rough idea of the paper's surface topography (Jirat-Wasiutynski, 1986).

Examination of paper under UV illumination is used in the paper conservation field to help detecting material differences, like alterations in media, presence of oils, varnishes, adhesives, foxing or mould, through characteristic UV fluorescence reflection or absorption (Jirat-Wasiutynski, 1986). Regarding foxing or mould, UV examination may reveal the presence and extent of paper deterioration resulting from these phenomena (Jirat-Wasiutynski, 1986; Corte et al., 2003). UV illumination may also help in the detection of early fungal development inside the paper matrix not visible to the naked eye (Bertalan et al., 1994; Florian et al., 1994).

Transmitted lighting is performed by illuminating the paper object from behind, on a light box or using a light sheet (for observation of binded sheets). This technique is used to detect variations

in sheet thickness or opacity (Jirat-Wasiutynski, 1986), which could be affected by fungal development.

Raking illumination consists in placing the light source on one side of the paper object at a low angle, causing the projection of light across the surface of the paper (Jirat-Wasiutynski, 1986). Any surface textures or reliefs are highlighted, revealing the detailed topography of the paper's surface. This could permit the detection of superficial development of fungal structures.

5.3.1.3. Materials and methods

Circular paper samples (5.5cm diameter) were placed in Petri dishes with PDA and inoculated with a mixture of five fungal species (*A. niger*, *Ch. globosum*, *Cl. cladosporioides*, *P. chrysogenum*, *P. corylophilum*, as discussed in Chapter 4). The samples were incubated at 22°C for 2 and 4 days.

The paper samples were photographed inside a laminar flow chamber, with the lids of the Petri dishes off. The lens of the camera was always kept at the same distance from the chamber's base and graph paper was used as background scale with a grey reference card (QPcard 101). A black square was used as a background to increase the contrast between fungi and the wet white paper substrate. For standard direct lighting, the fluorescent lamp from the laminar flow camera was used as illumination source (Figure 5.9 A). For raking lighting, a LED light (70 Lumen, 3000 Kelvin) was placed perpendicularly to the sample (Figure 5.9 B). UV lighting was undertaken with a UV Magnification Inspection Lamp placed at a 45°angle (Figure 5.9 C) (365nm, Luxo Vista UV Handle, Max 1x9W G23, Preservation Equipment, England). Transmitted lighting was obtained by placing a light sheet (Conservation light and equipment, London) below the Petri dish (Figure 5.9 D).

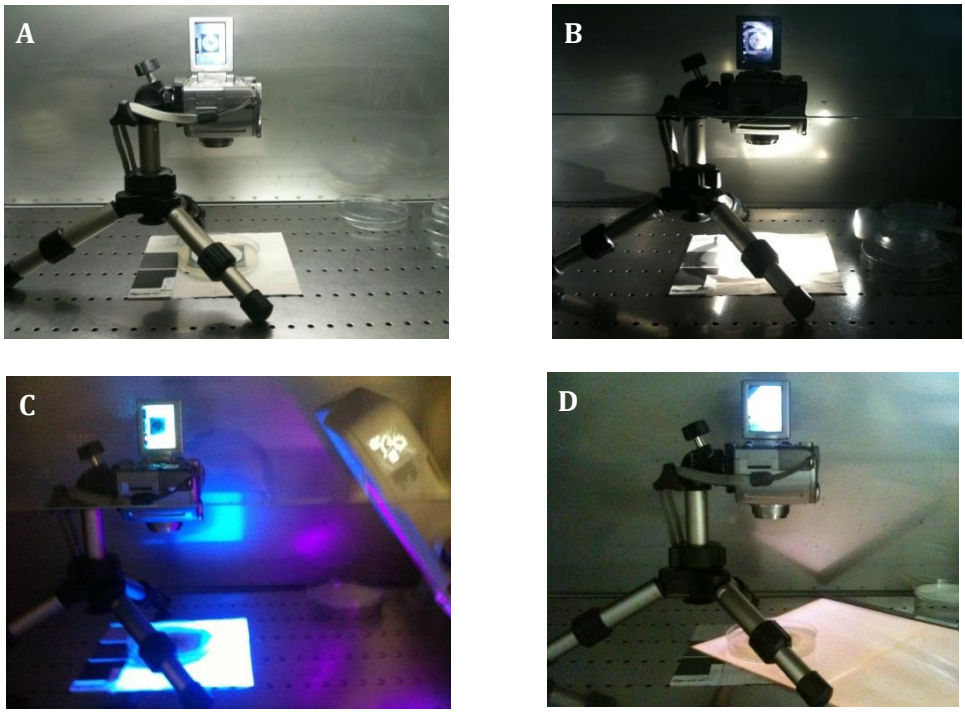


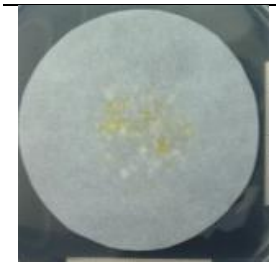
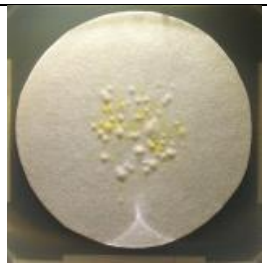
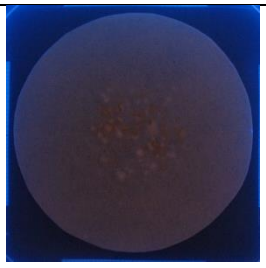
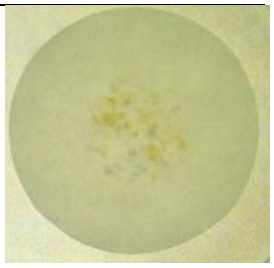
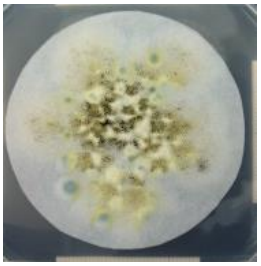
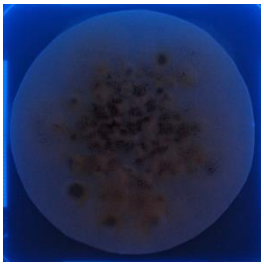
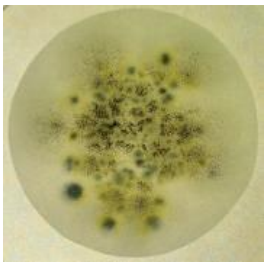
Figure 5.9: Photographic registry of fungal growth on paper samples with different lighting techniques: direct lighting (A); raking lighting (B); UV lighting (C); transmitted lighting (D).

After selection of the best lighting technique, the areas of fungal colonies were calculated using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA) (Schneider et al., 2012) with the freehand selection tool.

5.3.1.1. Results and discussion

After incubation, paper samples were photographed using four different illumination techniques: direct, raking, UV and transmitted lighting. The obtained images are presented in Table 5.4.

Table 5.4: Images of mixed inoculum growing over paper obtained with different illumination techniques: direct light, raking light, ultraviolet light and transmitted light. The photographs were taken 2 and 4 days after inoculation.

	Direct	Raking	UV	Transmitted
2d				
4d				

In the picture taken with direct lighting, the black background aids in the distinction between paper and white mycelium. However, in the tests where samples were incubated on top of nitrocellulose membranes for biomass quantification (see 5.3.2 Estimation of biomass dry weigh), the black background was not visible through the paper, and raking lighting was indispensable for fungal growth distinction (Figure 5.10). Also, the samples used for testing the antifungals as a curative treatment (see Chapter 6) were vacuum cleaned before treatment (see Chapter 6). With this process, the superficial colonies were removed, but any previous fungal staining was retained in the paper (Figure 5.10), and so, after antifungal treatment, any colouration in the paper could not be directly interpreted as fungal development.

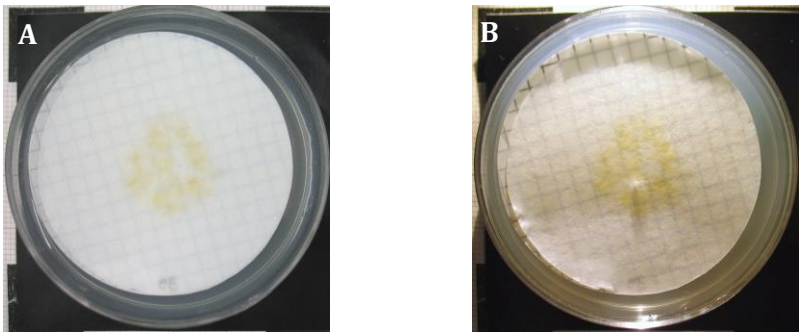


Figure 5.10: Direct (A) and raking lighting (B) pictures, respectively, of a paper sample incubated over a nitrocellulose membrane. A small colony developing at the centre of the sample is only discernible in the right image taken with raking lighting.

As shown in Table 5.4, UV lighting did not aid in the distinction between paper and fungi. The images captured with UV lighting in RGB mode were afterwards separated in the respective red, green and blue channels. The red channel alone permitted a better distinction between fungi and paper, as shown in Figure 5.11. However, the limits of the colonies were not as sharp as the ones obtained with raking lighting (Table 5.4).

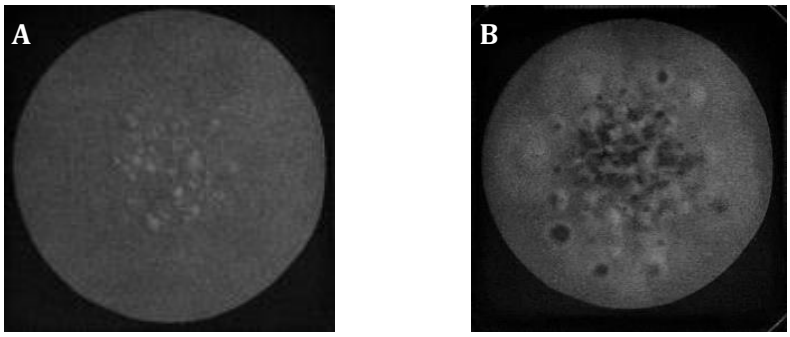


Figure 5.11: Red channel from the RGB images captured with UV lighting, at 2 and 4 days of incubation, (A) and (B) respectively.

UV lighting is used in the field of paper conservation, amongst other things, for the detection of fungal development (Jirat-Wasiutynski, 1986; Bertalan et al., 1994; Florian et al., 1994). Though in those cases, fungi have already aged and may have oxidized the paper substrate.

Transmitted lighting did not improve the detection of fungal presence on paper, when compared with direct lighting (Table 5.4). Probably in a further deterioration stage, this technique could aid in detecting areas where the paper matrix has been more deteriorated by fungi, due to an increase in paper transparency.

Raking lighting was the one that enabled a better differentiation between the substrate and the developing fungi (Table 5.4). The protrusion of the colonies developing on the paper's surface became highlighted, and could easily be distinguished from the flat non-colonized area. In further tests, colonies as small as 0.5mm diameter could be detected.

After selection of raking light as the most suitable lighting method, area measurements were performed using ImageJ software (Figure 5.12). Only the growth occurring over the paper samples was taken into account, not the growth extending to the medium, since the final aim of using fungal measurement in Chapters 6 and 7 was the evaluation of antifungal compounds present on the paper alone.

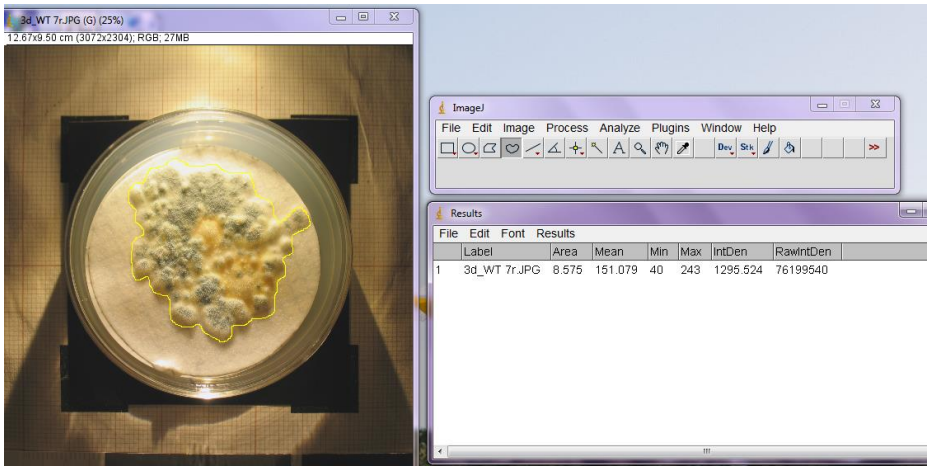


Figure 5.12: Example of a paper colonized area measurement with ImageJ on a picture taken with raking light. The yellow line is the measured area contour. At the right are the measured values.

To calculate the error associated with measuring the colonisation area by the developed method, the same image was analysed three times at distinct days. The obtained differences were in the 1% range.

Still, any early fungal growth inside the paper matrix, before visible development on the surface cannot be quantified by this method.

For a better estimation of fungal biomass, which besides the area would also take into account the volume of the colonies, 3D digitization could be an alternative to explore in the future.

5.3.2. Estimation of biomass dry weight

Dry weight biomass estimation is a commonly used method for quantification of microorganisms, especially for fungi growing in liquid systems. The main downside of this kind of measurement is that it requires the destruction of the sample, precluding the monitoring of the same culture along time, and replicates must be used instead. The sensitivity of dry weigh measurement is lower than colonies size measurement, as colonies smaller than 2 mm are not possible to detect accurately by this method (Taniwaki et al., 2006). However, it is the most direct technique for fungal growth quantification (Gow and Gadd, 1996:302), as any evolution or decline in fungal growth will be reflected on biomass.

According to Prosser (1996:301), fungal growth may be defined as the "orderly increase in cell components leading to an increase in biomass". Biomass quantification is usually regarded as the fundamental measure of fungal growth in biotechnology (Pitt and Hocking, 2009:34), and when new methods for fungal quantification are developed, the aim is to have a good correlation with biomass measurements (Marín et al., 2005).

While measuring dry weight biomass in liquid cultures is quite straightforward, when solid substrates are used, it gets more complicated due to extraction and accuracy problems (Gow and Gadd, 1996:302).

When the solid substrate is agar medium, cellophane membranes can be placed over the medium and inoculated, which enables an easy separation of the fungus after growth and weighting (Reeslev and Kjoller, 1995; Marín et al., 2005; Mesquita, 2013:85). Separation of mycelium from agar medium can also be performed by melting the agar (Taniwaki et al., 2006). However, when fungi are developing in the core of food or cultural heritage materials, the total extraction of fungal biomass from the substrate can be an impossible task (Marín et al., 2005). The alternative of scrapping or peeling fungal colonies from the substrate may lead to incomplete removal and underestimation of dry weight (Taniwaki et al., 2006).

In order to measure the biomass in paper samples for the present thesis, the existing methodologies were adapted and optimized.

5.3.2.1. Materials and methods

Whatman #1 paper discs with 45 mm diameter were previously numbered, weighted and sterilized by autoclave.

In order to avoid the penetration of fungal mycelium into the solid culture medium, paper samples were incubated on top of sterile 47mm nitrocellulose membranes (0.45 µm porosity, 47mm diameter, GVS, Spain) (Bottone et al., 1998; Barry et al., 2009), over PDA growth medium in 60mm Petri dishes (Figure 5.13a). Nitrocellulose membranes, also used for microbial sampling and isolation (Cappitelli et al., 2010), guarantee a high level of purity, when compared to commercial cellophane paper, and can be bought already sterilized at a relatively low price, when compared with cellulose acetate or nylon membranes.

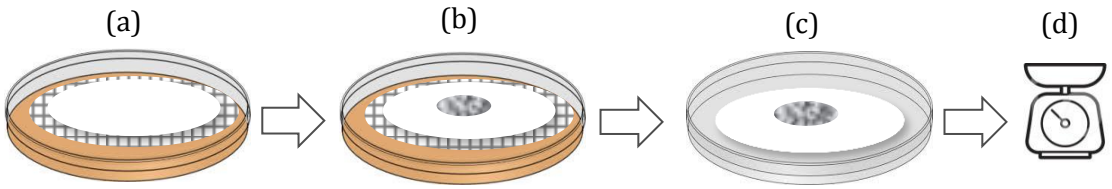


Figure 5.13: Illustration of experimental procedure used for biomass quantification: Paper sample (white disk) placed over nitrocellulose membrane (squared disk) on PDA inside Petri dish, before (a) and after (b) incubation; Colonized paper sample after removal from PDA and membrane on clean Petri dish (c); weighting of sample after drying (d).

After fungal development (Figure 5.13b), paper samples were separated from the membranes placed in clean Petri dishes (Figure 5.13c), dried and weighted (Figure 5.13d). All weight measurements (including the initial one, pre-sterilization) were preceded by drying the samples in vacuum (15±5 mBar) for 20 hours, and weight stabilization at the environmental relative humidity for one hour inside a laminar flow chamber (Petri dish lids open).

All samples were maintained in individual Petri dishes. For weighting, tared sterile petri dishes were used to transport the sample from the laminar flow chamber into the precision scale (Sartorius LE623S). Weight measurements were made in duplicate for each sample, in two weighting rounds, and the average values were calculated.

Biomass was calculated by subtracting the weight of each sample after incubation from its initial weight. As paper is highly hygroscopic and variations in relative humidity can result in oscillations in paper weight, the relative humidity inside the laminar flow chamber was registered in every assay, and reference uninoculated samples were used for weight compensation due to differences in humidity content. The average weight gains or losses due to relative humidity differences in the uninoculated reference samples were either subtracted or added, respectively, to the inoculated samples.

5.3.2.2. Results and discussion

Through the observation of inoculated samples incubated over nitrocellulose membranes it was possible to see that these membranes limited the penetration of fungal mycelium into the solid culture medium, while allowing the passage of nutrients and humidity from the medium to the paper samples (revealed by the successful fungal development)(Figure 5.14). The membranes also aided on the removal of paper samples from the medium, while they were physically weakened by humidification and fungal deterioration.

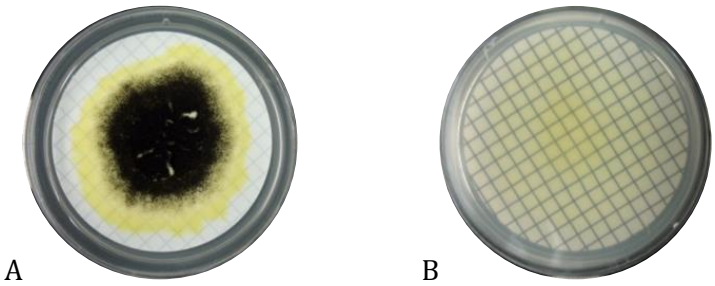


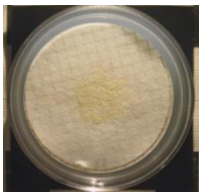
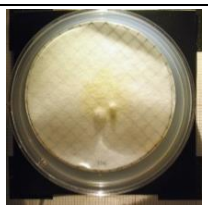
Figure 5.14: *A. niger* growing over nitrocellulose membrane: front (A) and back (B) of Petri dish.

A study performed on the effect that growing over cellophane membranes has on fungi, concluded that although radial growth was not profoundly affected by the presence of the membrane, biomass can exhibit differences in density in some of the tested fungal species (Reeslev and Kjoller, 1995). Whereas differences may occur on fungal growth over membranes, since all the samples being compared are maintained in the same conditions (with or without membranes), the validity of comparison is preserved.

The two replicate weight measurements performed in each sample showed a variation in the 0.001g range. This variation is within the uncertainty of the used scale, which measured to the nearest 0.001g.

Samples with no visible fungal growth could show a maximum of 1.5mg weight variation between initial and final weight (after correction with references), and samples with visible growth could show a maximum loss of 2 mg (Table 5.5). From 2 mg weight gain there was always a correspondence with visible fungal growth.

Table 5.5: Examples of paper samples showing weight gain with no visible fungal development (Sample A) and showing weight loss with visible fungal development (Sample B).

	Picture	Area (cm^2)	Δ weight (mg)
Sample A		0	+1.5
Sample B		0.234	-2.0

5.3.3. Correlation between colonized area and biomass

The validation of colonisation area measurements on paper samples was made through correlation with biomass measurements. Figure 5.15 presents the data from paper samples inoculated with a single species (*P. chrysogenum*) (Figure 5.15 A) and with a mixed inoculum (*A. niger, Ch. globosum, Cl. cladosporioides, P. chrysogenum, P. corylophilum*) (Figure 5.15 B).

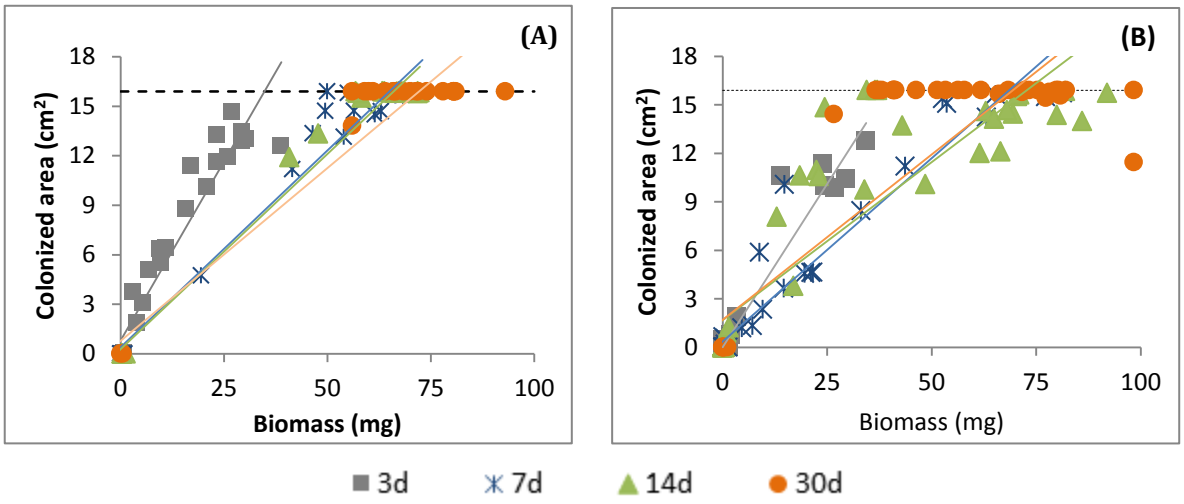


Figure 5.15: Correlation between colonized area and biomass for *P. chrysogenum* (A) and mixed inoculum (B)after treatment with antifungals, at 3, 7, 14 and 30 days of incubation. The upper dashed line corresponds to the maximum area of paper samples.

In Table 5.6, the ratio between the average biomass values and the average area values, as well as the correlation coefficient and *p* value of correlation between these two variables are presented.

Table 5.6: Ratio between biomass dry weight (mg) and colonisation area (cm²), correlation coefficient (R^2) and correlation *p* value obtained for paper samples inoculated with *P. chrysogenum* and mixed inoculum, at 3, 7, 14 and 30 days of incubation.

Inoculation (number of samples)	Time (days)	Ratio biomass/area	R^2	*P* value
P. chrysogenum (n=30)	3	1.96	0.929	0.0000
	7	4.01	0.973	0.0000
	14	4.11	0.983	0.0000
	30	4.41	0.956	0.0000
Mixed inoculum (n=45)	3	2.43	0.943	0.0000
	7	3.93	0.931	0.0000
	14	3.89	0.789	0.0000
	30	3.92	0.803	0.0000

As shown in Table 5.6, high correlation coefficients (R^2) were obtained between biomass dry weight and colonisation area. Marin et al. (2005), also found a good correlation between colonies size and biomass dry weight.

In all correlations, the first stage of incubation, 3 days, reveals a lower biomass dry weight/colonized area ratio than the following stages (7, 14 and 30 days). This points out to an initially higher radial growth with less dense colonies, followed by a more structured growth, accompanied by conidiophore development and sporulation. According to the literature, initially the radius of the colony increases exponentially, but becomes constant afterwards (Gow and Gadd, 1996:310).

Approximately constant biomass/area ratios were obtained for 7, 14 and 30 days of incubation when mixed inoculum was used. When a single species was growing (*P. chrysogenum*), this ratio slightly increased with time.

The correlation coefficients are generally lower for mixed inoculum samples, especially for longer periods of incubation (14d and 30d). This is a symptom of the heterogeneity of these samples, where five different species can be developing simultaneously or individually, and each species can present different behaviours with ageing, like sporulation or autolysis, with reflection on biomass. Taniwaki et al. (2006) also observed a reduction in correlation between colony size (diameter) and biomass with longer periods of incubation due to sporulation. Furthermore, the analysed samples were subjected to antifungal treatments that had an effect on the development of fungi, namely by delaying or even preventing it. The samples in Figure 5.15 that at 7, 14 or 30 days of incubation are closer to the 3 days trendline correspond to fungal colonies in the first stages of development, which growth was delayed by antifungals.

In Figure 5.15, it is also shown that the colonisation area is limited to the maximum area of the paper samples while biomass can still continue to increase when reaching this point, most likely due to a continuing formation of reproductive structures and increase in colony density.

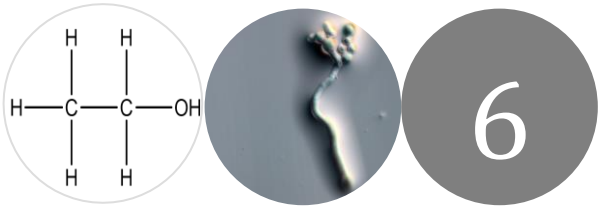
5.3.4. Conclusions

The aim of these experiments was to obtain reliable methods for quantifying fungal growth on paper samples to enable the evaluation of antifungals efficacy in Chapters 6 and 7.

The measurement of colonized area confirmed to be a simple, non-destructive method, with high sensitivity and strong correlation with biomass dry weight. The developed inspection and measurement protocol, using raking light to capture the images to be measured, enabled a good distinction between the fungal colonies developing in the surface of paper and the substrate. This method has the limitation of not taking into account the fungi growing inside the paper matrix or the density of colonies.

Dry biomass weight quantification, although less sensitive than colonization area measurement, performed better in evaluating fungal growth in colonies with longer incubation times, when the total area of samples had already been colonized. Also, the total biomass, including the one not visible on the surface, and the density of colonies are taken into account. The major limitation of this technique is its destructive character which invalidates a continuous monitoring of a same sample over time.

All fungal quantification techniques have limitations, and as stated by Taniwaki et al. (2006) the most reliable information is obtained by using more than one technique. Therefore, in Chapters 6 and 7, both dry biomass weight and colonized area measurements were used to evaluate the efficacy of antifungal treatments.



Ethanol as an antifungal treatment for paper: short-term and long-term effects

Published with minor alterations as: Sequeira, S. O., Phillips, A. J. L., Cabrita, E. J., Macedo, M.F. (2016) 'Ethanol as an antifungal treatment for paper: Short-term and long-term effects'. *Studies in Conservation* (*in press*). DOI: 10.1080/00393630.2015.1137428.

In paper conservation ethanol is used as an antifungal agent. However, the information on the antifungal efficacy of this alcohol is scarce and often inconsistent. In this study we clarify if ethanol is effective and safe to use in paper conservation in the short as well as in the long term.

None of the tested ethanol concentrations (5%-100%) promoted conidia germination, but rather delayed or entirely inhibited it, depending on alcohol concentration and contact time.

Treating paper preventively with ethanol did not reveal a significant antifungal effect. However, in a simulation of a curative treatment of samples colonized by fungi, all the tested ethanolic solutions (30%, 70% and 100%) presented antifungal activity. The best results were obtained with 70% ethanol, showing fungicidal properties on 4 of the 5 tested fungal species (*Aspergillus niger*, *Cladosporium cladosporioides*, *Penicillium chrysogenum* and *Penicillium corylophilum)*.

No deleterious effects of 70% ethanol on the tested paper were observed either in the short or in the long term.

6.1. Introduction

Ethanol is a universally acknowledged disinfectant, antiseptic and preservative. Due to its multiplicity of toxic effects, ethanol is generally considered to be a nonspecific antimicrobial, ranked among the membrane-active agents (Block, 2001:231; Paulus, 2004:444). Although the specific nature or site of action of ethanol is not fully known, the most widely adopted theory is that ethanol acts through coagulation/denaturation of proteins and membrane damage, interfering with metabolism and causing cell lysis (McDonnell and Russell, 1999; Block, 2001:231).

In the field of heritage conservation, among other applications, ethanol is often used by paper and book conservators as an antifungal (see Chapter 3). However, the literature on the actual efficacy of ethanol as an antifungal is scarce and often contradictory. While some authors recommend such treatment of affected paper (Florian et al., 1994; Brokerhof et al., 2007; Child, 2011) and others confirm the fungicidal activity of ethanol vapours (Bacílková, 2006), or demonstrate complete inhibition of fungal development on samples treated with ethanol at 70% (Valentin, 1986), still other researchers indicate that ethanol may act as a fungal spore activator

(Florian, 2002:37; Guild and MacDonald, 2007), or that 70% ethanol does not have sporicidal properties (Nittérus, 2000a). Consequently, it could be questioned if this treatment should continue to be used.

In this chapter we intended to clarify if ethanol is suitable for use as an antifungal in paper conservation practice. The main questions to answer were whether ethanol enhances or inhibits the germination of fungal spores, which water/ethanol concentration is the most efficient, what are the preventive and curative antifungal effects of ethanol, and the effect of ethanol on the chemical and physical properties of paper in the short and long term. The preventive antifungal treatment was aimed at testing the ability of ethanol to protect the paper from future fungal development. The curative treatment intended to simulate a situation where the paper has already been affected by fungi and is treated to stop fungal development.

First experiments were made using *Penicillium chrysogenum*, as it is the most frequently isolated fungal species from paper/book materials worldwide, according to the research presented in Chapter 4. It is also highly cellulolytic (Chinedu et al., 2011) and its colonies grow fast and sporulate in standard media (Pitt and Hocking, 2009:235). The ethanol concentration that provided best antifungal results with *P. chrysogenum*, was then tested as a curative treatment on paper samples with a mixture of fungal species composed of five of the most commonly identified fungal species on paper collections: *Aspergillus niger*, *Penicillium chrysogenum*, *Penicillium corylophilum*, *Chaetomium globosum* and *Cladosporium cladosporioides* (see Chapter 4). The mixed inoculum was used in order to simulate a real-case scenario where paper collections are exposed to contamination from several species and competition and suppression strategies can occur, and only the most well adapted organism(s) will develop.

To ascertain the safeness of the treatment of paper based cultural heritage materials with ethanol, the short and long term effects of this compound on the chemical and physical properties of paper (colour, pH, folding endurance, and molecular alterations) were evaluated using moist heat artificial ageing.

6.2. Materials and Methods

6.2.1. Paper

Whatman filter paper #1 was selected as the model paper as it is additive-free and has a high cellulose content (98% w/w), which reduces the number of variables in the results, and also due to its frequent use in paper conservation and biodeterioration research (Michaelsen et al., 2006; Zervos and Moropoulou, 2006), which makes it comparable to other studies. The paper has an average thickness of 180 μm, grammage of 88 g/m^2 and 0.06% ash content.

6.2.2. Fungal species

Penicillium chrysogenum Thom was selected for tests with a single species. For testing the most efficient ethanol:water concentration, a mixture of five fungal species was used. The mixed inoculum was composed of *Aspergillus niger* Tiegh., *Chaetomium globosum* Kunze, *Cladosporium cladosporioides* (Fresen.) G.A. de Vries, *Penicillium corylophilum* Dierckx and also *Penicillium chrysogenum*. The characteristics and origin of the selected species are described in Chapter 4, section 4.2.

6.2.3. Inoculum preparation

Fungal strains were plated on potato dextrose agar (PDA, Oxoid, UK) and incubated at 22 °C for 15 (*A. niger*, *P. chrysogenum*, *P. corylophilum* and *Cl. cladosporioides*) and 30 days (*Ch. globosum*). For inoculum preparation, spores were harvested by pipetting sterile 0.05% Tween 80 (Panreac) on the surface of colonies and collecting the suspension. For *Ch. globosum*, the suspension was gently crushed with a sterile glass rod to release ascospores from perithecia and asci. The concentration of spore suspension for each fungal species was determined with a haemocytometer and adjusted to $1x10^6$ spores/ml.

When the mixed inoculum was used to inoculate the samples (for treatments with EtOH 70%), aliquots of the inoculum from each single species (with the same concentration as above) were added to a flask followed by vortexing. To determine the viability of the single species, each individual inoculum was also plated on PDA.

6.2.4. Inhibition effect of ethanol on *Penicillium chrysogenum* in liquid medium

Malt extract medium (Scharlau, Spain) solutions (50 ml each) at 1.5% (w/v) were prepared in 100 ml Erlenmeyer flasks closed with cotton plugs with four different ethanol (99.5%, Carlo Erba, Spain) concentrations if 0%, 5%, 10% and 20% (v/v). These concentrations (lower than the ones tested in the following assays) were selected in order to assess the lowest concentration of ethanol that could inhibit fungal growth by constant contact with ethanol, which was expected to be inferior to temporary contact. Each malt extract solution was sterilized by autoclaving and the respective volume of filter-sterilized (0.2 µm cellulose acetate membrane, VWR) ethanol was added to the cooled medium to achieve the required ethanol concentration. The flasks were prepared in triplicate for each ethanol concentration. Each sample of the liquid growth medium was inoculated with 100 µl of *P. chrysogenum* conidial suspension ($1x10^6$ conidia/ml) and incubated statically with diurnal periodicity of light at 22 °C±2 °C. Three blank control samples of uninoculated growth medium were also prepared.

After 3, 7 and 14 days of incubation, fungal inhibition was qualitatively observed and the results recorded as: + (growth), - (no growth).

6.2.5. Microscopic observations of the effect of ethanol on the germination of *Penicillium chrysogenum* conidia

In order to investigate the effect of ethanol on the germination of fungi the following experiment was performed. Eight customized slides were prepared consisting in two overlaid microscope glass slides attached with autoclave tape. From the top slide a 1 cm^2 square was previously cut from the centre, creating a cavity (Figure 6.1). After sterilization of the slides, 50 µl of PDA were added to the cavity of each slide and allowed to solidify. 3 µl of a suspension of $1x10^6$ conidia/ml (*P. chrysogenum*) were poured over the PDA and let to dry. 10 µl of each ethanol solution were pipetted on two of the samples over the inoculum.

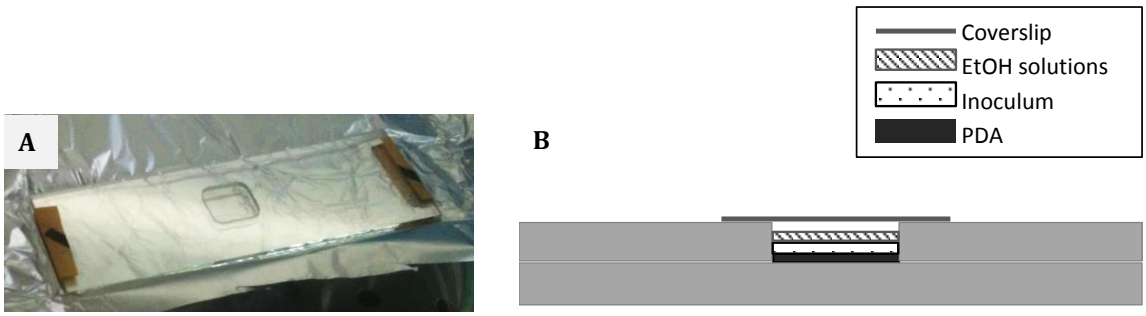


Figure 6.1: (A) Example of customized slide used for microscopic observation of the effect of ethanol on the germination of *P. chrysogenum* conidia; (B) Scheme of experimental set.

The tested ethanol solutions were: 100/0%; 70/30%; 30/70%; 0%/100% of water (distilled and sterilized)/ethanol (99.5%, Carlo Erba, Spain). These will be referred to as 0% EtOH, 30% EtOH, 70% EtOH, and 100% EtOH, respectively. On one half of the tested samples (one for each ethanol concentration) the treatment solutions were left to evaporate for 1.5 h in a laminar flow chamber before covering with a glass coverslip and sealing with autoclave tape, in order to study the effect of a temporary contact between ethanol and fungal spores. These are referred as Group A: evaporated specimens. The other half were immediately covered and sealed to observe the effect of ethanol in a continuous contact with fungal spores. These are the Group B: non-evaporated specimens. In the evaporation process of ethanolic solutions (Group A), the PDA medium also dried out, which impeded the development of the fungus. To these samples, 10 µl of liquid malt extract medium (Scharlau, Spain) were added.
The samples were observed under a Leica DMR microscope and photographed with a Leica DFC320 camera using Nomarski differential interference contrast optics. Three random areas of each sample were studied. Observations were made directly after preparation of the slides (0 days of incubation) and after 1, 2 and 5 days of incubation.

6.2.6. Effect of ethanol on the development of fungi on paper

Whatman #1 paper discs (45-mm diameter) were numbered, weighed with a Sartorius LE623S precision scale and sterilized by autoclaving.

6.2.6.1. Preventive treatment

Each paper sample was placed in an individual sterile 60 mm diameter Petri dish, and treated by pipetting 300 µl of the ethanol treatment solution (quantity determined empirically as the one required to soak the entire sample) along the periphery of the sample and allowing the solution to migrate to the centre. This method was chosen in order to replicate the one used in the curative assay, where the pipetting in the centre of the sample (location of the fungal colonies) would cause a dispersal of the spores.

The tested treatment solutions were the same as used in the previous assay: 0% EtOH, 30% EtOH, 70% EtOH, and 100% EtOH. Treated samples were kept in individual Petri dishes and dried under continuous vacuum in a desiccator, at 15±5 mBar for 2 h. Petri dishes (60-mm diameter) with PDA medium were prepared. In order to facilitate the extraction of the paper samples for biomass measurement and make sure that the fungal colonies were growing only on the paper and not immersed in the PDA, sterile nitrocellulose membranes (0.45-µm porosity, 47-mm diameter, GVS, Spain) were placed between the PDA and the paper samples (see Chapter 5). Each paper sample was inoculated with 10 µl of a $1x10^6$ conidia/ml suspension and incubated at

22 ± 2 °C, for 3, 7 and 14 in Petri dishes closed with Parafilm®. Three control samples for each experiment variation were kept uninoculated.

6.2.6.2. Curative treatment

Each paper sample was firstly put in individual Petri dishes with PDA, inoculated in the centre (10 µl of a 1x10^6 conidia/ml suspension) and incubated for 3 days (Figure 6.2 (a)). They were then removed from the PDA, placed in clean Petri dishes and dried under vacuum (15±5 mBar) for 20 h (Figure 6.2 (b)).

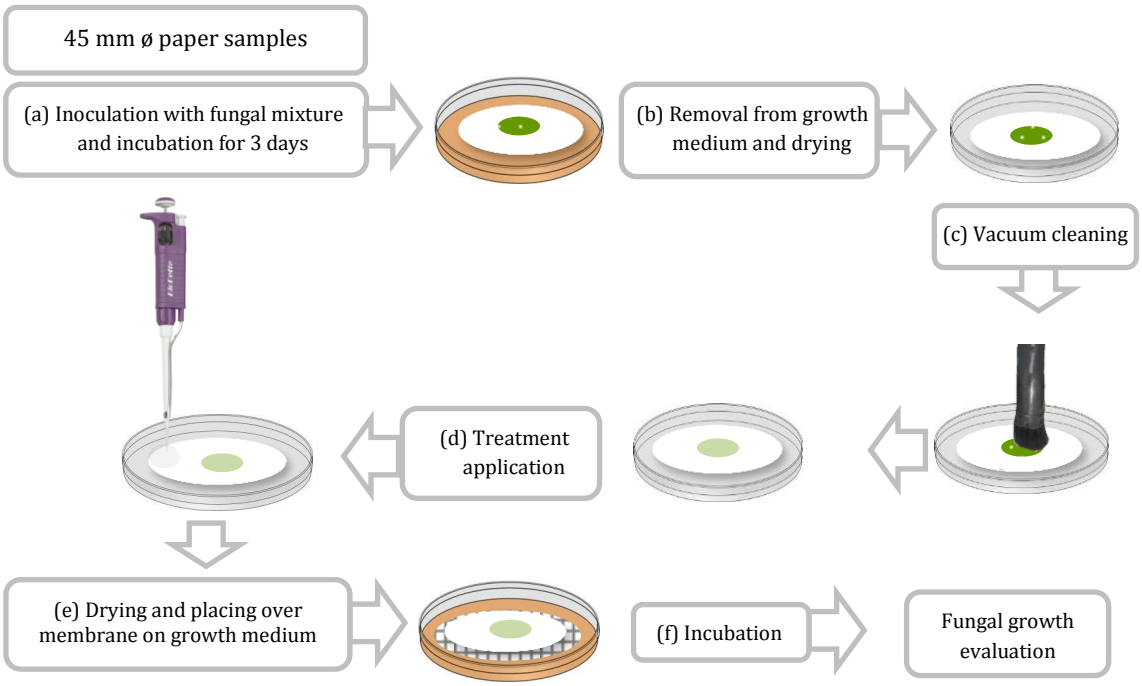


Figure 6.2: Flow chart of experimental setup used for the curative treatment. PDA is represented in brown, paper samples in white circles, fungal colonies in dotted green circles, cleaned fungal colonies in dull green and nitrocellulose membrane in squared white circles.

Prior to treatment, each sample was cleaned with vacuum cleaner (MUNTZ 555-MU-E with PHU-10 smaller brush) for 60 s (Figure 6.2 (c)) (only on the areas with visible fungal development) and weighted afterwards. This intended to simulate a generally used paper conservation practice where the application of ethanol as an antifungal is preceded by drying and superficial cleaning of the fungi (Nittérus, 2000a; Brokerhof et al., 2007). Prior to use, the vacuum cleaner brush attachment was immersed in ethanol at 70% (v/v) overnight and then let dry under laminar flow.

The ethanol solutions were applied in the same way as in the preventive assay (Figure 6.2 (d)). The samples were then transferred to new Petri dishes with fresh PDA, over sterile nitrocellulose membranes (Figure 6.2 (e)) and re-incubated at 22 ± 2°C for 3, 7, 14 and 30 days, in Petri dishes closed with Parafilm®. Three control samples for each experiment variation were kept uninoculated.

6.2.6.3. Fungal growth evaluation

Colony growth and biomass were determined after 3, 7, 14 and 30 days of incubation (in the preventive assay, the experiment was stopped at 14 days of incubation as all the samples were completely colonized at that stage). Since the measurement of biomass is a destructive analysis,

different samples (in triplicate) from each treatment variable were prepared for analysis at each incubation period and selected randomly. Colony growth was determined in the same samples used for biomass quantification at each incubation period.
To quantify colony growth by measuring colonization areas and biomass, the procedures described in Chapter 5 were used. Paper samples inoculated with the mixed inoculum were also observed with a stereo microscope (Leica MZ16) and recorded with a digital camera (Leica ICD) to identify which species were developing.

6.2.7. Evaluation of physical, chemical and optical alterations caused by 70%EtOH on paper samples

7x5 cm Whatman #1 paper samples treated with 660 μl of 70% EtOH (proportional volume to the area of these samples) were analysed before and after treatment and after artificial ageing. Untreated samples were used as controls in all analyses. Artificial ageing tests only give us relative results and cannot perfectly mimic slow natural degradation processes (Bansa, 2002), therefore they were used only with the intent to compare the stability of paper samples with and without the studied treatments without trying to predict lifetimes.

Figure 6.3: Climate chamber used for artificial ageing: closed (A) and open (B) with the suspended samples inside.

Moist heat artificial ageing was performed at 80^0C±0.5^0C and 65%±2% relative humidity (RH) (ISO 5630/3, 1986), for 329 h in a FITOCLIMA 150 EDTU climate chamber (Figure 6.3). Samples were free-hung separated from each other, with a linen thread, which was fastened to the chamber trays leaving the samples suspended (Figure 6.3 B). Before and after artificial ageing, the samples were kept at 22^0C ± 2^0C, 50% ± 1% RH in a desiccator.

6.2.7.1. pH determination

pH measurements were performed using the cold extraction method, according to TAPPI 509 (TAPPI, 2011), using a Docu-pH Meter, Sartorius, with a Py-P22 electrode.

6.2.7.2. Colourimetry

Colourimetry, a very sensitive and non-destructive technique, allows for an evaluation of aesthetic alterations caused by the antifungal treatments. At the same time, alterations in colour may indicate chemical alterations. A decrease in L* and an increase in b* coordinates (darkening and yellowing, respectively), are associated with hydrolysis and oxidation, on cellulose and on its degradation products (Zervos, 2007).

Colour measurements were carried out with a hand-held colorimeter Data Color International® (Figure 6.4 A) with a D65 Standard Illuminant and 10° Standard Observer, following the International Commission on Illumination (CIE) colourimetry system. The CIE L*a*b* colorimetric space is defined according to three coordinates: L*, a* and b*. L* is referred to the level of lightness (0=black, 100=white); a* is referred to the redness-greenness (red: a* > 0, green: a*<0); and b* to the yellowness-blueness (yellow: b* > 0, blue: b* < 0) (Bacci et al., 2003). Samples were measured on top of 4 layers of Whatman #1 paper, using a transparent polyester film template (Figure 6.4 B) to position the colorimeter in the same spots each time a measure was taken. Reported values are the average of 3 samples from which 3 distinct areas were analysed and each area was measured in triplicate.

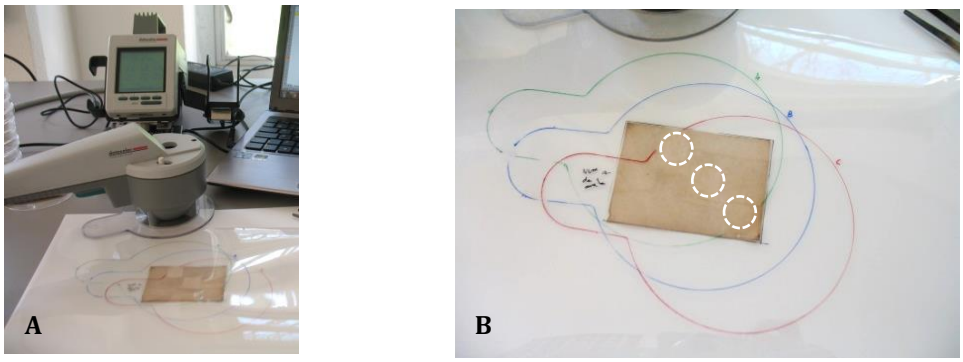


Figure 6.4: Data Color International® hand-held colorimeter (A) and sample under transparent polyester film mask, with the three analysed areas marked with dashed white circles (B).

6.2.7.3. Folding endurance

Folding endurance (log of number of double folds) (FE) has proven to be the most sensitive mechanical property for the detection of changes induced by artificial ageing on paper, and is also the one that better expresses the usability of paper (Zervos, 2007). FE is also highly correlated with the percentage of the hydrolysed glycosidic bonds on cellulose, which endorses its sensitivity to paper chemical decay (Zervos, 2007).

FE was determined according to ISO 5626:1993 (ISO 5626, 1993) with a Köhler-Molin instrument, Lorentzen & Wettre (Figure 6.5). The applied tension was 0.25 kg. 10 replicate samples (15 mm wide strips) were analysed for each type of paper treatment.

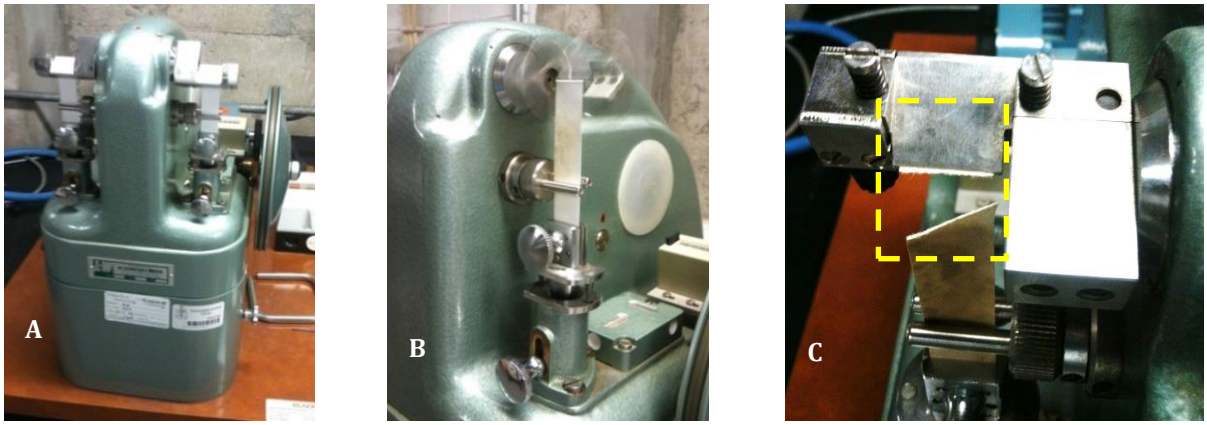


Figure 6.5: Köhler-Molin folding endurance instrument (A); instrument in folding action (B); and paper sample after breaking (C).

6.2.8. Weight variation

Weight variation on the samples was calculated from three replicates for each treatment. The samples were weighed with a Sartorius LE623S precision scale before treatment, one day after treatment and one day after removal from the climate chamber. Prior to weighting, the samples were removed from the desiccator at 50%RH and left to reach equilibrium with the ambient RH in the scale room for 2 h. The RH in the scale room on the 3 different days of analysis was 35%, 38% and 36%RH, respectively.

6.2.9. Statistical analysis

Data were analysed using a t-test when only two treatments were being compared and one-way analysis of variance (ANOVA) when comparing more than two treatments. When significant differences were detected, Fisher's least significant difference (LDS) post-hoc test was used for multiple comparisons (Massart et al., 1998). A significance level of 0.05 was applied using Statistica software v12 (StatSoft, Inc., 1984–2013, Tulsa, OK, USA).

6.3. Results and discussion

6.3.1. Inhibiting effect of ethanol on *Penicillium chrysogenum* in liquid medium

As shown inTable 6.1, all tested concentrations of ethanol in malt extract liquid medium completely inhibited the development of *P. chrysogenum* during the 14 days period. In control samples, without ethanol, fungal development was already observed after 3 days of incubation. These results show that for a direct continuous contact with *P. chrysogenum* spores, percentages of ethanol as low as 5% are enough to inhibit fungal development.

Table 6.1: *Penicillium chrysogenum* development in liquid medium with different ethanol concentrations (v/v).

	Incubation (days)		
[EtOH]	3	7	14
0%	+	++	++
5%	-	-	-
10%	-	-	-
20%	-	-	-

Evaluation: -, no growth; +, growth; ++, intense growth

Similar results were obtained for this species by Dantigny et al. (2005 a), where the constant exposure of *P. chrysogenum* conidia to ethanol vapours at 4% (w/w) concentrations (~5.02% v/v) resulted in a total inhibition of germination during 3 weeks of incubation.

Dantigny et al. (2005 b) determined the minimum inhibitory concentration (MIC) for liquid treatments with ethanol on twelve fungal species, and for all of them the estimated MIC was close to the one obtained for *P. chrysogenum*, in the range 2.14%-6.43% (w/w) (~2.75%-7.82% v/v).

6.3.2. Microscopic observation of the effect of ethanol on the germination of *Penicillium chrysogenum* spores

Table 6.2 summarizes the results from the observation of the germination of *P. chrysogenum* spores under the influence of ethanol. The captured images are presented in Table III.1–Appendix III.

Table 6.2: Effect of different concentrations of ethanol on the germination and development of *Penicillium chrysogenum*.

		Incubation (days)			
Sample		0	1	2	5
A: Evaporated	0%	-	++	+++	+++
	30%	-	++	+++	+++
	70%	-	+	+++	+++
	100%	-	+	+++	+++
B: Non-evaporated	0%	-	++	+++	+++
	30%	-	-	-	+
	70%	-	-	-	-
	100%	-	-	-	-

Evaluation: -, no germination; + germination; ++, hyphae development; +++, intense mycelium development

In samples where the contact with ethanol was only temporary (evaporated samples), initially fewer conidia germinated and mycelial development was less intense in samples treated with 70% EtOH and 100% EtOH than with 0% and 30% EtOH. Nevertheless, the hyphae ultimately colonized the entire samples.

When conidia were in constant contact with ethanol (non-evaporated samples), germination was delayed in the 30%EtOH samples (occurring only after 5 days of incubation and in only a few conidia) and was totally inhibited by 70% and 100% EtOH.

In both cases, there was no promotion of germination caused by ethanol but instead the opposite: conidia germination was either delayed or totally inhibited, depending on the percentage and contact time with ethanol. According to a previous study, only very diluted levels of ethanol (0.5% - 1.5% ethanol) showed a stimulatory effect on *Trichoderma reesei* and acted as conidial activator, whilst in ethanol concentrations greater than 2%, germination was completely inhibited (Sharma, 1992).

The fact that low levels of ethanol are required for complete inhibition of fungal development (as shown above in 6.3.1) in addition to the reversibility of inhibition suggest that the alcohol may be functioning by inhibiting the enzyme(s) required for germination as proposed by Trujillo and Laible (1970).

Gurtovenko and Anwar (2009) studied the interaction of ethanol with biological membranes, and at concentrations below 30.5% (v/v) ethanol induces the expansion of the membranes together with a reduction of their thickness, as well as causing disorders and enhancement of the interdigitation of lipid acyl chains. However, the bilayer structure of the membranes is maintained.

6.3.3. Preventive antifungal effect of different ethanol concentrations on paper inoculated with *Penicillium chrysogenum*

The same ethanol concentrations and fungal species used in the previous experiment were tested on paper samples, where the paper was firstly treated and then contaminated, simulating a preventive paper conservation treatment.

Figure 6.6 shows that the treatment of paper samples with ethanol did not have a significant effect on *P. chrysogenum* development.

The only significant differences in fungal growth were obtained after 3 days of incubation in the samples treated with 0%EtOH, which show a significantly higher (p<0.05) colonized area than all the other treatments. This greater colonized area in samples treated with 0%EtOH was not accompanied by a significantly higher biomass value. It was likely due to a spreading of the spores in the more hydrated and swollen paper fibres structure caused by this aqueous treatment.

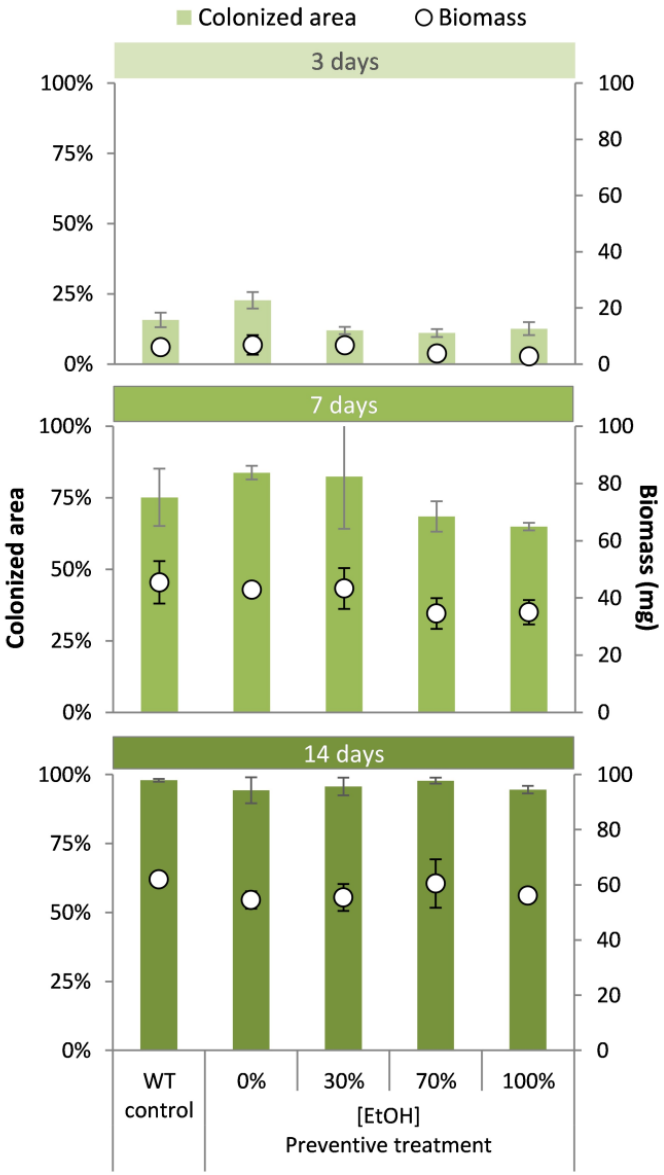


Figure 6.6: Biomass (circles) and colonized area (bars) measurements at 3, 7 and 14 days of incubation for *P. chrysogenum* growing on paper treated preventively with different ethanol concentrations. WT stands for without treatment control. Values are the average of 3 replicates per treatment ±SD.

After 7 and 14 days of incubation no significant differences between treatments could be detected in colonized area and biomass results (p>0.05). At 14 days of incubation all samples were completely colonized as shown in Table 6.3.

Ethanol under appropriate conditions can form complexes with cellulose, which can remain stable for long periods in anhydrous conditions or under a high vacuum (Arney and Pollack, 1980). However, these complexes are not stable when moist, and merely the moisture in the air can completely displace the solvent (Arney and Pollack, 1980). The process of drying the samples under vacuum after treatment, together with the elevated moisture from the PDA, must have been displaced the ethanol molecules from the paper and no significant antifungal effect is retained.

6.3.4. Curative antifungal effect of different ethanol concentrations on paper colonized by *Penicillium chrysogenum*

The results obtained from the simulation of a curative treatment, where paper samples were firstly colonized by fungi and subsequently treated with ethanol, are shown in Figure 6.7.

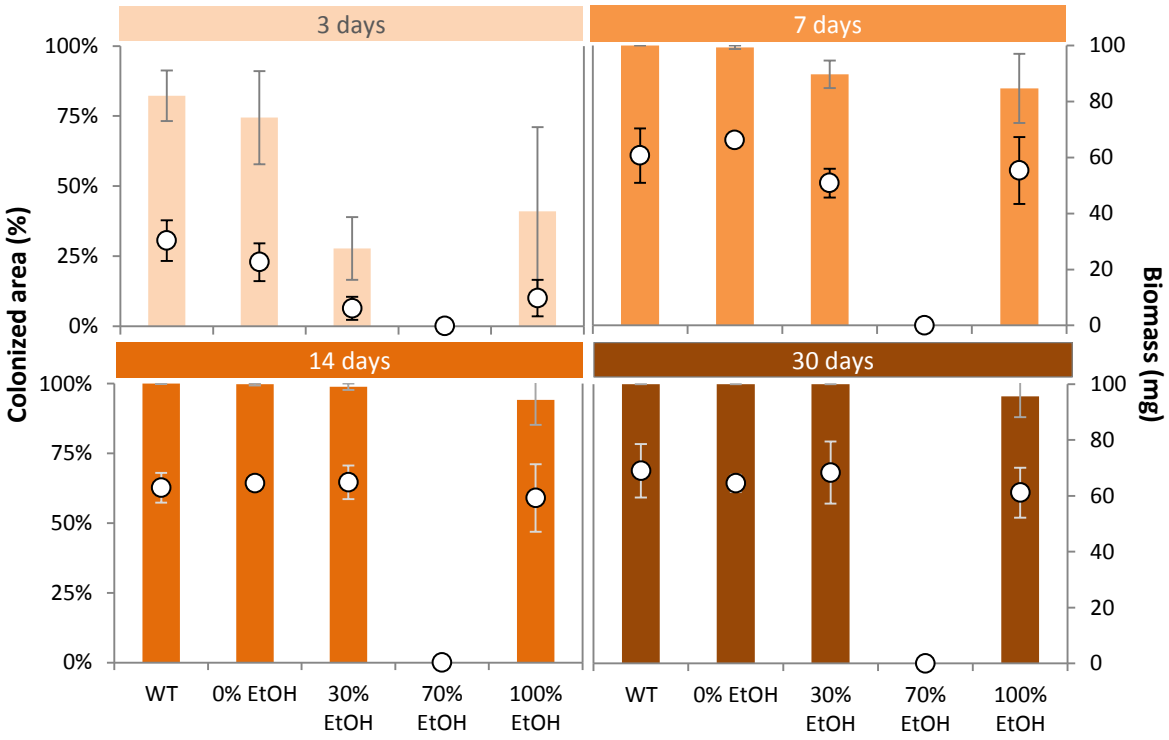


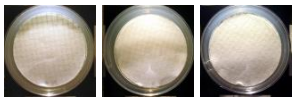
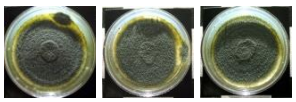
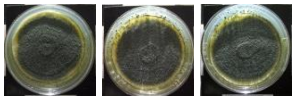
Figure 6.7: Results from the curative treatment of ethanol on paper colonized by *P. chrysogenum* at 3, 7, 14 and 30 days of incubation. Colonized area measurements represented in bars and biomass measurements in circles. Values are the average of 3 replicates per treatment ± SD.

While in the control samples without treatment (WT) and in the samples treated with 0%EtOH, *P. chrysogenum* quickly resumed growth, the samples treated with the different ethanol concentrations showed a delay or a total absence of fungal development (Figure 6.7). This is most likely due to an irreversible inactivation of vegetative cells caused by the alcohol (Trujillo and Laible, 1970), whereas the subsequent growth probably originates from spores.

The best antifungal effect was obtained with 70%EtOH where no fungal growth was observed along the duration of this assay (30 days). As the spores of *P. chrysogenum* were not able to

develop at all when returned to conducive conditions (Figure 6.7 and Table 6.3), this points out to a sporicidal effect of 70%EtOH on this particular species.

Table 6.3: Photographic comparison between the preventive and curative treatments of paper samples inoculated with *Penicillium chrysogenum* and mixed inoculum at 14 days of incubation.

Treatment	*P. chrysogenum*		Mixed inoculum
	Preventive	Curative	Curative
Uninoculated control			
Without treatment			
0% EtOH			
30% EtOH			
70% EtOH			
100% EtOH			

The antifungal effect of 30%EtOH was similar to the one obtained with 100% EtOH. The differences in colonized area and biomass obtained from these two treatments were not statistically significant at any incubation period (p>0.05). The antifungal effect of both these treatments was only partial, as there were surviving fungal structures that were able to subsequently colonize the samples.

These results are in accordance with the literature, where it is reported that the greatest microbiocidal effect of ethanol is reached at concentrations of 60-70% (Block, 2001:236; Paulus, 2004:445; Fraise et al., 2013:37). This optimal concentration is likely related to the mechanism of action of ethanol as an antimicrobial, which is believed to derive mainly from protein coagulation/denaturation, resulting in disruptions of cytoplasmic integrity, denaturation of essential proteins, interference with metabolism, and cell lysis (Block, 2001:231; Fraise et al., 2013:145). Protein coagulation/denaturation occurs only within certain concentration limits around an optimal alcohol level. In the absence of water, proteins are not as readily denaturated (Block, 2001:231; Fraise et al., 2013:145). These factors may afford an explanation why absolute ethanol, a dehydrating agent, is not as effective as water-ethanol mixtures. Accordingly, 70% concentration must be within the mentioned optimal alcohol level range for microbial protein denaturation.

When comparing the curative and the preventive treatments, we can conclude that after an initial colonization, even if the samples are thoroughly cleaned, the re-growth is much faster and intense than the initial colonization. This observation is clear when we compare the fungal

growth after 3 days of incubation in the WT samples in Figure 6.6 (preventive) and in Figure 6.7 (curative), where the colonized area and biomass are 5 times higher in this last one. Several factors can justify this effect: a higher number of spores in the paper matrix after colonization than the one present in the initial inoculum; a spreading of the spores with the cleaning process; the resumption of growth of already established mycelium; and/or physical-chemical alterations in the substrate rendering it more susceptible to fungal growth.

Also, as shown in Table 6.3, the colonized surface of the samples is more heterogeneous after the curative treatment, likely due to the development of more individual colonies with different phenotypes, and/or antagonizing interaction between individual colonies.

6.3.5. 70%-EtOH treatment of paper inoculated with a mixture of 5 fungal species

The ethanol concentration that led to optimal antifungal results with *P. chrysogenum* (70% EtOH), was tested with a mixed inoculum consisting of five fungal species (*A. niger*, *P. chrysogenum*, *P. corylophilum*, *Ch. globosum* and *Cl. cladosporioides*).

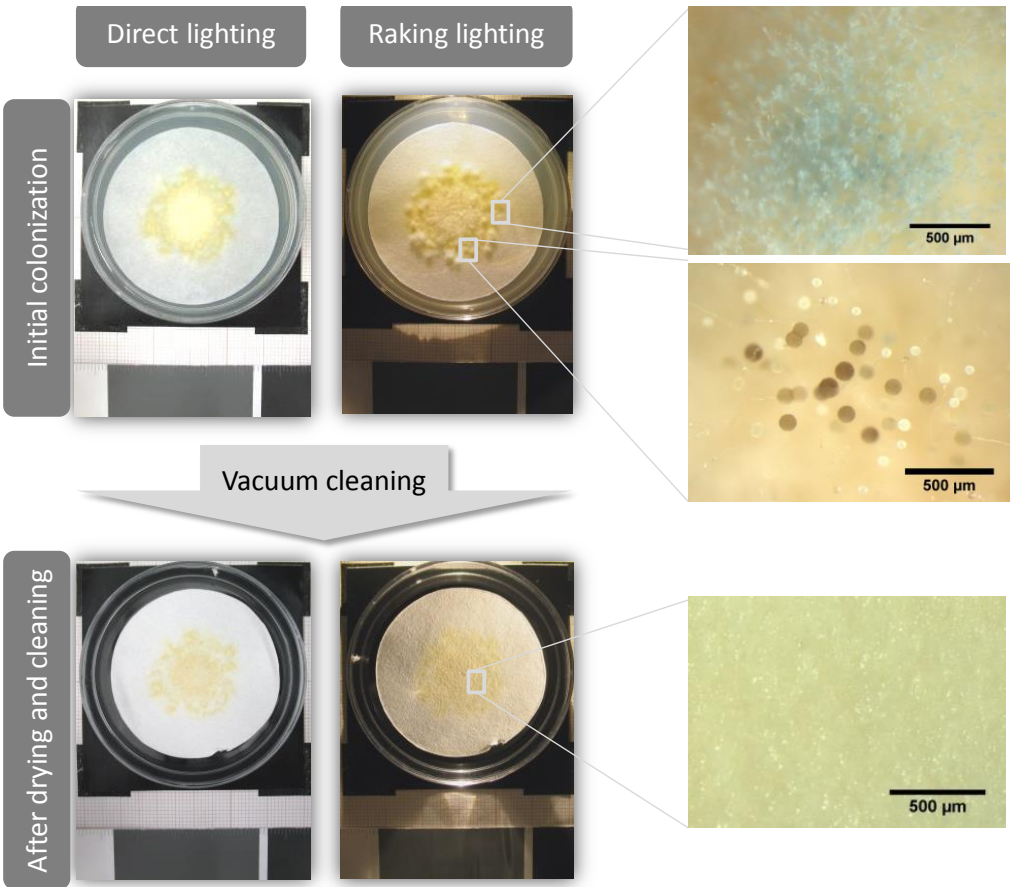


Figure 6.8: Example of paper samples inoculated with the fungal mixture before and after vacuum cleaning. On the right: binocular microscope images, scale bar=500 µm.

Paper samples inoculated with the mixed inoculum were firstly incubated for 3 days. The only species that were distinctly growing at that stage were *Penicillium* species (*P. corylophilum* and *P. chrysogenum* were not visually distinguishable at this stage) and occasionally *A. niger* (Figure 6.8). These species grow faster than *Ch. globosum* and *Cl. cladosporioides* (as observed in our

viability control sets, where each individual inoculum was plated before mixing) and through the faster colonization and/or production of inhibiting metabolites, they outgrew the other species. After removal from growth medium, the samples were dried, cleaned (Figure 6.8) and treated before re-incubation.

The results from fungal colonization on samples treated curatively with 70% EtOH are presented in Figure 6.9.

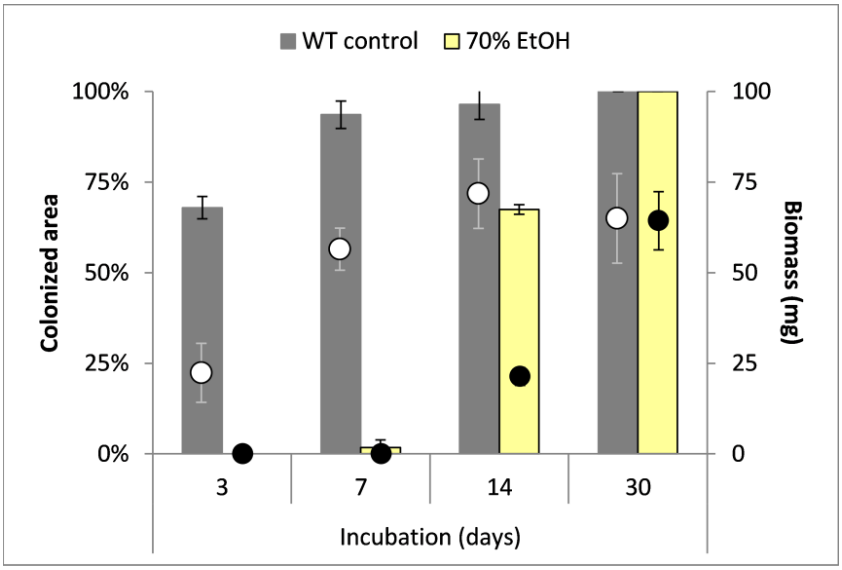


Figure 6.9: Results from the curative treatment with 70% ethanol on paper inoculated with a mixed inoculum composed by *A. niger*, *P. chrysogenum*, *P. corylophilum*, *Ch. globosum* and *Cl. cladosporioides*, at 3, 7, 14 and 30 days of incubation. WT stands for control without treatment. Colonized area is represented in bars and biomass values in circles. Values are the average of 3 replicates per treatment ± SD.

No fungal development was observed on the samples treated with 70% EtOH after 3 days of incubation (Figure 6.9), as in the previous assay with only *P. chrysogenum*. Nevertheless, after 7 days of incubation, fungal growth was already detected (Figure 6.9) although the only fungal species that was developing on the ethanol treated samples was *Ch. globosum* (Figure 6.10).

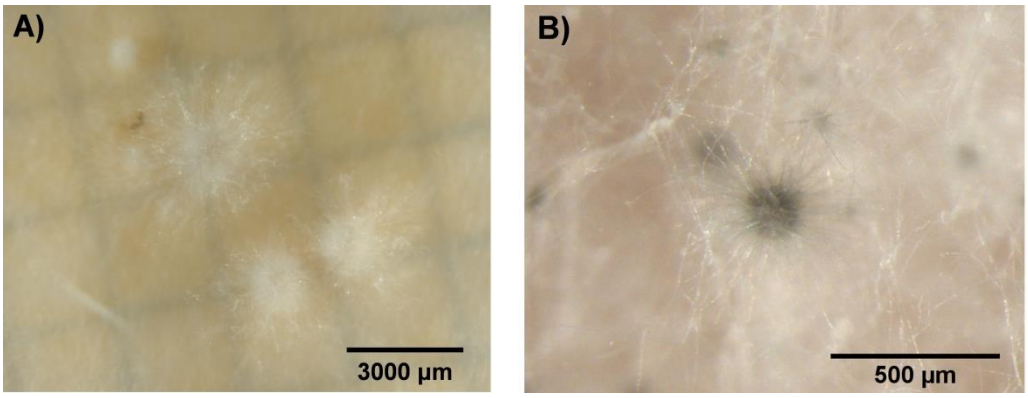


Figure 6.10: Stereo microscope images from paper samples inoculated with fungal mixture after cleaning and curative treatment with 70% ethanol. A): *Ch. globosum* at 7 days of incubation; B): *Ch. globosum* (perithecia detail) at 14 days of incubation.

Ch. globosum initially grows slower than *A. niger*, *P. chrysogenum* or *P. corylophilum* and the samples have not been colonized by *Ch. globosum* before treatment. This could explain why the

control WT samples were already thoroughly colonized by *Aspergillus* and *Penicillium* after 3 days of incubation and no fungal growth was detected on the samples treated with 70% EtOH.

Nittérus (2000a) also observed the survival of *Ch. globosum* and a total inactivation of *A. niger* after a 70% ethanol immersion treatment on paper samples. Bacílková (2006) also reported the inactivation of *A.niger* on paper samples treated with ethanol vapour at concentrations between 30 and 90%, and no re-growth was observed for at least 14 days after the evaporation of the alcohol.

Ch. globosum is the only fungus from the tested mixed inoculum that has ascospores instead of conidia, and ascospores are often resistant to heat, pressure and chemicals (Pitt and Hocking, 2009:14).

Cl. cladosporioides did not grow on these samples inoculated with the mixed inoculum, either before or after treatment, although its viability was positive in the viability control plates. This species was probably inhibited by the other more competitive colonizing species.

6.3.6. The effect of 70% ethanol on paper stability

Colour, pH and folding endurance were measured to evaluate the influence of the 70% EtOH treatment on the optical, chemical and physical properties of paper. The obtained results before and after artificial ageing are presented in Table 6.4.

Table 6.4: Results from the analysis of paper samples without treatment (WT) and treated with 70% ethanol (70%EtOH), before and after artificial ageing.

	Before artificial ageing			*After artificial ageing*		
	WT	**70% EtOH**	*P* value	**WT**	**70% EtOH**	*P* value
CIE L*a*b* colourimetry	L*=97.37±0.04 a*=-0.07±0.01 b*=1.21±0.04	L*=97.35±0.02 a*=-0.06±0.001 b*=1.17±0.06	0.519 0.190 0.462	L*=95.69±0.09 a*=-0.29±0.03 b*=4.23±0.23	L*=95.92±0.24 a*=-0.20±0.06 b*=3.93±0.40	0.193 0.064 0.317
Δ weight (%)	0.27±0.25	1.24±0.18	<u>0.005</u>	0.05±0.19	0.16±0.33	0.641
pH	6.31±0.02	6.31±0.05	1.00	5.05±0.02	5.25±0.02	<u>0.0001</u>
Folding endurance	2.58±0.18	2.57±0.16	0.968	1.82±0.09	1.98±0.15	<u>0.008</u>

The values represent the average of 3 replicates in the case of colourimetry, pH and weight variation and 10 replicates in the case of folding endurance ±SD. *P* values below the confidence level 0.05 are underlined.

Colour measurements (Table 6.4) reveal that artificial ageing caused darkening and yellowing of all the samples. However, no significant differences (p>0.05) could be detected between the colour of the untreated samples and the samples treated with ethanol, either before or after the artificial ageing.

The differences in weight presented in Table 6.4 are the result of the subtraction of the weight after treatment or after degradation from the initial weight. The samples treated with 70% EtOH showed a significantly higher weight gain than the WT ones after treatment. Taking into account

that, according to the literature (Arney and Pollack, 1980), after 10 h at 50% RH ethanol is completely evaporated from this kind of paper, and in this case, the paper was at that RH for ca 20 h; two hypotheses may be presented to justify these results. Firstly, the fact that an ethanol-water mixture was used instead of pure ethanol could alter the evaporation rate from paper; or the water present in the ethanol solution could have reacted with cellulose, creating thermodynamically stable structures that slow down evaporation. Secondly, cellulose, due the hydroxyl groups in its structure, has a strong affinity for materials containing hydroxyl groups, such as water. As a result of the interaction between cellulose and water, the volume of the polymer increases, due to growth of amorphous regions and expansion of crystalline regions, which is associated with weight gain (Khazraji and Robert, 2013). After artificial ageing, the weight of samples returned to initial values, meaning that the changes caused by the treatment were reversed.

The pH values of the WT and 70% EtOH samples were identical on non-aged samples (Table 6.4). With ageing, both samples acidified, although the pH of the WT samples (pH 5.05) was slightly lower than that of the 70%EtOH samples (pH 5.25).
The folding endurance results before degradation for the WT and 70%EtOH samples were analogous. The degradation process caused a mechanical resistance decrease in both types of samples, although the ethanol treated samples show a slightly smaller decline than the untreated samples (Table 6.4). The 70% EtOH solution may have washed out part of the degradation products of paper during treatment, preventing, although at a low scale, its acidification and loss of folding endurance at a long term.

6.4. Conclusions

The present work was undertaken to clarify whether the use of ethanol as an antifungal treatment for paper is appropriate.

According to our results, *P. chrysogenum* was totally inhibited when in continuous contact with concentrations of ethanol as low as 5%. All of the tested ethanolic solutions had a negative effect on conidia germination of *P. chrysogenum*, depending on ethanol concentration and contact time.

The treatments of paper samples with different ethanol concentrations simulating a real paper conservation scenario led to distinct results. While the preventive treatment revealed no significant antifungal effect on treated paper samples, the curative treatment was very effective in impeding fungal development.

All the tested ethanol solutions (30%, 70% and 100%) showed antifungal properties when used as a curative treatment for paper colonized by *P. chrysogenum*. Antifungal activity of the 30% and 100% EtOH solutions was similar, spore germination was initially reduced but not completely inhibited. In the samples treated with 70% EtOH, the antifungal effect was total and no growth of *P. chrysogenum* was observed throughout the 30 days of re-incubation. This indicates a fungicidal activity of the 70% solution as the fungus did not grow after returning to suitable conditions.

When tested as a curative treatment on paper inoculated with a mixture of five of the most common fungal species found on paper based collections, 70% EtOH totally prevented the development of four of the five tested species, although *Ch. globosum* was able to grow after the treatment.

Ethanol at a concentration of 70% caused no deleterious effects on Whatman #1 paper either prior to or after artificial ageing, compared to untreated samples. High purity ethanol (99.5%) was used in these tests. When using commercial ethanol solutions, conservators need to be aware of its purity, as undesired residues may be introduced to the paper matrix.
The presented analyses were performed on standard paper without additives and no writing or painting media were tested. In a real paper document or work of art different materials may be present and undesirable reactions may occur, such as dissolution. Therefore each material should be thoroughly tested before treatment.

Ethanol at 70% has advantageous properties compared to other antifungal treatments generally used in paper conservation. It is readily available in any conservation studio and evaporates quickly leaving no residues that could negatively affect the treated material or human health. The development of resistance of fungi to ethanol is not a significant issue, especially at concentrations used for disinfection (Block, 2001:234). Nonetheless, inhalation of ethanol can cause respiratory tract irritation, and direct contact may cause skin irritation and dehydration, therefore it must be handled using protective clothing and masks or in a fume hood.

7

Study of antifungal and deacidificant formulations to inhibit fungal biodeterioration of paper

The present chapter is divided in three subsections. In each subsection, different formulations with antifungal and deacidificant properties were evaluated.

In Chapter 7A, the formulation using calcium propionate and parabens presented excellent antifungal properties. The preventive treatment of paper samples completely inhibited fungal development on four of the five tested fungal species, and the curative treatment prevented the growth of all five tested species. The long term effects of this formulation on paper were an effective deacidification and prevention of the loss of mechanical resistance, although a slight paper discoloration was observed. This was the only treatment capable of significantly preventing the deterioration caused by fungal metabolites.

In Chapter 7B, the antifungal activity of clotrimazole and limonene was tested against *Penicillium chrysogenum*. While clotrimazole could inhibit over 80% of fungal growth, limonene had no inhibitory capacity and could even enhance the growth of the tested species.

In Chapter 7C, a formulation using clotrimazole as antifungal and calcium hydroxide nanoparticles as a deacidificant was evaluated. Clotrimazole showed antifungal activity against all tested species but its effectiveness varied according to microorganism. With the curative treatment, *Chaetomium globosum* was the only species able to grow and only in part of the tested samples. Although causing a slight discoloration on paper, the deacidification properties of this formulation were confirmed and protected the paper from acidification and loss of mechanical properties at a long term.

7.1. Introduction

According to the literature reviewed in Chapter 2, all existent methods for prevention and treatment of fungal biodeterioration of paper have limitations. When considering chemical methods, treatment with calcium propionate, esters of *p*-hydroxybenzoic acid (parabens), or ethanol, are the ones that combine better antifungal properties with minor health effects and less negative impact in the chemical and physical properties of paper.

Nevertheless, a more thorough research into the effectiveness of these methods and long term effects on paper materials was needed to assure the safeness of its application in paper based cultural heritage objects. The study of ethanol was already presented in Chapter 6. In Chapter 7A, calcium propionate and parabens (methylparaben and propylparaben) were tested against five of the most common fungal species found on paper collections (see Chapter 4). Their

preventive and curative antifungal activities were evaluated, simulating real case scenarios on paper samples. The durability of the antifungal preventive effect was also accessed.

According to the questionnaire presented in Chapter 2, paper conservators consider the study of new non-toxic antifungals as the topic that most needs further research in the field of paper biodeterioration by fungi. This highlights the fact that currently used antifungal methods are not fulfilling all the requirements set by paper conservators, especially regarding their effectiveness and toxicity levels. Taking this perspective into account, this thesis was also aimed at contributing to the development of safer and effective treatments to prevent and treat fungal biodeterioration of paper.

The development of a safer chemical antifungal treatment for paper based cultural heritage had as a main goal the use of low toxicity/low negative health effects chemicals, which could also have a positive impact on paper preservation. The selection of antifungal compounds had as a starting point the already existent antifungal compounds used in the cosmetics and pharmaceutical industries. In cosmetics, antifungals are used as preservatives to prevent product spoilage by development of microorganisms. In the pharmaceutical industry, antifungals have been developed for mycoses (fungal infections) developed in the skin (topical treatment) or in internal organs (systemic treatment). In both industries, these compounds are extensively tested for toxicity before being approved.

Antifungals used as cosmetics preservatives and for topical mycosis treatment are applied to the skin. As dermal contact would also be the primary route of contact between antifungal compounds added to paper documents and the people handling those documents, topical antifungal agents were preferred to oral agents as their mode of reaction would be more similar to the intended treatment of paper materials.

The criteria used for the selection of antifungals for test on paper samples were: low toxicity; paper compatible colouration (colourless or white); colour stability; non-acidity; and solubility in low toxicity non-aqueous solvents. An aqueous treatment was not desired, as it would increase water activity levels in the treated paper, and contribute to raise the relative humidity in the surrounding environment. It could also easily cause tidelines and would take longer to evaporate, increasing the duration of treatment, which is a disadvantage since time is a key factor in an emergency situation.

From the most relevant antifungals considered (see Appendix IV), the most promising ones were clotrimazole and limonene. These two compounds were therefore tested on paper samples at different concentrations, to determine their minimum inhibitory concentration. The results of this study are presented in Chapter 7B. The most effective compound was afterwards studied against a higher number of fungal species, both preventively and curatively in Chapter 7C. To help neutralize the deterioration caused by fungal acidic metabolites (Hastrup et al., 2011), calcium hydroxide, an alkaline compound commonly used for paper deacidification in paper conservation practice, was added to the antifungal compound. The antifungal and deacidificant efficacies of this mixture were evaluated, and the results are presented in Chapter 7C.

To ascertain the safeness of the tested treatments for application on paper based cultural heritage, the short and long term effects of each one of the tested compounds on the chemical and physical properties of paper (colour, pH, folding endurance, and molecular alterations) were evaluated using moist heat artificial ageing. Besides plain paper, paper previously

biodeteriorated by fungi was also tested in order to evaluate the potential of each compound in preventing further biodeterioration by excreted fungal metabolites. *Aspergillus niger* was the species used for colonizing the paper samples since it is a powerful producer of organic acids (Andersen et al., 2009; Borrego et al., 2012), and therefore a good subject for testing the deacidification capacity of the tested deacidificant compounds. To the best of found knowledge, this is the first time that artificial ageing is performed on paper previously colonized by fungi, to evaluate the effect of compounds on fungal biodeterioration.

As mentioned before, this Chapter is divided in three subsections – Chapter 7A; Chapter 7B and Chapter 7C. In each of these subchapters, the results concerning different tested formulations are reported, but the common materials and methods to all the three subchapters are described below.

7.2. Materials and Methods

The paper used as test samples, the fungal strains and the procedure used for inoculum preparation was the same as in Chapter 6.

7.2.1. Preventive treatment of paper samples

For the preventive antifungal activity tests, the same type of paper samples and Petri dishes, the number of samples per Petri dish, and inoculum concentration used by Neves et al. (2009) were replicated to allow for the comparison with this study.

Whatman #1 paper discs (55 mm diameter) were sterilized by autoclave, dried for 2 hours at 65^0C, immersed in the respective treatment solution for 60 seconds and let to dry inside a vertical laminar flow chamber for 1 hour. In Neves et al. (2009), the samples treated with solvent alone, contrarily to the other samples, were not let to dry, but immediately inoculated and incubated. In the present experiments, the solvent treated samples were dried in the same way as all other samples. Control samples were kept untreated.

The antifungal activity of the treatments was assessed against each fungal species individually and against a mixture of the five fungal species (Figure 7.1).

Sterile glass Petri dishes (ø=15 cm) with PDA were prepared. In each dish 3 replicate samples were placed over the PDA and inoculated at the centre with 50 µl of the respective $1x10^4$ spores/ml inoculum (Neves, 2006; Neves et al., 2009). Petri dishes were closed with plastic paraffin film (Parafilm M®, Bemis NA, USA) and incubated at 22°C with diurnal periodicity of light.

Fungal growth was assessed by measuring the area of the colonies developing over the paper (as described in Chapter 5) at 2, 4, 8, 15 and 30 days of incubation.

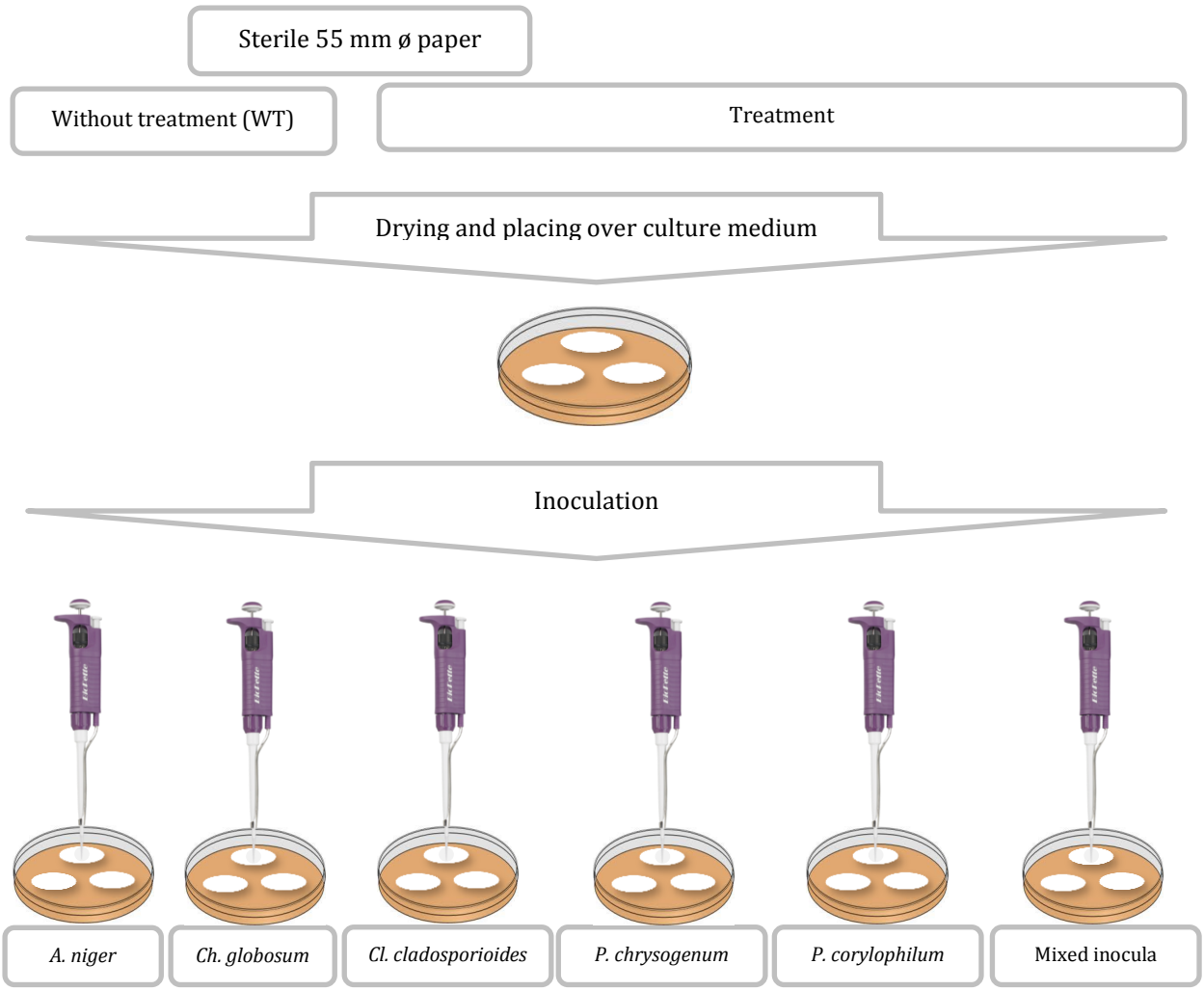


Figure 7.1: Flow chart of experimental setup used for the preventive treatment.

7.2.2. Curative treatment of paper samples

Regarding the curative treatment, the protocol followed by Neves et al. (2009) to simulate a real-life situation, consisted in grouping all samples in a Petri dish and applying a high volume of inoculum (300 µl) randomly over the stacked samples. The incubation was performed without culture medium in a Petri dish inside a desiccator at high relative humidity (97%±2) until fungal development was visible. The treatments were applied afterwards in the same way as in the preventive treatment. Although the protocol by Neves et al. (2009) can simulate a real situation of fungal development on paper, it was difficult to replicate rigorously, as the inoculum quantity and distribution varied among samples and the incubation period is not specified. Therefore, since a direct comparison with the data obtained by that study could not be obtained, a slightly different protocol was developed to enable the quantification of fungal growth by determination of dry weight biomass, besides radial growth. This protocol was identical to the one used in Chapter 6, with a minor change: the 10 µl inoculum concentration was $5x10^4$ spores/ml in order to match the number of fungal spores used in the preventive treatment assays.

The samples used in the curative treatment tests (45 mm diameter) were smaller than the ones used for the preventive treatments (55 mm diameter), in order to fit the 47 mm membranes necessary for biomass measurement, as described in Chapter 5.

7.2.3. Fungal growth measurement

Fungal growth was assessed by measuring the area of the colonies and/or measuring fungal biomass, as described in Chapter 5.

The percentage of growth inhibition was calculated from the following formula (Equation 7.1), adapted from Ansari et al. (1990), where A_{WT} is the average colonized area on the control sample without treatment (WT) and A_T is the average colonized area on the treated sample:

$$\% \text{ inhibition} = \frac{A_{WT} - A_T}{A_{WT}} 100$$

(Equation 7.1)

7.2.4. Detection of physical-chemical alterations on treated papers

7.2.4.1. Sample preparation

7x5 cm Whatman #1 paper rectangles were divided in two groups: pristine samples and biodeteriorated samples. To prepare the biodeteriorated samples, each paper rectangle was previously numbered with grafite pencil and sterilized by autoclave, placed over PDA in a 9 cm diameter Petri dish, inoculated with *A. niger* (50 µl, $1x10^4$ spores/ml) and incubated for 7 days at at 22 ± 2°C (Figure 7.2 A). Control samples were kept uninoculated as a reference for the influence of the culture medium alone in the results. Afterwards, the samples were removed from the growth medium (Figure 7.2 B), dried overnight inside a laminar flow chamber and vacuum cleaned for 60 seconds each (Figure 7.2 C).

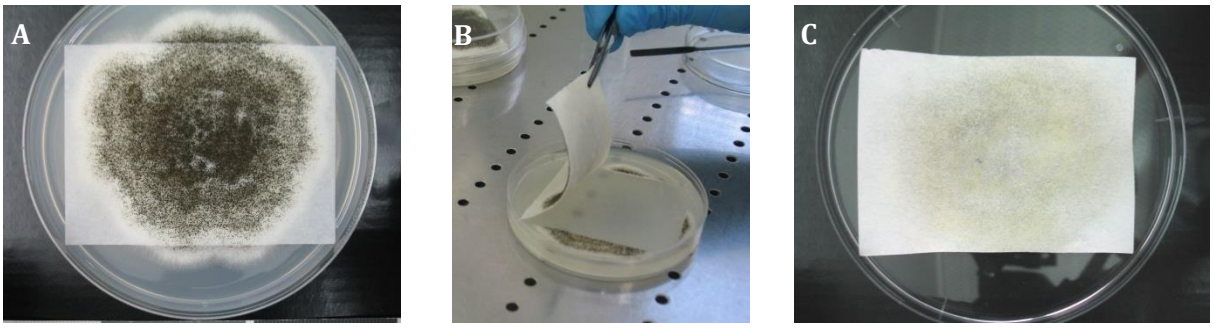


Figure 7.2: Example of biodeteriorated sample preparation: after inoculation with *A. niger* and incubation (A); removal from growth medium (B); after drying and vacuum cleaning (C).

Each pristine or biodeteriorated sample was treated with 660 µl of treatment solution. Control samples were kept untreated (WT samples).

7.2.4.2. Artificial ageing, pH measurement, colourimetry, folding endurance and statistical analyses

Artificial ageing, pH measurements, colourimetry, folding endurance and statistical analyses were performed as described in Chapter 6.

The colour difference (ΔE) was calculated by the formula:

$$\Delta E_{Lab} = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$$

According to (Mokrzycki and Tatol, 2011), a standard observer does not notice the difference in colour when $0<\Delta E<1$; only an experienced observer can notice the difference in colour when $1<\Delta E<2$; an unexperienced observer already notices the difference when $2<\Delta E<3.5$; a clear difference in colour is noticed when $3.5<\Delta E<5$; and when $\Delta E>5$, the observer notices two different colours.

7.2.4.3. Infrared spectra

Molecular alterations were monitored by Fourier Transform Infrared Spectroscopy (FTIR). Infrared spectra from paper samples were obtained with a Bruker FTIR spectrometer (Alpha), using a diamond single reflection attenuated total reflectance (ATR) device, to allow for a non-destructive localized analysis. Triplicate spectra per sample collected on different areas were acquired with 24 scans per spectrum at a spectral resolution of 4cm-1 in the wavenumber range from 4000 to 400 cm^{-1}. Three replicate samples were analysed for each treatment variant. Baseline correction was performed at 4000, 3700, 1962, 1800, 1486, 1182 and 400 cm^{-1}. The spectra were normalized at the 1159 cm^{-1} band (pristine samples) or 1147 cm^{-1} band (biodeteriorated samples) using OriginPro software, version 9.0.

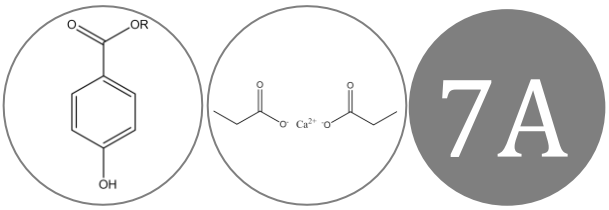
Infrared spectra from reference compounds in powder form were obtained with an Agilent Technologies 4300 Handheld FTIR in ATR mode. Spectra were acquired with 24 scans per spectrum with spectral resolution of $4cm^{-1}$, covering the 4000 to 650 cm^{-1} range.

7.2.4.4. X-ray diffraction

X-ray diffraction (XRD) technique was used to identify any crystalline products formed by reaction between the various compounds, the paper and the fungal metabolites. XRD analyses were performed with a PANalytical X'Pert PRO MPD X-ray diffractometer equipped with X'Celerator detector, with a CuKα radiation source (wavelength 1.540598 Å).

7.2.4.5. X-ray fluorescence spectrometry

Micro-energy dispersive X-ray fluorescence spectrometry (μ-EDXRF) was an auxiliary method for punctual detection of elemental composition of the used materials. μ-EDXRF analyses were carried out using a portable ArtTAX 800, Bruker spectrometer , with a Mo target X-ray tube. The primary X-ray beam diameter is approximately 70 μm, focused by means of polycapillary X-ray optics. Spectra were acquired for 120s, with excitation at 40 kV and a 300 μA current, and He purging.



Parabens and calcium propionate

A deacidificant/antifungal mixture composed of 5% calcium propionate, 0.5% methyl paraben and 1% propyl paraben was tested against *A. niger*, *Cl. cladosporioides*, *Ch. globosum*, *P. chrysogenum* and *P. corylophilum*. The preventive treatment of paper samples resulted in a complete inhibition of fungal growth on 4 of the 5 tested species. The antifungal properties of the formulation remained unaffected for a minimum period of one year. The curative treatment was even more successful, with a total elimination of all tested fungal species.

Artificial ageing on treated paper samples revealed an effective deacidification and long term prevention of mechanical resistance loss, although causing paper discoloration. However, on paper previously colonized by fungi, the treatment effectively prevented the deterioration caused by fungal metabolites,.

7A.1. Introduction

In 2009, Neves et al. (2009) tested the use of a multipurpose deacidificant/antifungal mixture composed of 5% calcium propionate, 0.5% methyl paraben and 1% propyl paraben to treat fungal biodeterioration of paper. The results were very promising as a total inhibition of fungal development on the two tested species (by parabens) and an effective deacidification effect (by calcium propionate) were obtained. This formulation could therefore constitute a multi-purpose solution for the treatment of paper documents affected by acidity and fungal colonization. Nevertheless, further studies needed to be performed to assure the effectiveness and safeness of this method on paper collections: more paper colonizing fungal species should be tested and the long term effects on paper should be evaluated.

Calcium propionate (CP) (Figure 7.3) is one of the most used antimicrobial preservatives in the fermented foods industry, especially in bread, since it does not inhibit yeast growth.

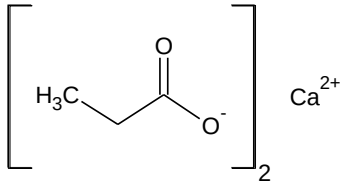


Figure 7.3: Chemical structure of calcium propionate

In aqueous solution, calcium propionate dissociates in propionic acid (the active antifungal ingredient) and calcium ions. The calcium salt is more commonly used than propionic acid itself since it is readily soluble and easier to handle (Hutkins, 2006:275). Propionic acid can only exert its antimicrobial action in its undissociated neutral form, since the fungal cell is impermeable to charged polar molecules such as the anionic deprotonated form of the acid (Hutkins, 2006:275).

The higher the pH above the pKa of propionic acid (4.87) (Suhr and Nielsen, 2004), the higher percentage of the anionic form of the acid is present and therefore, the lower will be its antifungal activity. Propionic acid acts by interfering with the electrochemical gradients in the cell membrane, disrupting the transport processes, and inhibiting the uptake of substrate molecules, such as phosphate and amino acids (O'Connell and Dollimore, 2000).

Calcium propionate is also known for its paper deacidification properties and has been used as a deacidificant agent for paper collections (Zappalà, 1990; Iannuccelli and Sotgiu, 2010; Botti et al., 2011; Bicchieri et al., 2012).

Calcium propionate is considered by the United States Environmental Protection Office to have no teratogenic activity (ability to cause birth defects) or reproductive toxicity (US-EPA, 1991). Even when administered in large doses, propionic acid is excreted in the urine and there is no risk of accumulation in the human body (Paulus, 2004:291).

Parabens are esters of *p*-hydroxybenzoic acid and are among the most common antimicrobial agents in pharmacy and cosmetics' industries, due to their low toxicity, pH range of activity, good stability and minimum secondary effects (Nguyen et al., 2005; Zhang et al., 2005).

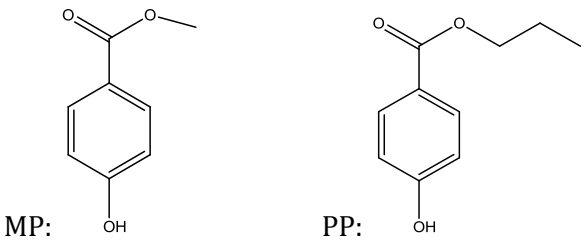


Figure 7.4: Chemical structures of methylparaben (MP) and propylparaben (PP).

At low concentrations, parabens selectively inhibit the proton motive force across the microbial cell membrane, and at high concentrations they affect the membrane permeability, causing leakage of intracellular constituents (Russell, 2003). The antimicrobial efficacy of parabens increases with increasing chain length, while its water solubility decreases correspondingly. Also, parabens can become inactive by micellization with non-ionic surfactants (Paulus, 2004:277).

Parabens can be used singly on in combinations and the most used ones are methyl and propyl paraben (Figure 7.4) (Soni et al., 2005).

As calcium propionate, paraben also exert their anti-microbial effect in an undissociated state. Since the pKa of methylparaben and propylparaben are 8.2 and 8.4, respectively, they are still active in slight alkaline media (Soni et al., 2005). This makes them suitable for use in paper conservation practice, where acidic treatments are not appropriate, due to the high susceptibility of cellulose to acid hydrolysis.

According to the Scientific Committee on Consumer Products (SCCP) from the European Commission, several studies have proven parabens to be practically non-toxic, not carcinogenic, not genotoxic and not teratogenic (SCCP, 2005). However, in the past few years, the detection of parabens in some breast tumour tissue samples has engaged a controversy on the possible oestrogenic hazards of these compounds. Some studies conducted thereafter claim that the previous ones fail to consider the metabolism and elimination rates of parabens, which are dose,

route, and species dependent (Soni et al., 2005). The Scientific Committee on Consumer Products - European Commission (SCCP), reported in 2005 that "viewing the current knowledge, there is no evidence of demonstrable risk for the development of breast cancer caused by the use of underarm cosmetics" (SCCP, 2005). Meanwhile, SCCP continued to repeatedly review scientific data regarding the potential health effects of parabens over the years (SCCP, 2008; SCCS, 2011, 2013). Currently, it is of general agreement that parabens have a weak estrogenic activity, and this activity appears to increase with increasing chain length (SCCS, 2011). However this activity is thousands to millions of times weaker than the activity of natural hormones (Routledge et al., 1998; SCCS, 2011), and as such, it is considered biologically implausible that parabens increase the risk of any oestrogen-mediated endpoint, including effects on the male reproductive tract or breast cancer (Golden et al., 2005). All the *in vivo* studies performed until now suggest that the impact of parabens on the organism reproduction, development and homeostasis seems to be of marginal importance, and currently parabens continue to be widely used not only as preservatives in cosmetics and pharmaceutical products, but also in food commodities and industrial products (Błędzka et al., 2014). For all these reasons, it seemed plausible to continue the study of parabens for antifungal treatment of paper in the present work.

A review on the use of parabens and calcium propionate as antifungals in paper materials is already presented in Chapter 2. The present work focused on evaluating both the preventive and curative effect of the antifungal/deacidificant mixture on 5 fungal species commonly isolated from paper collections; testing different methods of application; evaluating the durability of the antifungal activity on paper; and testing the chemical and physical short and long term effects on paper.

7A.2. Materials and Methods

7A.2.1. Tested formulations

The supplied reagents were used without further purification: ethanol (CAS No. 64-17-5, 99.5% for analysis, Carlo Erba Reagents); calcium propionate (CAS No. 4075-81-4, 95% Sigma-Aldrich Chemie, GmbH, Germany); propylparaben (n-propyl ester of p-hydroxybenzoic acid, CAS No. 94-13-3, 99% AlfaAesar, Germany); and methylparaben (methyl ester of p-hydroxybenzoic acid, CAS No. 99-76-3, 99% AlfaAesar, Germany).

The concentrations used were the ones that achieved the best results in a previous study by Neves et al. (2009): calcium propionate at 5% (w/v); methylparaben at 0.5% (w/v); and propylparaben at 1% (w/v).

The solvent used for the formulation was ethanol at a 72.15% (v/v) concentration. In Neves et al. (2009) the ethanol concentration is incorrectly designated as 85% (w/w). According to the solvent preparation description in Neves (2006:Anexo IV), the ethanolic solution used was 67.15% w/w, which corresponds to 72.15% (v/v).

The components of the antifungal/deacidificant formulation were tested together and also individually, to evaluate the contribution of each compound to the results obtained with the formulation.

The conventional notations for applied treatments are as follows:

- WT: control samples without treatment
- Solv: Treatment with the ethanolic solvent
- CP: Treatment with calcium propionate solution at 5%
- PBs: Treatment with a mixture of methylparaben (MP) and propylparaben (PP)
- PBs+CP: Treatment with a mixture of calcium propionate, MP and PP

7A.2.2. Influence of different application methodologies

Besides the treatment by immersion for 60 seconds (Im60) (Neves et al., 2009), other treatment variants were tested on paper samples to analyse the variation of chemical compounds intake with time of immersion:

- Pp: treatment by pipetting 450 µl (quantity determined empirically to soak the entire sample) of treatment solution;
- Im0: immersion and immediate removal from solution;
- Im30: immersion for 30 seconds

The differences between treatment variants were analysed by measurements of compound retention (weight variation) and fungal growth (samples inoculated with mixed inoculum).

7A.2.3. Permanence of preventive antifungal effect

18 paper samples (ø=55mm) per treatment were treated following the protocol described in section 6.2.3 (Chapter 6) and kept inside sterile petri dishes (separated by treatment) in a closed desiccator maintained at a 50±5% relative humidity and 22±3°C temperature. Relative humidity control was achieved by a saturated solution of calcium nitrate placed on the bottom of the desiccator. Temperature was controlled with air conditioning equipment. The conditions inside the desiccator were monitored using an EasyLog UBS datalogger, Lascar Electronics.

After 0, 3, 7, 14 and 30 days, and 1 year, three samples from each type of treatment were collected. Each sample was individually placed over PDA in a 90 mm polystyrene petri dish, inoculated with the mixture of five fungal species and incubated at 22°C. Control samples were left non-inoculated. Fungal growth was assessed after 2, 4, 8, 15 and 30 days of incubation.

7A.3. Results and discussion

7A.3.1. Preventive antifungal effect on paper

The results of fungal growth inhibition on paper samples treated preventively (first treated and inoculated afterwards) with Solv, CP, PBs and PBs+CP are presented in Table 7.1.

Table 7.1: Calculated fungal growth inhibition (%) at 4, 8, 15 and 30 days of incubation caused by the different treatments on paper samples. Each value is an average of three replicates ± SD.

		Fungal growth inhibition (%)					
Incubation (days)		*A. niger*	*Ch. globosum*	*Cl. cladosporioides*	*P. chrysogenum*	*P. corylophilum*	Mixed inoculum
Solv	**4**	1 ± 10	4 ± 7	17 ± 9	9 ± 2	-6 ± 5	2 ± 11
	8	10 ± 4	0 ± 0	4 ± 2	3 ± 1	-22 ± 4	9 ± 3
	15	0 ± 0	0 ± 0	-1 ± 7	0 ± 0	0 ± 0	0 ± 0
	30	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
CP	**4**	54 ± 6	-28 ± 6	53 ± 10	26 ± 11	-9 ± 4	17 ± 4
	8	35 ± 2	0 ± 0	46 ± 6	5 ± 8	-13 ± 5	12 ± 4
	15	1 ± 2	0 ± 0	41 ± 4	0 ± 0	0 ± 0	9 ± 8
	30	0 ± 0	0 ± 0	34 ± 5	0 ± 0	0 ± 0	1 ± 2
PBs	**4**	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	8	100 ± 0	100 ± 0	100 ± 0	100 ± 0	93 ± 8	100 ± 0
	15	100 ± 0	100 ± 0	100 ± 0	100 ± 0	58 ± 11	99 ± 1
	30	100 ± 0	100 ± 0	100 ± 0	100 ± 0	8 ± 5	45 ± 48
PBs+CP	**4**	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	8	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0
	15	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	88 ± 11
	30	100 ± 0	100 ± 0	100 ± 0	100 ± 0	84 ± 14	55 ± 20

According to the obtained results, the solvent alone (Solv) did not exhibit a preventive antifungal activity in the paper samples. This is in accordance with the results from Chapter 6, where a similar ethanol concentration was tested.

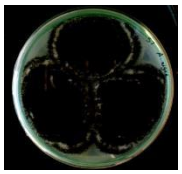
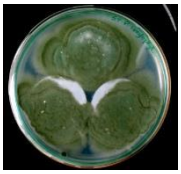
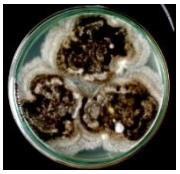
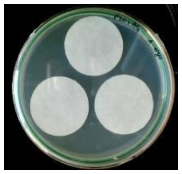
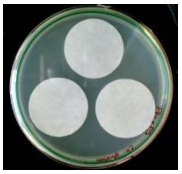
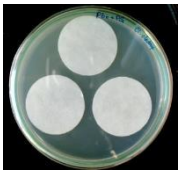
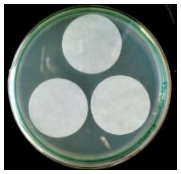
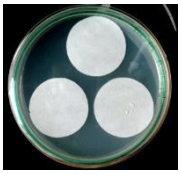
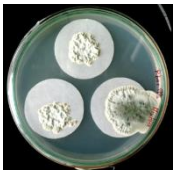
Calcium propionate at 5% (CP) caused an initial delay in *A. niger* development and partially inhibited *Cl. cladosporioides* growth (Table 7.1). For *P. chrysogenum* and *P. corylophilum* no clear antifungal effects were visible with CP treatment, and even an initial enhancement of *Ch. globosum* growth was observed. Neves (2006:30) also obtained a partial inhibition of *Cladosporium* (*Cladosporium* sp.) with CP, although lower (19% at 15 days) than one obtained here (41% at 15 days), and a null inhibition of *P. corylophilum*.

As stated in Chapter 2, the antimicrobial activity of CP occurs in its undissociated state, as only in that form is propionic acid lipophilic and readily soluble in fungal cell membranes where it can exert its antifungal effects (Biggs, 1999). Since the pKa of CP is 4.87 (Suhr and Nielsen, 2004), its activity greatly decreases at pH values above 4.87.

The solution of CP at 5% had a pH~9.0 and the PBs+CP solution a pH~8.4. The paper treated with these solutions, had a pH~7.7 and pH~7.3, for CP and PBs+CP, respectively. At these pH levels, CP is mainly in its ionized state and therefore its antifungal capacity is greatly diminished (Suhr and Nielsen, 2004). Although a lower pH would improve the antifungal activity of CP, treating paper with acidic compounds is not an option in paper conservation practice due to the resulting cellulose degradation by acid hydrolysis.

The antifungal effect of parabens (PBs and PBs+CP) is visible for all fungal species, resulting in a total fungal inhibition of *A. niger*, *Ch. globosum*, *Cl. cladosporioides* and *P. chrysogenum* along the 30 days of incubation (Table 7.1, Table 7.2).

Table 7.2: Pictures of paper samples at 30 days of incubation after preventive treatment.

	A. niger	*Ch. globosum*	*Cl. cladosporioides*	*P. chrysogenum*	*P. corylophilum*	Mixed inoculum
WT						
PBs+CP						

The only species showing tolerance to PBs is *P. corylophilum*, which was able to start colonizing the paper after 8 days of incubation, when inoculated individually, and after 15 days when the mixed inoculum was used. Nevertheless, inhibition of *P. corylophilum* by PBs was still observed, with slower development and smaller colonies than the untreated controls. When tested by Neves et al. (2009), *P. corylophilum* was completely inhibited by the preventive treatment with parabens. This may be due to a strain difference, as individual strains can show some degree of tolerance to antifungals (Vandeputte et al., 2012).

7A.3.1.1 Influence of different methods of preventive treatment application

Different application methods of PBs+CP formulation were tested to evaluate the retention of the compounds by the paper and its influence on antifungal activity. Treatment solutions were either pipetted, or applied by immersion. The immersion varied between a swift immersion (I0), immersion for 30 seconds (I30), or immersion for 60 seconds (I60 – the same immersion time used by Neves et al. (2009)).

The results from the first set of samples where the retention of the compounds was evaluated (non-inoculated samples) are presented in Figure 7.5.

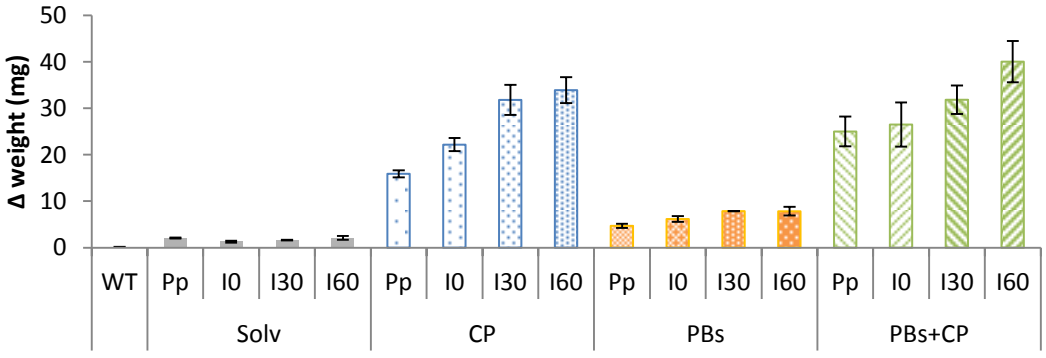


Figure 7.5: Weight variation according to each treatment application method: WT - without treatment; Pp – treatment by pipetting; I0 – swift immersion; I30 – immersion for 30 seconds; I60 – immersion for 60 seconds. Each value is an average of three replicates ± SD.

As shown, CP and PBs retention increases with immersion time. This increase is more pronounced for CP. A swift immersion (I0) is still more effective than application of treatment by pipetting (Pp), which shows a good affinity between CP and PBs and the paper.

The influence of the different retention of compounds according to methodology of treatment observed above was evaluated on the antifungal activity. Figure 7.6 shows the results of fungal growth, measured as colonized area.

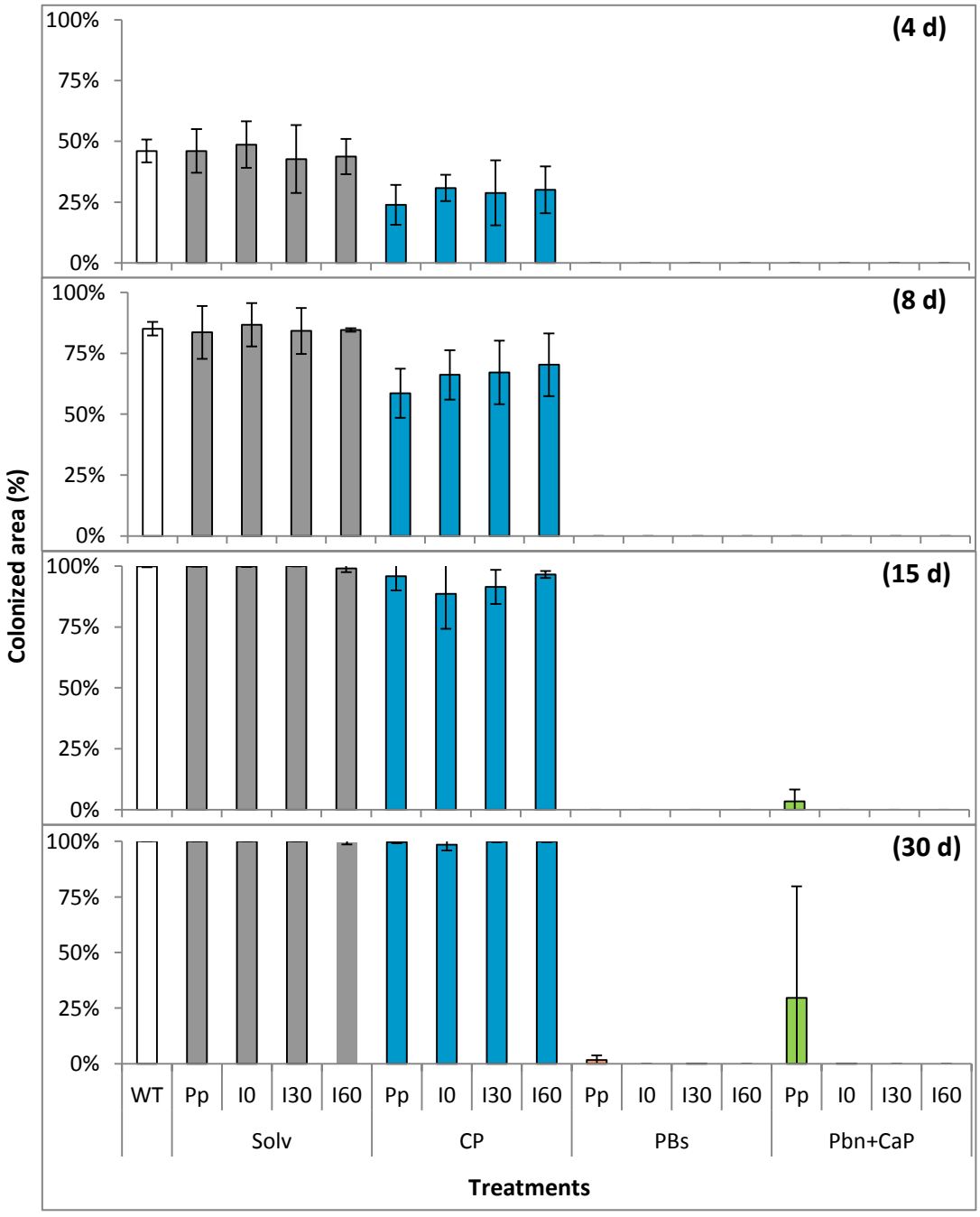


Figure 7.6: Fungal growth measurements at 4, 8, 15 and 30 days of incubation, according to each treatment application method: WT - without treatment; Pp – treatment by pipetting; I0 – swift immersion; I30 – immersion for 30 seconds; I60 – immersion for 60 seconds. Each value is an average of three replicates ± SD.

The variation in Solv or CP application method had no statistically significant effect on fungal growth (mixed inoculum), at every incubation period (p>0.05). The preventive fungal inhibition by the maximum quantity of these compounds (immersion for 60 seconds) was already observed to be null in Table 7.1.

Regarding the variations in application method of PBs and PBs+ CP, although some samples exhibit minor fungal colonization by *P. corylophilum* at 15 and 30 days of incubation (Figure 7.6), the differences between all methods are not statistically significantly (p>0.05).

These results show that, although increasing contact time with formulations results in increasing product retention, this increase does not have a significant reflection on antifungal efficacy.

7A.3.1.2 Permanence of the antifungal effect

The permanence of the antifungal effect of the tested formulations was evaluated over a one year period, against the mixed inoculum. Treated samples were inoculated in the day of treatment (t0) and 3, 7, 14, 30 days and 1 year after treatment (t3, t7, t14, t30, t1year, respectively). The results are shown in Figure 7.7.

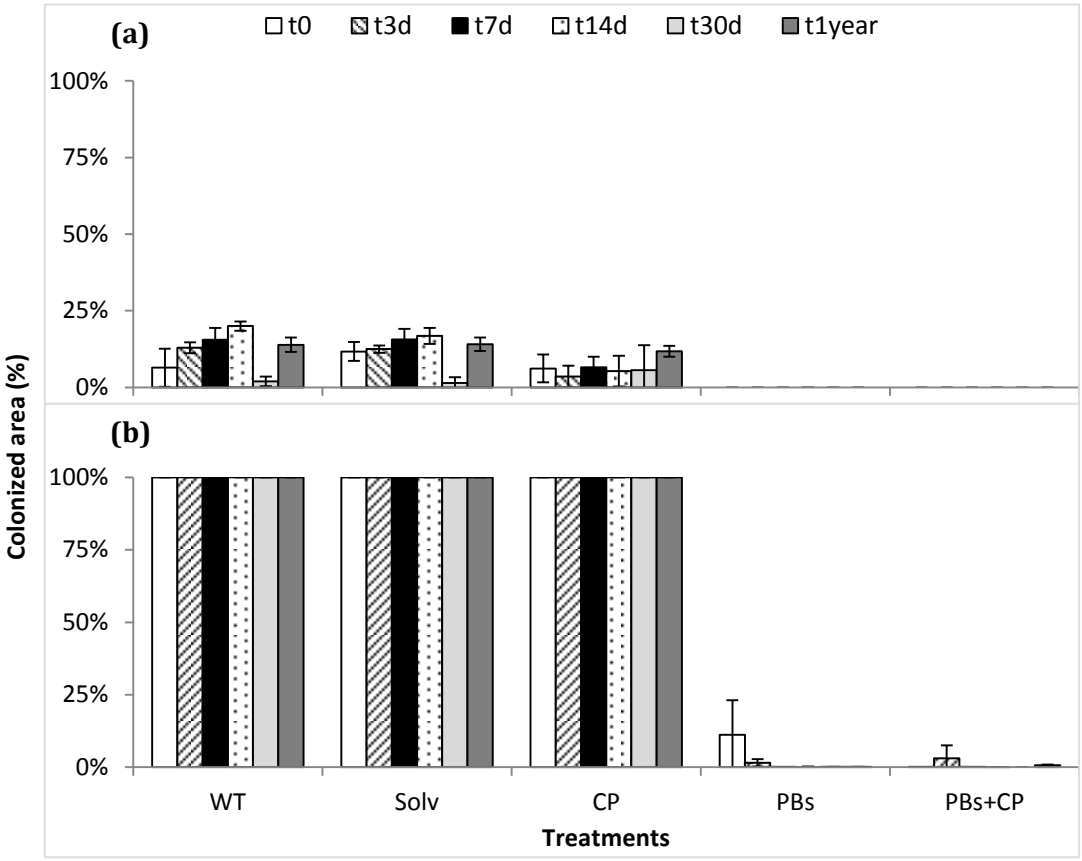


Figure 7.7: Fungal growth occurring at 2 (a) and 30(b) days of incubation, in samples inoculated with mixed inoculum 0, 3, 7, 14, 30 days and 1 year after being treated with solvent alone (Solv), calcium propionate (CP), parabens (PBs) and parabens/calcium propionate mixture (PBs+CP). Each value is an average of three replicates ± SD.

As presented in Figure 7.7, there is no visible tendency of higher fungal growth with higher post-treatment time. One year after treatment there is not a discernible loss of antifungal properties on treated samples. This shows that the applied products maintain their antifungal properties for at least one year.

7A.3.2. Curative antifungal effect on paper

Paper samples inoculated with the mixed inoculum composed of 5 species were firstly incubated for 3 days, after which *A. niger* and *P. chrysogenum* and/or *P. corylophilum* already showed the formation of conidiophores. After removal from growth medium, drying, cleaning and application of treatment solutions, in a simulation of a curative treatment, the samples were re-incubated. The results of fungal growth after re-incubation are presented in Figure 7.8.

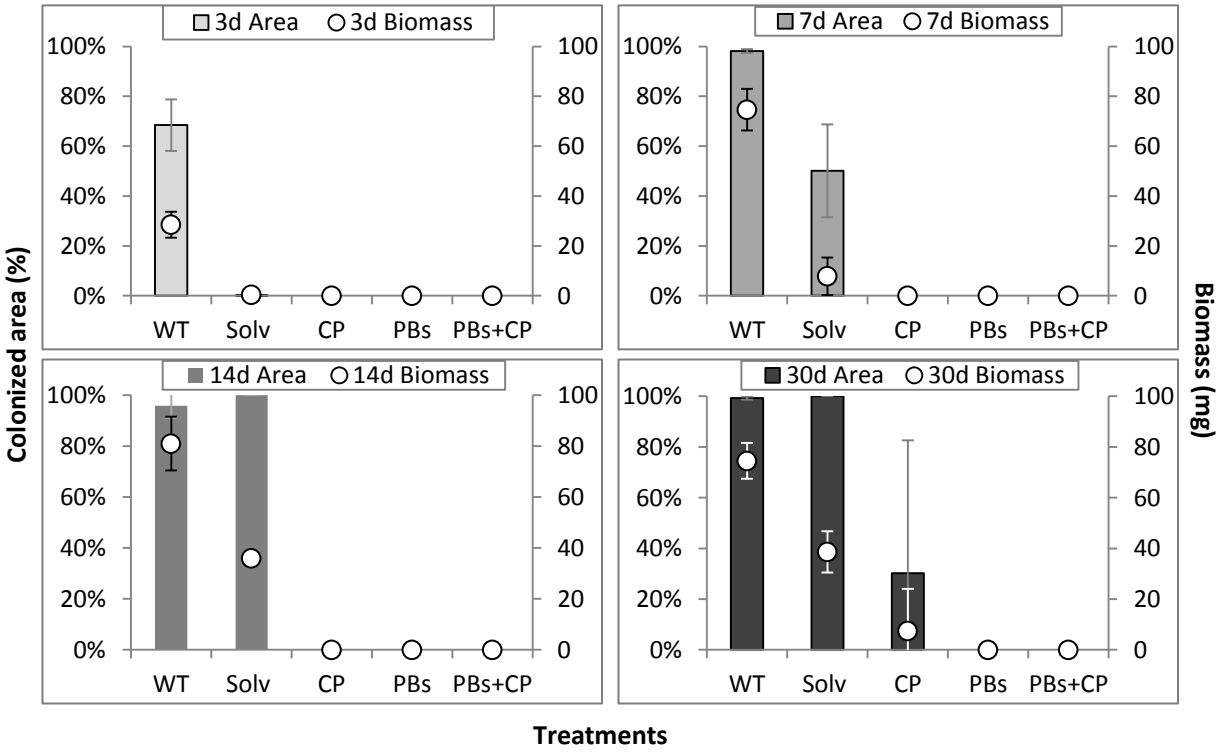


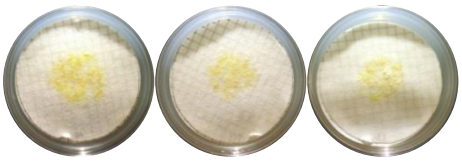
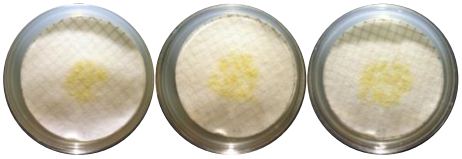
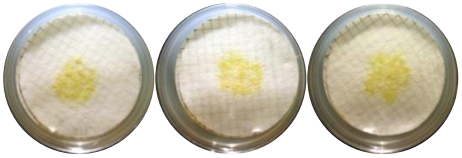
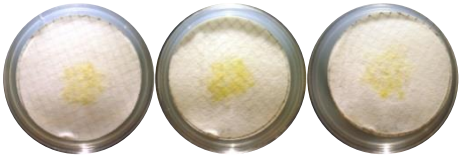
Figure 7.8: Results from the curative treatment of paper inoculated by a mixed inoculum with *A. niger*, *P. chrysogenum*, *P. corylophilum*, *Ch. globosum* and *Cl. cladosporioides*, at 3, 7, 14 and 30 days of re-incubation. Colonized area (represented in columns) and biomass values (represented in white circles) are the average of 3 replicates ± SD.

All curative treatments (Solv, CP, PBs and PBs+CP) exhibit an antifungal effect on the tested species, when compared with WT samples. This is particularly noticeable in the first periods of incubation. Even the solvent alone (72.15% ethanol) had a negative impact on the initial stages of fungal development. Solv samples were statistically different (p<0.05) from WT controls at 3 and 7 days of incubation. These results points out to a harmful effect of ethanol on the mycelium: while in the WT samples, the mycelium quickly resumed growth, in the treated samples new colonies probably had to develop from the surviving spores, with its inherent lag phase in development. This is in agreement with the observations made in Chapter 6.

While on untreated controls (WT) *A. niger* and *Penicillium* were the colonizing species, on treated samples *Ch. globosum* was the only species able to grow (Table 7.3). *P. corylophilum*, which showed tolerance to parabens in the preventive assay, did not survive the curative treatment. This higher resistance of *Ch. globosum* to ethanol and the negative impact of ethanol on fungal mycelium and spores were already discussed in greater detail in Chapter 6.

Calcium propionate, which did not present a significant preventive antifungal effect against the mixed inoculum before, when applied as a curative treatment had a notable antifungal impact. Especially at 7 and 14 days of incubation, the antifungal effect of CP is evident when compared with the solvent alone. This result is likely due to an enhancement of the antifungal efficacy of CP caused by ethanol, which may be acting as a carrier of CP throughout the fungal cell (Zotti et al., 2007).

Table 7.3: Pictures taken with raking light of fungal growth on paper samples at 30 days of incubation after curative treatment.

Treatment	0 days	30 days
WT		
Solv		
CP		
PBs		
PBs+CP		

The samples treated curatively with parabens (PBs and PBs+CP) were completely free of fungal recolonization along the 30 days of re-incubation (Figure 7.8 and Table 7.3). The yellow stains at the centre of the samples shown in Table 7.3 are the result of fungal staining from the prior colonization, which was not removed by the performed mechanical cleaning (see Chapter 6, Figure 6.8).

7A.3.3. Evaluation of physical, chemical and optical alterations on treated paper

Alterations in pH, colour, mechanical resistance and molecular changes on treated paper samples were evaluated to assess any positive or negative impact of the applied treatments on the preservation properties of paper, in the short and long term.

7A.3.3.1. pH results

The pH results of pristine paper (non-biodeteriorated) and biodeteriorated paper treated with the antifungal/deacidificant formulation and its individual components are presented in Figure 7.9.

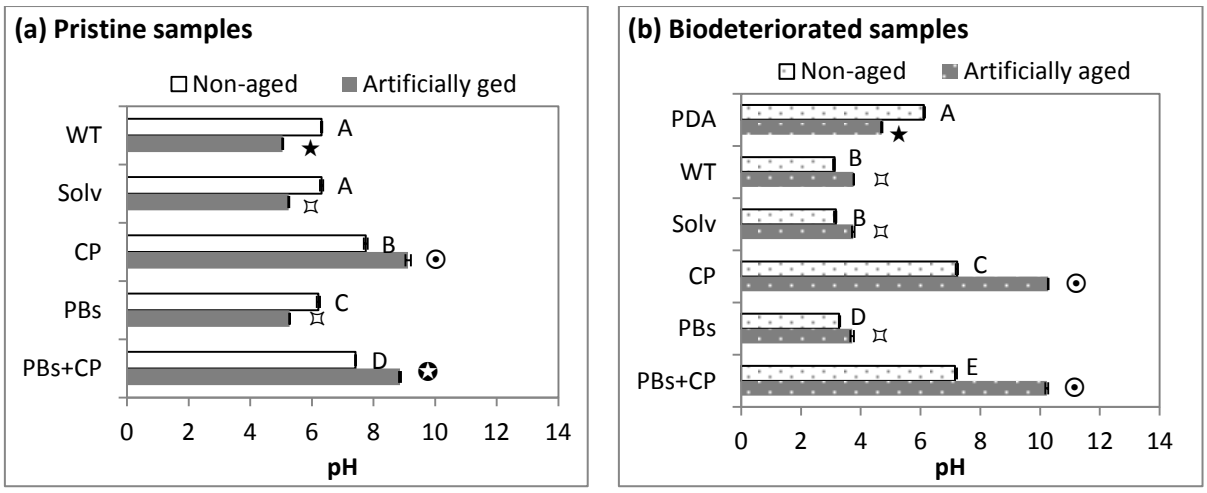


Figure 7.9: Results from pH measurements on pristine (a) and biodeteriorated (b) samples, non-aged and artificially aged. Each value is the average of 3 replicates ± SD. Bars of the same colour followed the same letters (Non-aged) or symbols (Artificially aged) are not significantly different by Fisher's LSD test at α=0.05.

On biodeteriorated samples (Figure 7.9 b) the acidification caused by *A. niger* is notorious when compared with PDA control samples (pH~6), with WT controls having pH values near 3. The treatment with CP successfully increased the pH of biodeteriorated samples to neutral values. Calcium propionate was added to parabens in the tested antifungal formulation mainly due to its already demonstrated deacidification properties on paper materials (Zappalà, 1990; Neves et al., 2009). On both pristine (Figure 7.9 a) and biodeteriorated (Figure 7.9 b) non-aged samples, CP significantly raised the pH of the paper (pH=7.2-7.8), confirming its deacidification properties.

Surprisingly, after ageing CP treated samples show an increase in alkalinity, reaching values near pH=9 on pristine samples and above 10 on biodeteriorated samples. Yanjuan et al. (2013), on the contrary, observed a decrease in the pH of paper treated with CP with artificial ageing, although the performed ageing was different, without humidity (dry-heat degradation at 105 °C). As the pH values obtained on the samples treated with CP after ageing are higher than those of a saturated solution of CP (pH=8.56+/-0.01), this points out to the formation of chemical species with higher alkalinity. This formation is likelly favoured by the high temperature and humidity conditions existent in the artificial ageing chamber (80°C, 65%HR), and by the presence of the excreted fungal metabolites, as the pH is higher in biodeteriorated samples.

In aqueous solution, CP dissociates in propionate and calcium ions (Hutkins, 2006). In the presence of protons, propionic acid is formed (Hutkins, 2006) and hydroxyde ions are produced, as presented in Equation 2.

Equation 2: Mechanism of calcium propionate dissociation in solution and formation of hydroxide ions.

$$\text{(a)} \qquad Ca(CH_3CH_2COO)_2 \leftrightharpoons Ca^{2+} + 2\,CH_3CH_2COO^-$$

$$\text{(b)} \qquad CH_3CH_2COO^- + H_2O \leftrightharpoons CH_3CH_2COOH + OH^-$$

At the artificial ageing conditions the amount of water in the environment is high and may favour the dissociation of CP on the paper. To justify the observed increase in pH, reaction b) from Equation 2 must be displaced to the right. So, either one or both these mechanisms probably occur: free calcium ions may form other complexes, more stable than CP, thus promoting the dissociation of CP according to reacton (a); due to the high humidity and heat, propionate reacts with water forming propionic acid, which evaporates, impeding the reversion of reaction (b). The formation of different calcium compounds was indeed detected by FT-IR analyses on aged samples, accompanied by a reduction in CP characteristic bands (see section 7A.3.3.4). However, further research needs to be done in order to clarify these mechanisms.

Regarding the results obtained with PBs applied individually, these compounds slightly decreased the pH of pristine samples but increased the pH of biodeteriorated samples (Figure 7.9). When artificially aged, both pristine and biodeteriorated samples treated with PBs exhibit a similar pH to samples treated with the solvent, illustrating a neutral effect of PBs on the pH stability of paper with ageing.

Contrarily to what was expected, all biodeteriorated aged samples show higher pH values than non-aged samples (Figure 7.9 b). This is only observed in inoculated samples: non-inoculated control samples (PDA) show lower pH values on aged samples, which points out to an influence of the fungal structures/metabolites on the obtained results. The high temperature and humidity in the ageing chamber could have altered/degraded the acidic fungal metabolites, diminishing their acidic potential.

7A.3.3.2. Colorimetric results

The overall colour alterations (ΔE) for pristine samples are presented in Figure 7.10 and the differences in the individual colour coordinates L*, a* and b* in Table 7.4. The actual coordinates and pictures of the samples are presented in Appendix V.

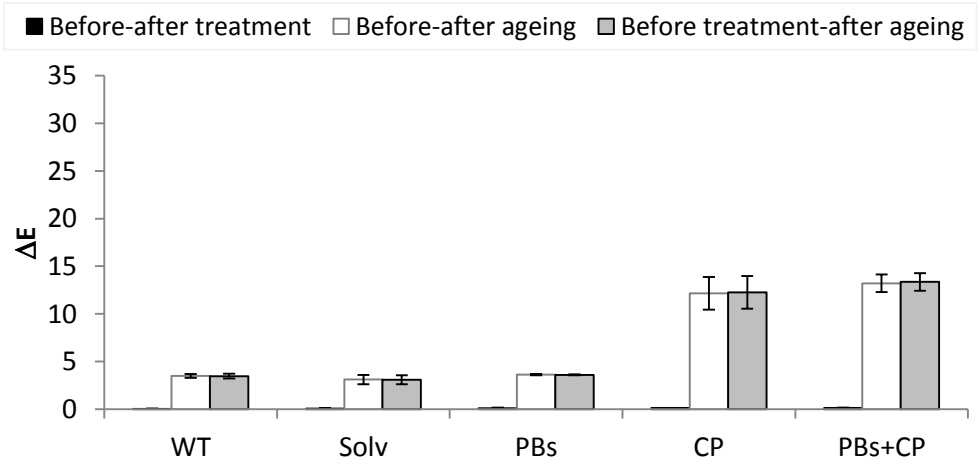


Figure 7.10: Calculated ΔE values before/after treatment; before/after ageing; and before treatment /after ageing, for pristine samples. Each value is the average of 3 replicates ± SD.

After treatment, a slight increase in ΔE was observed for CP, PBs and PBs+CP samples, although these colour changes (ΔE <0.025) are not discernible to the naked eye (Mokrzycki and Tatol, 2011). Neves et al. (2009) also obtained a slight increase in ΔE with the PBs+CP treatment, however they obtained a much stronger yellowing with the PBs component alone. In the present study PBs alone did not cause yellowing (see Δb*, Table 7.4). This difference may be due to a purity or different production matter, since two different product brands were used in both studies.

Table 7.4: Colorimetric alterations calculated for pristine Whatman #1 paper before and after treatments and after artificial ageing. Each value is the average of 3 replicates ±SD. In each column, values followed by the same letters (a, b, c) are not significantly different by the Fisher's LSD test at α=0.05.

	Pristine samples					
	Before - After treatment			Before Treatment – After artificial ageing		
	ΔL*	Δa*	Δb*	ΔL*	Δa*	Δb*
WT	0.03 ±0.04 a	0.00 ±0.01 a	-0.01 ±0.04 a, b	-1.68 ±0.09 a	0.36 ±0.03 a	3.02 ±0.23 a
Solv	-0.02 ± 0.02 a, b	0.01 ±0.00 a	-0.04 ±0.06 a	-1.44 ±0.24 a	0.27 ±0.06 a	2.72 ±0.40 a
PBs	-0.07 ±0.05 b, c	0.03 ±0.00 b	-0.07 ±0.05 a	-1.77 ±0.03 a	0.34 ±0.02 a	3.13 ±0.05 a
CP	-0.12 ±0.01 c	-0.01 ±0.00 c	0.06 ±0.01 b, c	-5.35 ±0.91 b	1.15 ±0.33 b	10.97 ±1.43 b
PBs+CP	-0.08 ±0.06 b, c	0.00 ±0.01 a	0.12 ±0.01 c	-6.54 ±0.39 c	1.72 ±0.07 c	11.53 ±0.85 b

After artificial ageing there were noticeable colour alterations on samples treated with CP (CP and PBs+CP). Looking into Table 7.4 it can be seen that these colour alterations result from a statistically significant increase in all three L* a* b* coordinates for CP and PBs+CP samples, particularly in L* and b*, which translates darkening and yellowing of the treated paper. Yanjuan et al. (2013) also observed darkening and yellowing of paper (newspaper) treated with CP, which were intensified with artificial ageing.

The colour alterations in samples treated with CP were likely caused by oxidation reactions on these alkaline samples, since oxidation is the dominant degradation pathway in alkaline paper (Kolar, 1997; Strlic and Kolar, 2005:121). Oxidized functional groups in cellulose may act as chromophores, being responsible for paper yellowing (Mosca Conte et al., 2012).

Regarding the biodeteriorated samples, the overall colour alteration (ΔE) is presented in Figure 7.11 and the differences in L*, a* and b* are presented in Table 7.5. The values for PDA controls were calculated having as reference untreated pristine paper samples. Like with pristine samples, the actual coordinates and pictures of the samples are presented in Appendix V, for future comparison.

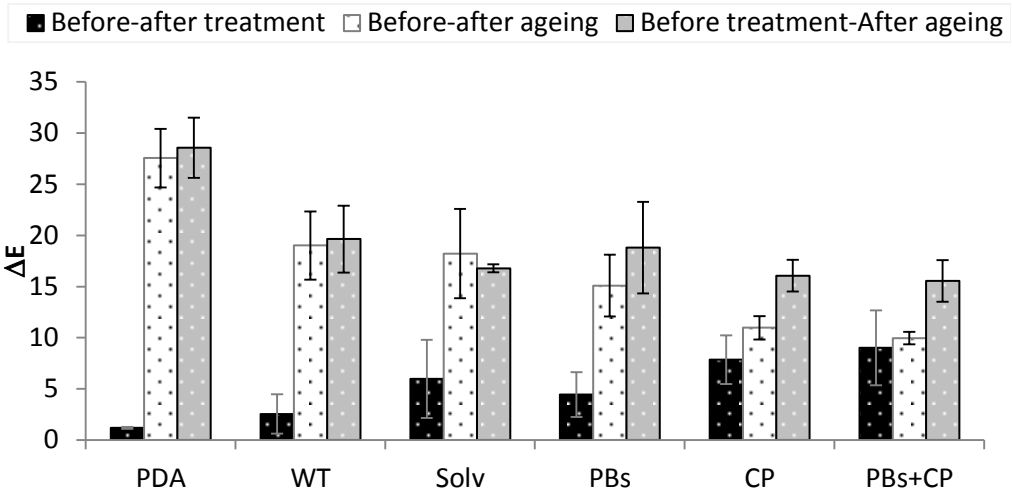


Figure 7.11: Calculated ΔE values for Biodeteriorated samples. Each value is the average of 3 replicates ± SD.

The colour alteration caused by the treatments on biodeteriorated samples (Figure 7.11) was only statistically significant on CP and PBs + CP samples. However, these same samples were the ones that least suffered colour changes with ageing, meaning that the CP deacidificant provided a long term protection against discoloration.

As shown in Table 7.5, CP and PBs+CP samples show a significantly lower darkening (-ΔL*) and a lower increase in magenta (+Δa*) with ageing than the remaining samples. The yellowing (+Δb*) was similar to the one obtained in WT controls.

PBs alone did not cause significant colour changes on biodeteriorated samples as it was similar to Solv and WT controls.

Table 7.5: Colorimetric alterations calculated for Whatman #1 paper biodeteriorated by *A. niger* before and after treatments and after artificial ageing. Each value is the average of 3 replicates ± SD. In each column, values followed by the same letters (a, b, c, d) are not significantly different by the Fisher's LSD test at α=0.05.

	Biodeteriorated samples					
	After treatment - before			After artificial ageing - Before Treatment		
	ΔL*	Δa*	Δb*	ΔL*	Δa*	Δb*
PDA	-0.47 ±0.05 a	-0.08 ±0.01 a	1.09 ±0.07 a, b	-21.69 ±1.25 a	5.86 ±0.50 a, b	17.64 ±1.05 a
WT	-0.71 ±3.15 a	0.27 ±0.24 a, c	0.04 ±1.61 a	-15.09 ±2.13 b	6.15 ±0.32 a	10.91 ±2.82 b, c
Solv	-0.74 ±7.62 a	-0.72 ±0.33 b	0.94 ±2.66 a, b	15.42 ±0.29 b	6.40 ±0.35 a	8.88 ±0.94 b
PBs	-3.84 ±1.81 a, b	-0.69 ±0.43 b	2.07 ±1.32 a, b	-15.26 ±4.46 b	6.61 ±1.05 a	8.53 ±2.40 b
CP	-7.06 ± 1.62 a, b	0.66 ± 0.15 c, d	2.97 ± 2.62 a, b	-8.07 ± 0.79 c	4.91 ± 0.32 b	12.96 ± 1.77 c
PBs+CP	-7.88 ± 2.92 b	1.01 ± 0.29 d	4.09 ± 2.58 b	-7.17 ± 1.01 c	5.38 ± 0.83 a, b	12.69 ± 1.77 c

The colour alteration in non-inoculated controls (PDA controls) after ageing is noteworthy, showing a considerable darkening (-ΔL*), yellowing (+Δb*) and overall high ΔE (Table 7.5, Figure 7.11). These results point out to a degradation of the PDA retained in the paper, which could have contributed to the colour alterations measured in all biodeteriorated samples.

7A.3.3.3. Folding endurance results

Table 7.6 summarizes the folding endurance (FE) results on pristine and biodeteriorated samples.

Table 7.6: Folding endurance results from pristine and biodeteriorated samples. Each value is an average of ten replicates ± SD. In each column, values followed by the same letters (a, b, c, d) are not significantly different by the Fisher's LSD test at α=0.05.

	Folding endurance			
	Pristine		Biodeteriorated	
	Before ageing	After ageing	Before ageing	After ageing
WT control	2.58 ± 0.18 a	1.82 ± 0.09 a	1.97 ± 0.14 a	0.35 ± 0.33 a
Solv	2.57 ± 0.16 a	1.98 ± 0.15 b	1.87 ± 0.13 a, c	0.51 ± 0.29 a, c
PBs	2.00 ± 0.11 c	1.80 ± 0.15 a	1.60 ± 0.12 b	0.72 ± 0.25 c
CP	2.33 ± 0.13 b	2.36 ± 0.27 c	1.97 ± 0.28 a	1.78 ± 0.29 b
PBs+CP	1.97 ± 0.12 c	2.32 ± 0.16 c	1.75 ± 0.20 b, c	1.78 ± 0.23 b
PDA controls			2.77 ± 0.23 d	2.12 ± 0.17 d

PBs treatment caused a slight decrease in FE of paper samples. However, with ageing FE values for PBs are similar or higher than WT controls, for pristine samples or biodeteriorated samples, respectively.

CP and PBs+CP treatments considerably prevented the loss of FE with ageing, for both pristine and biodeteriorated samples. This protective effect is especially remarkable in biodeteriorated samples, where the deleterious effects of fungal metabolites on untreated samples are evident. FE in untreated biodeteriorated controls was practically null after ageing, which illustrates the degradative effect of *A. niger* metabolites at a long term.

One of the main causes for the loss of FE on biodeteriorated samples was probably degradation by acid-catalysed hydrolysis. Oxalic acid, one of the acids produced by *A. niger* (Andersen et al., 2009), has already proven to significantly depolymerize cellulose (Hastrup et al., 2011).

7A.3.3.4. ATR FTIR and XRD results

Molecular alterations on cellulose, or degradation/alteration of the applied antifungal and deacidificant compounds were followed by FTIR and XRD analyses.

Pristine untreated paper samples in Figure 7.13 show the typical infrared spectrum of cellulose (Figure 7.12), with bands at 3332 cm^{-1} (ν OH); 3280 cm^{-1} (ν C-H); 2900 cm^{-1} (ν CH_2); 1635 (adsorbed water); 1427 cm^{-1} (δ C-H); 1314 cm^{-1} (δ C-H); 1219 cm^{-1} (δ C-OH); 1159 cm^{-1} (ν C-C);

1108 cm^{-1} (ν C-O-C); 1052 cm^{-1} (ν C-OH, 2nd alcohol); 1028 cm^{-1} (ν C-OH, 1st alcohol); 998-982 cm^{-1} (ρ -CH-); and 897 cm^{-1} (ν C-O-C) (Garside and Wyeth, 2003; Cocca et al., 2011).

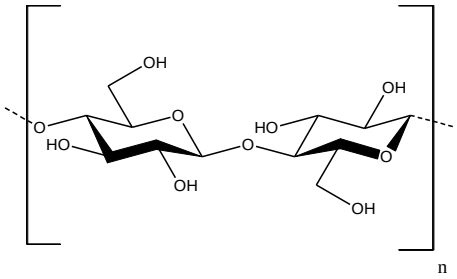


Figure 7.12: Chemical structure of cellulose.

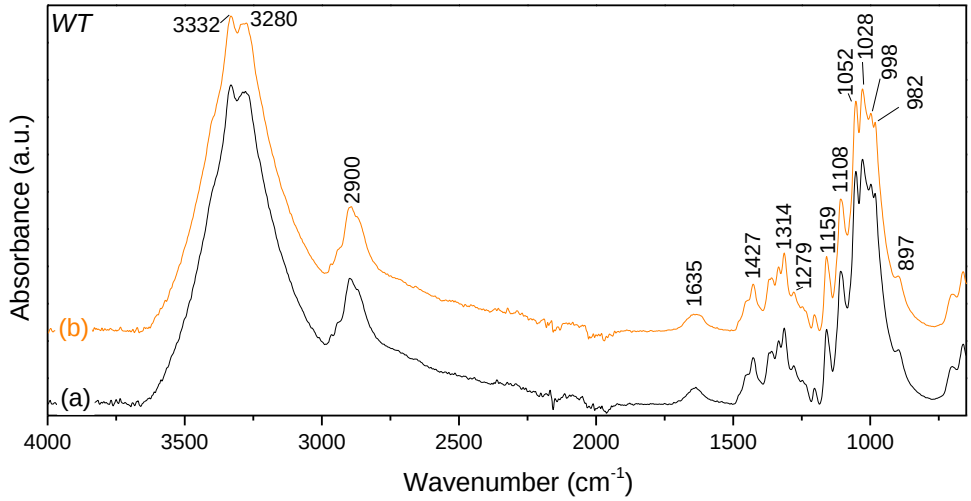


Figure 7.13: Infrared spectra from untreated pristine paper samples (WT) before (a) and after artificial ageing (b).

The infrared spectra of artificially aged WT samples did not differ significantly from that of unaged samples, as shown in Figure 7.13. Notwithstanding that the performed artificial ageing induces a certain molecular degradation on these paper samples, as detected above by the lowering of pH, yellowing and loss of mechanical properties, the results of infrared spectroscopy do not reflect that deterioration. The degradation of cellulose is expected to result in molecular alterations like polymeric scission and formation of carbonyl and carboxyl groups (Cocca et al., 2011). Nonetheless, the detection of carbonyl bands on cellulose infrared spectra is difficult or sometimes impossible since they lie in the same spectral region of the broad band of absorbed and bonded water (1720-1550 cm^{-1} with centre at ~1640 cm^{-1}) (Łojewska et al., 2005; Bicchieri et al., 2006). Since in this case paper degradation was not severe, the carbonyl and carboxyl bands were probably not sufficiently intense to be detected.

The only molecular alterations detected by FTIR in the present study are related with the PBs or CP degradation, and also the presence of fungi in biodeteriorated samples, as will be described below. Higher sensitivity techniques, like Gel Permeation Chromatography (GPC) or Size Exclusion Chromatography (SEC), could be performed in the future to better evaluate the degradation of cellulose polymer in terms of chain scission or reticulation (Zervos and Moropoulou, 2006).

PBs treated samples (Figure 7.14 c) show characteristic bands of propylparaben and methylparaben according to the references in Figure 7.14 a) and b), respectively: 1684 cm^{-1}

(νC=O); 1608 cm^{-1} - 1593 cm^{-1} (νC=C); 1515 cm^{-1} (νC-O); 1435 cm^{-1}(νC-O); 853 cm^{-1} (γC-H); 772 cm^{-1} (γC=O) (Vidyulatha et al., 2012; Kuş et al., 2013). After artificial ageing, those bands disappear and a spectrum similar to untreated samples is obtained (Figure 7.14 d). This indicates a degradation of methyl and propyl paraben when exposed to the applied ageing conditions.

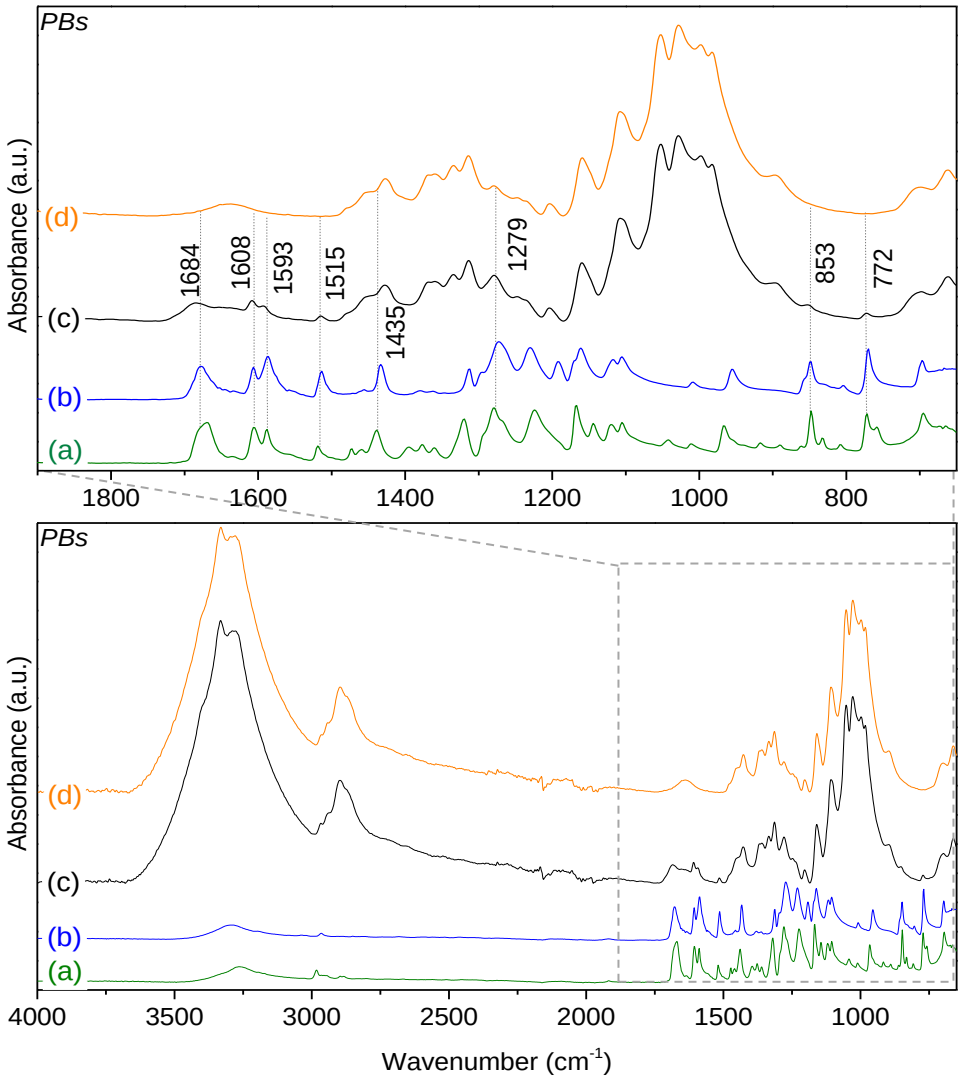


Figure 7.14: Infrared spectra of propylparaben (a) and methylparaben (b) references (both 99% AlfaAesar, Germany) and paper samples treated with parabens before (c) and after artificial ageing (d).

Infrared spectrum of non-aged samples treated with calcium propionate (CP) (Figure 7.15 b), in addition to cellulose absorption bands, reveals characteristic bands of calcium propionate, by comparison with the reference spectrum (Figure 7.15 a): a strong band at 1541 cm^{-1} with a shoulder at 1571 cm^{-1} (both from the carboxylate group) and a small band at 813 cm^{-1} (Rose et al., 2012). The remaining bands of CP reference are coincident with cellulose bands.

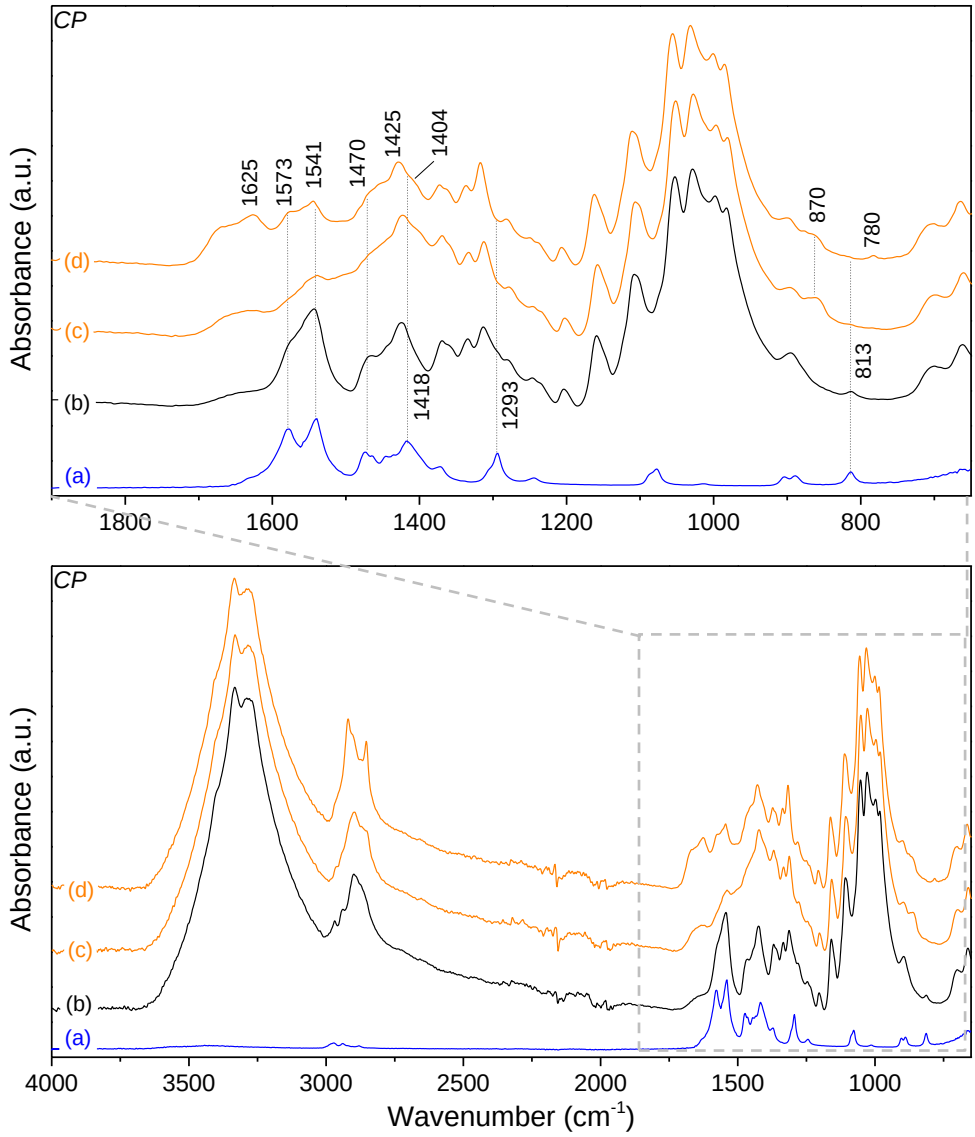


Figure 7.15: Infrared spectra of calcium propionate reference (95%, Sigma-Aldrich Chemie, GmbH, Germany) (a), and paper samples treated with calcium propionate before (b) and after artificial ageing (c; d).

After artificial ageing a decrease of calcium propionate bands was accompanied by the appearance of a band at ~870 cm^{-1} and the increase and broadening of the band centred at 1425 cm^{-1} with a shoulder near 1404 cm^{-1} (Figure 7.15 c). These two bands may be attributed to calcium carbonate (Figure 7.16 a), and would therefore indicate the dissociation of CP and formation of carbonate with the free calcium ions. According to the literature, thermal degradation of CP results in the formation of calcium carbonate, at temperatures above 300 °C in dry conditions (O'Connell and Dollimore, 2000; Valor et al., 2002). However, the presence of humidity could have contributed to the formation of $CaCO_3$ at the lower temperatures used in the artificial ageing assay (80°C).

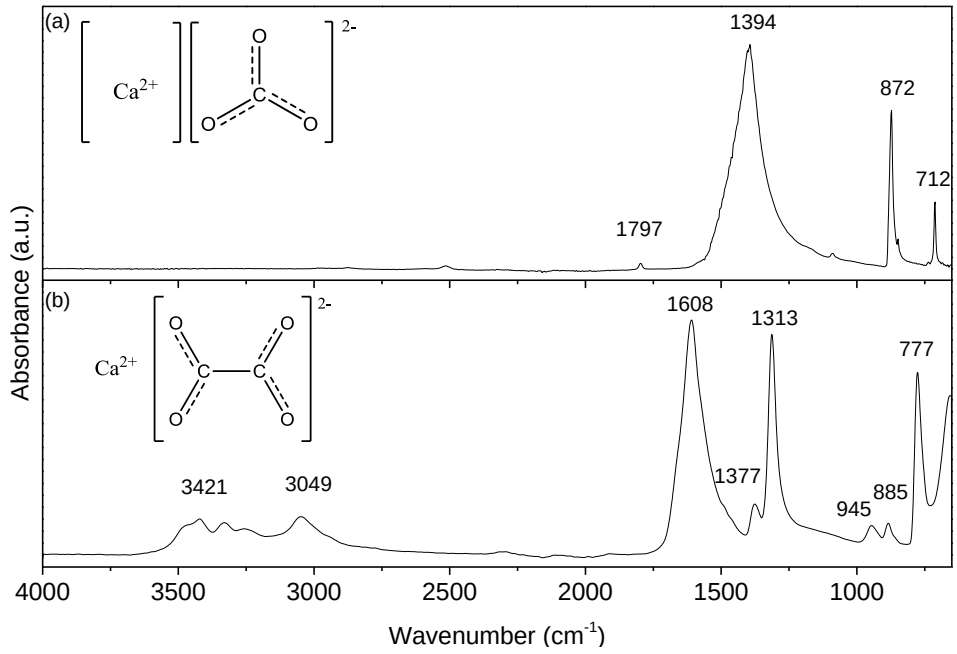


Figure 7.16: Infrared spectra of reference samples of (a) calcium carbonate (98%, Chalk from Champagne, Kremer Pigments); and (b) Calcium oxalate monohydrate (≥98.0%, Fluka).

On part of the analysed areas of samples treated with CP and aged, an interesting phenomenon was observed, the appearance of small bands at 1625 cm^{-1} and 780 cm^{-1} (Figure 7.15 d). These two bands may be attributed to calcium oxalate (Pinzari et al., 2010) (Figure 7.16 b), as a product of the reaction between calcium from CP and oxalic acid. Oxalic acid can result from the degradation of cellulose itself (Bansa, 2002; Jiang et al., 2016) and can also be copiously produced by *A. niger* (Andersen et al., 2009). Although pristine samples were not in direct contact with biodeteriorated samples, they were all in the same environment during artificial ageing. Oxalic acid, aided by high temperature, relative humidity and air circulation in the ageing chamber may have volatilized and migrated to the pristine samples.

These results could illustrate the influence that a fungal contaminated object can have on the surrounding uncontaminated objects, even after the inactivation of the fungi, where the volatile metabolites can migrate and react, causing deterioration. This is the first time such phenomenon is detected; however, further analyses should be performed at environmental temperatures to confirm the occurrence of such migration at environmental conditions.

To confirm the presence of $CaCO_3$ and calcium oxalate in CP treated samples, X-ray diffraction (XRD) analyses were performed. However, only the diffraction pattern of cellulose (Park et al., 2010) was obtained in CP samples (Figure 7.17) as demonstrated by the typical diffraction peaks at 14.5-14.9°, 16.5° and at 22.7°(Hajji et al., 2016). Not even calcium propionate was detected, probably due to a matter of crystallinity as this analytical technique is used for identification of the crystalline phase composition of materials.

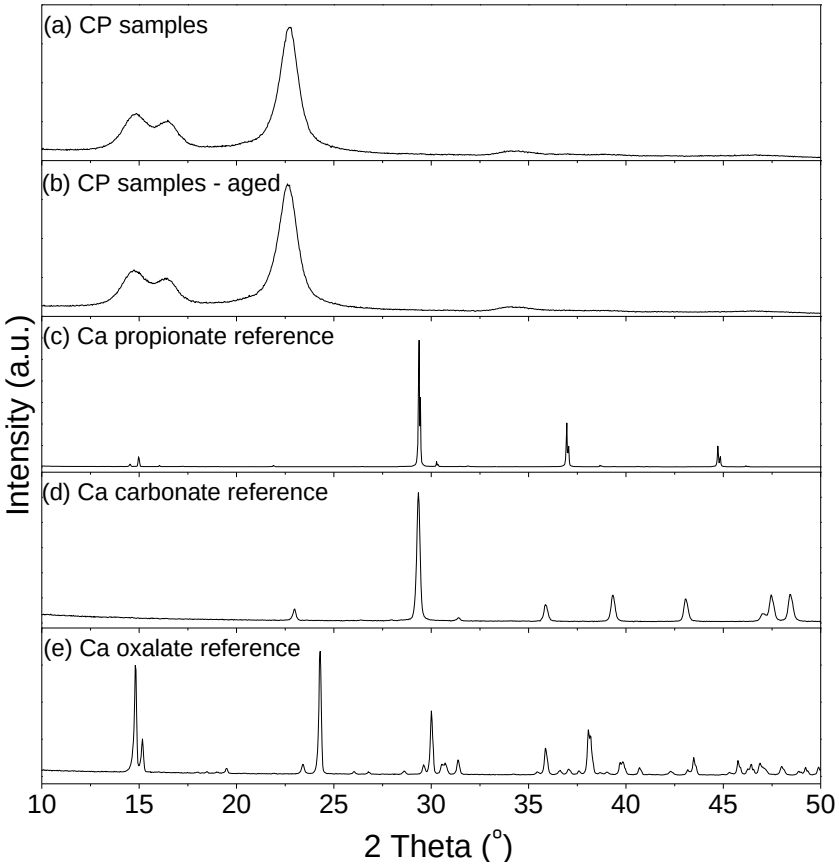


Figure 7.17: X-ray diffractograms obtained from CP samples before (a) and after artificial ageing (b) and reference compounds: (c) calcium propionate (95%, Sigma-Aldrich Chemie); (d)calcium carbonate (≥99%, Scharlau, S. L); and (e) calcium oxalate monohydrate (≥98.0%, Fluka).

The spectrum of paper samples treated with the parabens and calcium propionate formulation (PBs+CP), (Figure 7.18 a) show bands of both PBs (1681 cm^{-1}; 1605 cm^{-1}; 850 cm^{-1}; 773 cm^{-1}) and CP (1573 cm^{-1}, 1541 cm^{-1} and 814 cm^{-1}). With ageing, the bands attributed to parabens disappear, and similarly to what was obtained with CP samples, CP bands diminish their intensity and bands attributed to $CaCO_3$ (~870 cm^{-1}) and calcium oxalate (1625 cm^{-1} and 780 cm^{-1}) appear (Figure 7.18 b).

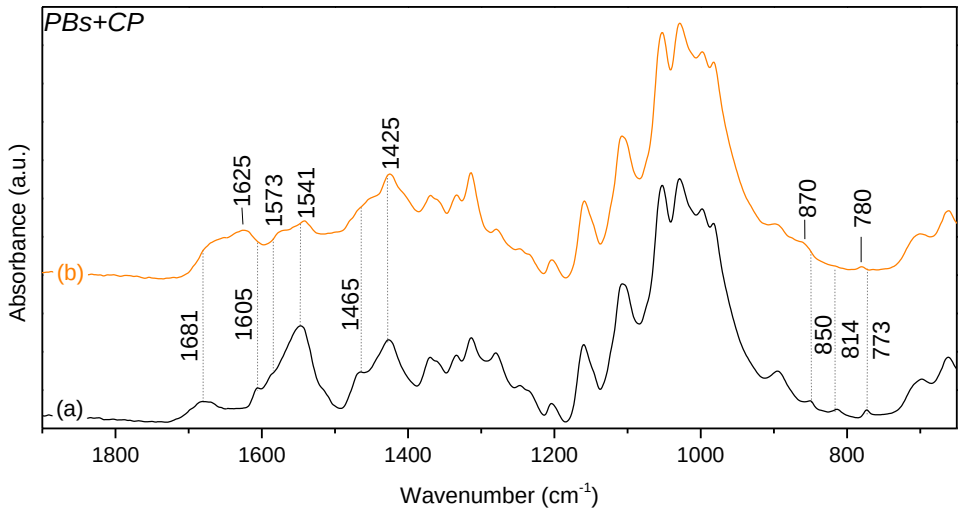


Figure 7.18: Infrared spectra (1900-650 cm^{-1} detail) of paper samples treated with PBS+CP before (a) and after artificial ageing (b).

On biodeteriorated samples (Figure 7.20 b, c), the characteristic bands of chitin (Figure 7.19) from the fungus were observed at 3273 cm^{-1} (νN-H); 2924 and 2854 cm^{-1} (νCH); 1641 cm^{-1} (Amide I); 1544 cm^{-1} (Amide II); 1424 and 1370 cm^{-1} (CHx deformations in β-chitin); 1075 cm^{-1} (νC-O); and 1018 cm^{-1} (νC-O-C in β-chitin), (Focher et al., 1992; Cárdenas et al., 2004; Kumirska et al., 2010) as in the reference spectrum in Figure 7.21.

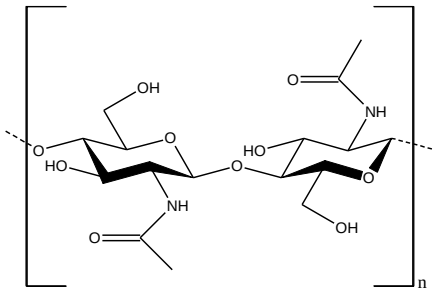


Figure 7.19: Chemical structure of chitin.

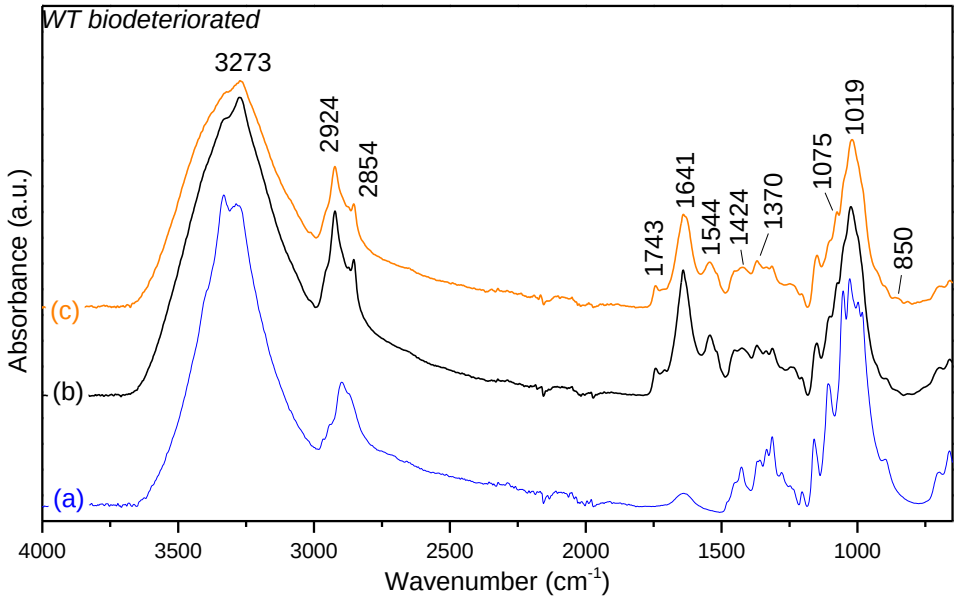


Figure 7.20: Infrared spectra of (a) artificially aged control PDA samples; and untreated samples colonized by *A. niger*, (b) before and (c) after artificial ageing.

Additionally, a band centred at 1743 cm^{-1} was obtained (Figure 7.20). This band was also observed in the *A. niger* reference sample in Figure 7.21 and can be assigned to carbonyl groups in melanin (Tavzes et al., 2013), one of the pigments responsible for the dark coloured conidia of *A. niger* (Jorgensen et al., 2011).

When comparing non-aged and aged samples (Figure 7.20 (b) and (c), respectively), no relevant differences are observed. The carbonyl region, which could give information on the oxidation level of cellulose, unfortunately lies in the same region as carbonyls from melanin and amide bands from the fungus itself.

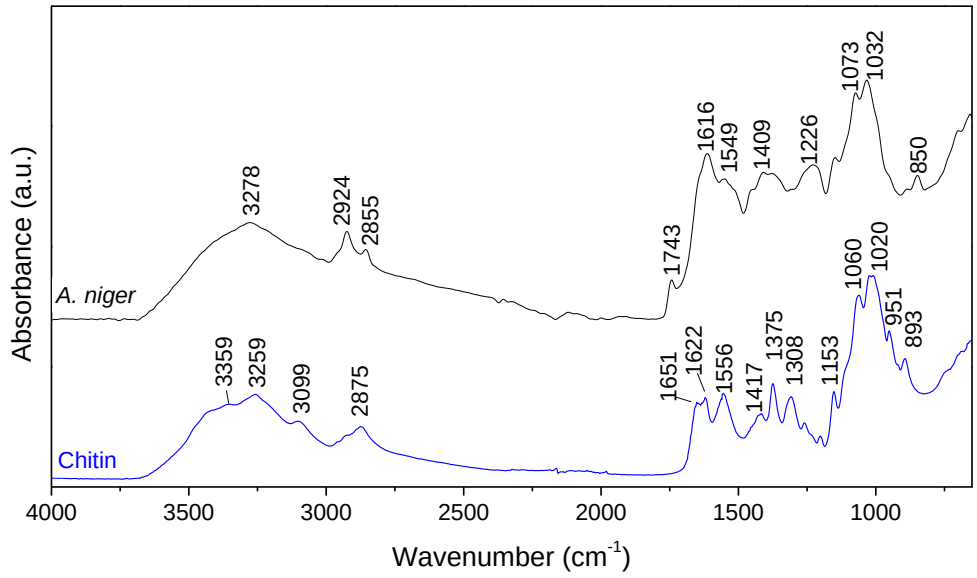


Figure 7.21: Infrared spectra of chitin (Chitin from shrimp shells, practical grade, Sigma-Aldrich, USA) and *A. niger* sample collected from PDA growth medium.

Though PDA control samples exhibited a strong discoloration with ageing, no carbonyl or carboxyl bands were detected in the infrared spectra. The infrared spectra of aged PDA samples Figure 7.20 (a) is similar to the one obtained with pristine WT samples (Figure 7.13 a).

On the infrared spectrum of non-aged biodeteriorated PBs+CP samples (Figure 7.22 a), only one of the bands attributed to PBs or CP are distinguishable (852 cm^{-1} and 813 cm^{-1}, respectively) with the bands attributed to *A. niger* masking the remaining ones. With ageing, those PBs and CP bands lose intensity or totally disappear. The presence of calcium oxalate is already noticed on non-aged samples by the bands at 1624 cm^{-1} and 780 cm^{-1}. On aged samples, contrarily to what happened in pristine samples, no band at 870 cm^{-1} attributed to $CaCO_3$ appears, but instead, the bands attributed to calcium oxalate at 1624 cm^{-1}, 1315 cm^{-1} and 780 cm^{-1} are intensified. The presence of the fungus in the paper and the correspondent excretion of oxalic acid seem therefore to take over the route of degradation and transformation of calcium propionate.

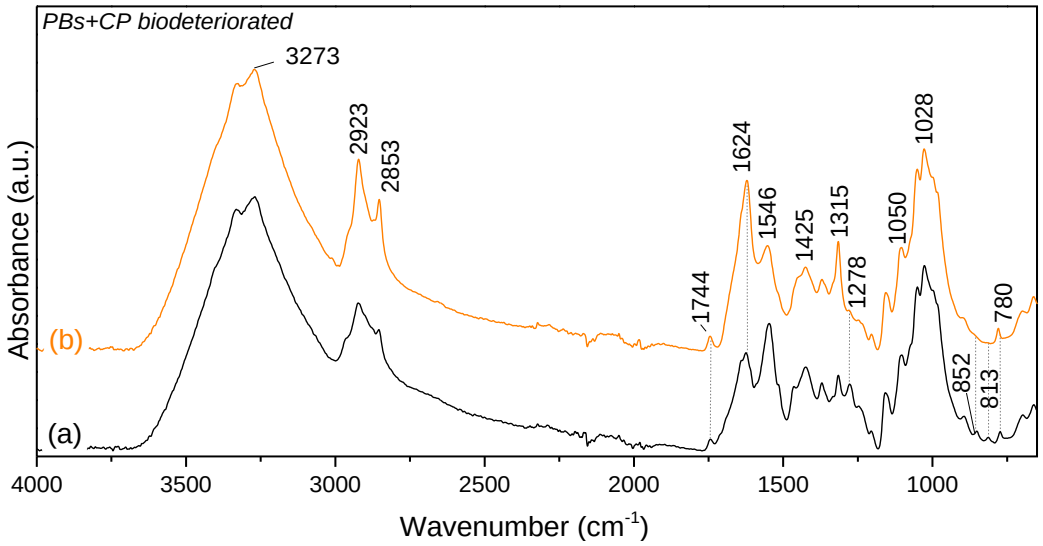


Figure 7.22: Infrared spectra of paper samples previously colonized by *A. niger*, treated with parabens and calcium propionate (PBs+CP), before (a) and after artificial ageing (b).

Although the presence of calcium oxalate on aged CP and PBs+CP pristine samples detected by FTIR was not identified by XRD, as shown in Figure 7.17, on biodeteriorated CP and PBs+CP samples XRD did reveal the presence of calcium oxalate (Figure 7.23 c). This is confirmed by the peaks at 14.8° and 30°, as in the reference difractogram of calcium oxalate monohydrate (whewellite) shown before in Figure 7.17 (e). Whewellite is the most stable form of calcium oxalate and the most common calcium oxalate hydrate found in association with fungi (Pinzari et al., 2010).

The XRD analyses also revealed an unexpected result: calcium oxalate was detected in all biodeteriorated samples, not just the ones treated with calcium (Figure 7.23 b). The quantity of calcium oxalate on those samples is below the detection limits of FTIR as no bands characteristic of calcium oxalate were detected on the infrared spectra (Figure 7.20).

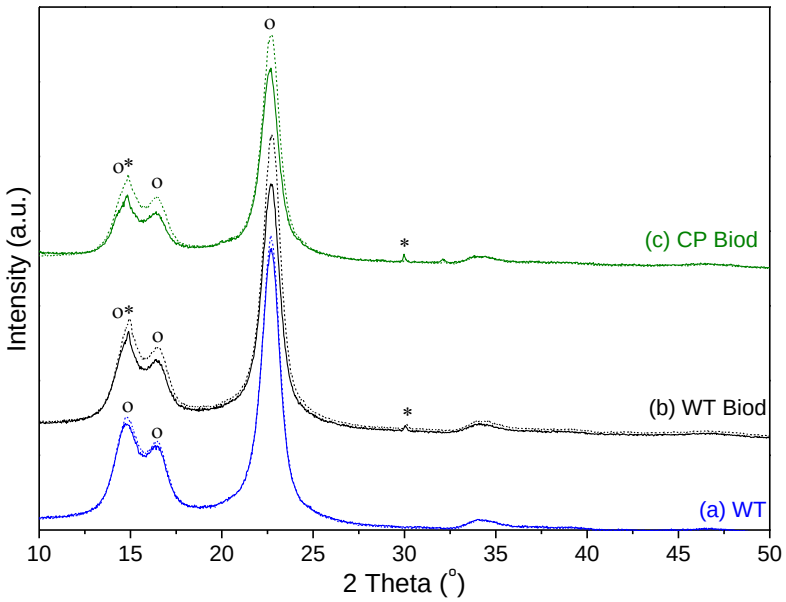


Figure 7.23: X-ray diffractograms obtained from pristine paper samples without treatment (a); biodeteriorated samples without treatment (b); and biodeteriorated samples treated with calcium propionate (c). Solid lines represent non-aged samples and dotted lines represent artificial aged samples. Peaks attributed to cellulose are marked with "*" and peaks attributed to calcium oxalate are marked with "o".

The presence of calcium on WT biodeteriorated samples was confirmed by Micro-energy dispersive X-ray fluorescence spectrometry (μ-EDXRF) (Figure 7.24 b). The fact that calcium oxalate was forming on samples that were not treated with calcium raised the question on where was the calcium coming from. μ-EDXRF analyses on the paper used as substrate in the present study, Whatman #1, demonstrated that the paper itself had minute amounts of calcium (Figure 7.24 a). Moreover, PDA powder also revealed the presence of calcium, along with potassium, sulphur, chlorine and phosphorus and iron (Figure 7.24 c), the same elements detected on biodeteriorated samples (Figure 7.24 b). A small peak of argon (2.96 KeV) appears in both samples since the measurements were not performed in vacuum.

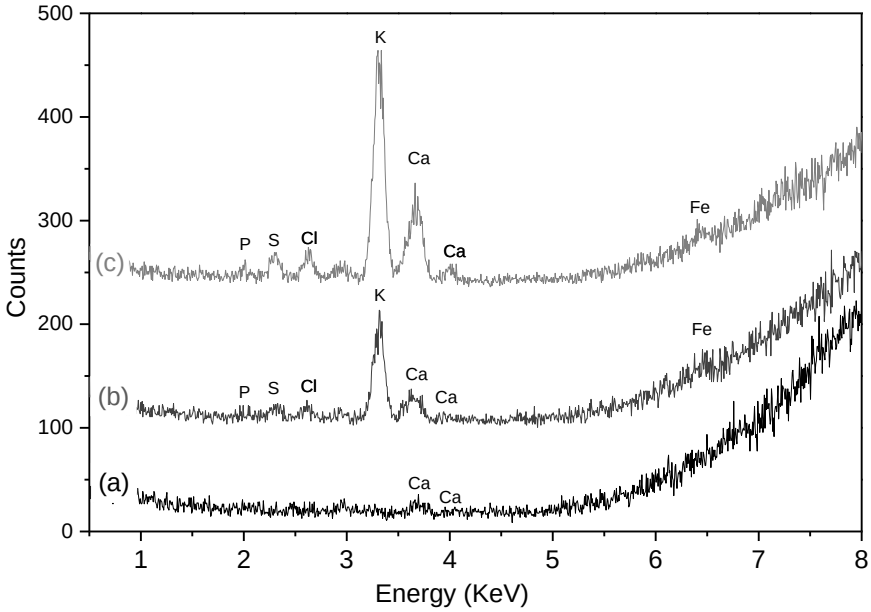


Figure 7.24: X-ray fluorescence spectra of pristine WT samples (a); biodeteriorated WT samples (b); and potato dextrose agar (PDA, Oxoid, UK) (c).

It seems therefore that calcium oxalate formed in biodeteriorated samples was in a crystalline state; while in pristine samples it was in an amorphous state, and consequently could not be detected by XRD. The formation of calcium oxalate crystals is a slow and chemically complex phenomenon that requires specific conditions to occur and is very dependent on the super-saturation and stoichiometry of the reagents (Jung et al., 2004). According to Pinzari et al. (2010), the addition of an oxalic acid solution onto paper containing calcium carbonate would not lead to the same crystal formation obtained with the fungal growth in paper. Although fungi require calcium as micronutrient, an excess of calcium in the environment may interfere with fungal metabolic processes, like pH regulation. The precipitation of calcium via reaction with oxalic acid is often a mechanism used by fungi to render exchangeable calcium insoluble, impeding it to participate in other reactions (Jellison et al., 1997).

The crystalline calcium oxalate detected by XRD in biodeteriorated samples treated with CP was probably formed during fungal colonization, not after treatment, as the XRD spectra from biodeteriorated WT samples are similar to the ones from CP samples (Figure 7.23 b and c, respectively). In accordance, the high quantity of calcium oxalate detected by FTIR in biodeteriorated CP samples could have resulted *a posteriori* from the reaction between calcium propionate and oxalic acid present in the paper, and be in an amorphous form, similar to the one obtained in pristine samples.

Calcium oxalate has a superior stability constant to calcium propionate (log K_1=3.0 and log K_1=0.5, respectively) (Furia, 1972:277) and is practically insoluble in water, which could justify the high rate of transformation of calcium propionate into calcium oxalate observed by FTIR.

Calcium oxalate has already been detected on paper affected by fungi by Pinzari (2006), but the actual chemical and mechanical effects calcium oxalate may have on paper stability have not yet been studied. Further studies are required, especially since the conditions required for calcium oxalate formation are quite common: paper with calcium based alkaline reserve and fungal biodeterioration.

7A.4. Conclusions

The present research was aimed at further testing the effectiveness and safeness of a parabens and calcium propionate formulation to prevent and treat biodeterioration of paper by fungi.

The preventive treatment of paper samples revealed a high antifungal effectiveness of the PBs+CP formulation. A total (100%) fungal inhibition was obtained on four of the five tested species along 30 days of incubation (*A. niger*, *Ch. globosum*, *Cl. cladosporioides*, *P. chrysogenum*). Only *P. corylophilum* showed some degree of tolerance to the antifungal solution, although the development of this species on treated paper was still partially inhibited. The antifungal properties of the PBs+CP formulation on paper samples were maintained for at least one year.

The curative treatment of paper samples previously colonized by a mixture of five fungal species had excellent antifungal results, with absolutely no fungal growth occurring after application of PBs+CP, along the 30 days of re-incubation.

The difference between the preventive and curative treatments relies on the fact that the fungi become in contact with the ethanolic solvent used in the formulation during the curative procedure, whilst in the preventive treatment, the formulation is already dry within the paper fibres. The contact with ethanol seems therefore to contribute to the antifungal effect of parabens and calcium propionate.

The results on the antifungal efficacy of PBs+CP corroborate the results obtained by Neves et al. (2009), although it has been proved that some degree of antifungal tolerance can be achieved by different fungal strains.

Before being applied on cultural heritage materials, new conservation treatments have to be thoroughly tested to assess any immediate or long term damage potential. In this case, it was also desirable to assess whether the PBs+CP formulation could stop an already ongoing fungal biodeterioration on colonized paper. As follows, both pristine paper and paper previously colonized by *A. niger* were treated and the chemical and/or physical alterations were analysed before and after moist-heat artificial ageing.

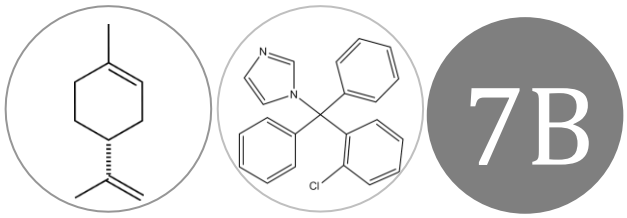
On pristine samples, PBs+CP effectively deacidified the paper and protected it from long term acidification. PBs+CP also successfully protected the paper from losing mechanical resistance with ageing. Regardless, although no distinguishable colour alterations were observed on the paper immediately after treatment, a statistically significant discoloration was detected with artificial ageing.

On biodeteriorated samples, a successful deacidification was also obtained with PBs+CP, which is noteworthy against such a potent acidifier like *A. niger*. Although the treated biodeteriorated paper achieved pH values close to 10 after ageing, no deteriorating effects of such alkalinity were observed. Instead, the darkening of the samples occurring with ageing was significantly prevented and the yellowing was similar to the untreated controls. Also, the PBs+CP treatment totally protected the loss of mechanical resistance on treated samples, as their folding endurance remained nearly unchanged after ageing, whilst untreated controls entirely lost their folding endurance.

Calcium oxalate was detected on biodeteriorated samples treated with PBs+CP and the quantity of this compound increased with ageing, at the cost of CP decomposition. Calcium oxalate was

also detected by XRD in all biodeteriorated samples, although in smaller quantities, not detected by FTIR.

Taking into account the obtained results, the studied treatment is very effective in preventing and stopping fungal development and also in preventing fungal biodeterioration on already colonized paper. The colour alterations observed in pristine samples, tough, discourages the application of such treatment as a prophylactic measure. Further tests should be performed to study the effects of calcium oxalate on the deterioration of samples.



Limonene vs Clotrimazole

A first screening test was performed on two low toxicity antifungals, limonene and clotrimazole, against *Penicillium chrysogenum,* one of the most common fungal species found on paper collections, in order to determine which compound had the best potential to be further developed.

Limonene, a natural antifungal component found on several plants, which has chemical characteristics potentially suited for inhibiting paper biodeterioration and lowering the toxicity of paper colonizing fungi, presented no inhibitory capacity on *Penicillium chrysogenum*, and could even enhance its growth.

Clotrimazole, one of the earliest azoles used for treating cutaneous fungal infections, also having antioxidant properties, showed a good inhibition of *P. chrysogenum* (over 80%) at every incubation stages and was therefore selected for further development.

7B.1. Introduction

Limonene (Lm) (Figure 7.25) is one of the most common terpenes in nature, being a component of several plants' essential oils (Chee et al., 2009). It is a colourless liquid with citreous aroma that has shown to have several purposes as a flavour or fragrance additive, solvent, or fungal inhibitor (IARC, 1999a; Dambolena et al., 2008; Chee et al., 2009; Yazar et al., 2011). Regarding the other antifungals presented in Appendix IV, limonene has the advantage of having very low toxicity, antioxidant properties, a strong inhibitory effect on cellulase activity (with potential to neutralize one of the most deleterious effects of fungus on paper – enzymatic decomposition), a broad fungitoxic spectrum and the ability to strongly inhibit aflatoxin production (one of the most hazardous toxins produced by fungi, with carcinogenic, mutagenic and immunosuppressive properties) (Dambolena et al., 2008; Singh et al., 2010; Marei et al., 2012). These properties made Lm a good candidate for further study, since it had potential to simultaneously inhibit paper biodeterioration and lower the toxicity of the fungi colonizing paper.

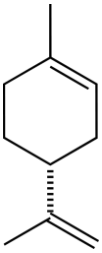


Figure 7.25: Chemical structure of limonene.

Clotrimazole (CLT) (Figure 7.26) is an imidazole derivative with a broad spectrum of antimycotic activity that was first synthesized in the late 1960s (Kadavakollu et al., 2014) and is currently listed in the World Health Organization List of essential medicines (WHO, 2015). Being firstly commercialized in the early 1970s, CLT is one of the earliest azoles used for treating cutaneous fungal infections (Lattif and Swindell, 2009).

Regarding the other antifungals presented inppendix IV, CLT has the advantage of being white in the solid state, a weak base, soluble in ethanol and isopropanol, and having only minor negative health effects with positive additional characteristics like anti-malarial, anti-cancer and neuroprotective properties (Isaev et al., 2002; Sweetman, 2009; Borhade et al., 2012; Furtado et al., 2012). Moreover, CLT has the potential to prevent one of the main degradation routes of paper – oxidation - as it has radical scavenging and metal corrosion inhibition properties (Obot et al., 2009; Iannelli et al., 2011). These characteristics, together with its long certification period, where CLT has proven to have unremarkable health side effects (Kadavakollu et al., 2014), have made this azole compound a good candidate for testing in paper samples.

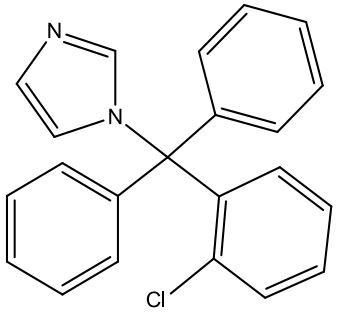


Figure 7.26: Chemical structure of clotrimazole.

The antifungal activity of CLT is attributed to inhibition of ergosterol biosynthesis in microbial cell membranes, affecting cell membrane integrity and function (Kleyi et al., 2012). It also inhibits the movement of Ca^{2+} and K^{+} ions across the plasma membrane constraining cell proliferation, and interferes with Ca^{2+} binding and Ca^{2+} dependent cellular processes (Bartolommei et al., 2006).

The antifungal potential of Lm and CLT on this first screening phase was evaluated against one of the most common fungal species found on paper/book materials worldwide (see Chapter 4), *Penicillium chrysogenum*. This species is also highly cellulolytic (Chinedu et al., 2011) and its colonies grow fast and sporulate in standard culture media (Pitt & Hocking, 2009:235).

7B.2. Materials and Methods

Paper samples, fungal species, inoculum preparation and treatment method were used as described in Chapter 7- Introduction.

7B.2.1. Tested formulations

The initially selected solvent was the same as used in the previous chapter (Chapter 7A), 72.15% (v/v) ethanol. However, LM was not soluble in this ethanol concentration and therefore 85% ethanol was used in this assay instead for both compounds.

Limonene and clotrimazole individual formulations were prepared by diluting limonene ((R)-(+)-Limonene, 97%, stab. Alfa Aesar, USA) or clotrimazole (CLT, CAS No. 23593-75-1, Alfa Aesar,

USA) in 85% ethanol (99.5%, Carlo Erba, Spain). The tested concentrations (w/v) were the following: 0.1%, 0.5%, 1.0%, 2.0%, 2.5% for limonene; and 0.5%, 1%, 1.5%, 2%, 2.5% for clotrimazole. These concentrations were selected according to the ones used commonly in commercial products containing these compounds (0.5-2% Lm, 1-2% CLT, see Appendix IV).

7B.3. Results and discussion

Fungal growth inhibition was calculated as described in Chapter 7- Introduction and the results are shown in Figure 7.27.

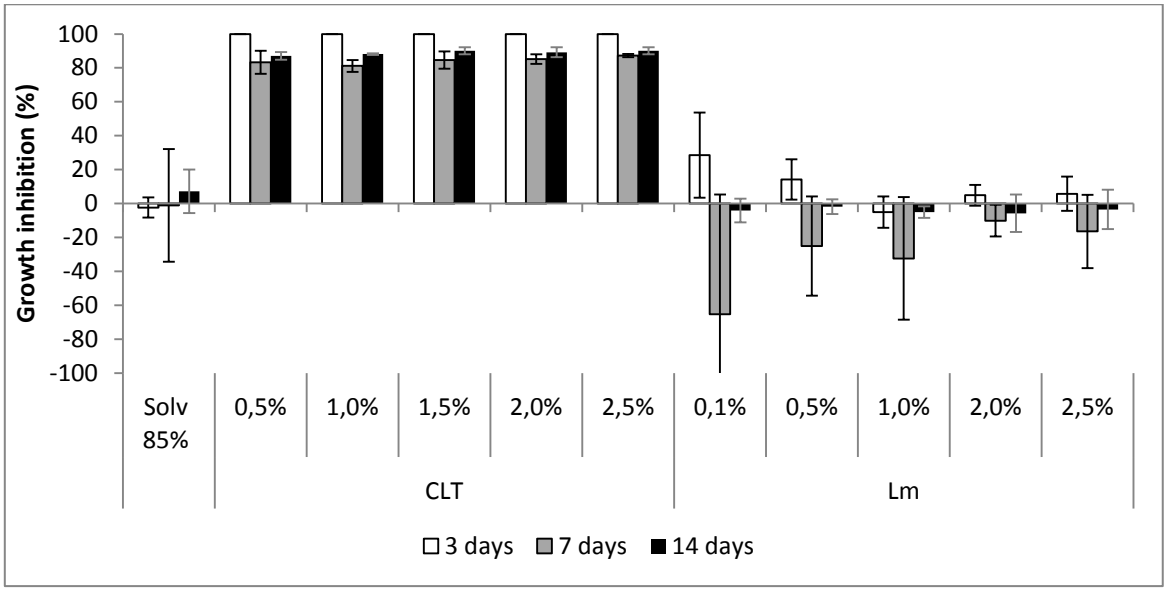


Figure 7.27: *P. chrysogenum* growth inhibition by clotrimazole and limonene in 85% ethanol, at 3, 7 and 14 days of incubation.

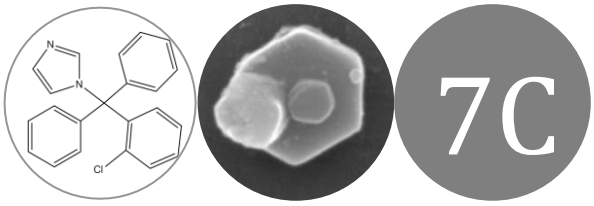
According to Figure 7.27, CLT strongly inhibited the growth of *P. chrysogenum.* The achieved fungal growth inhibition was higher than 80% at every incubation period and for all concentrations tested.

Lm, on the other hand, generally revealed a negative growth inhibition, especially at 7 days of incubation, meaning that it favoured the growth of *P. chrysogenum*.

7B.4. Conclusions

This first screening for antifungal activity on the two low toxicity selected compounds revealed that CLT had a good antifungal potential and therefore was eligible for further testing.

Lm, albeit presenting a good potential for inhibiting paper biodeterioration and lowering the toxicity of the fungi colonizing paper, did not show any antifungal activity against *P. chrysogenum* and could even enhance its development. As *P. chrysogenum* is very common in paper collections, and given the results obtained with Lm on it, no more tests were performed with this compound. The following tests performed with CLT are presented in the next chapter.



Clotrimazole and $Ca(OH)_2$ nanoparticles

The antifungal potential of clotrimazole, a well-known antimycotic agent, was tested against five of the most common fungal species affecting paper collections. The addition of a deacidificant agent, calcium hydroxide nanoparticles, resulted in a multipurpose formulation intended to also neutralize the deleterious effects of acids excreted by the fungi.

The preventive and curative antifungal activity of the formulation was evaluated on paper samples. Clotrimazole showed antifungal activity against all tested fungal species and its effectiveness varied according to microorganism. *Chaetomium globosum* was the only species able to grow after the curative treatment and only in part of the tested samples.

The safeness of this treatment for a possible use with paper artefacts was tested on pristine paper and on paper already biodeteriorated by fungi. The deacidification properties of the formulation were confirmed concerning the long term protection of paper from acidification and loss of mechanical properties, although causing a minor paper discoloration. The protective effect of the treatment on previously biodeteriorated samples was less evident, due to the strong deteriorating power of *A. niger*.

7C.1 Introduction

Given the good antifungal results obtained with CLT against *P. chrysogenum* in the previous chapter, this compound was tested against a higher number of fungal species (*A. niger*, *Ch. globosum*, *Cl. cladosporioides*, *P. corylophilum*) individually and as a mixed inoculum.

With the aim of preparing a multipurpose formulation that would simultaneously inhibit fungal development and neutralize the deleterious effects of already excreted acidic fungal metabolites, the addition of calcium hydroxide to CLT was also tested.

Calcium hydroxide ($Ca(OH)_2$) is one of the most used deacidification compounds in paper conservation due to the long-term physicochemical stability between calcium and cellulose (Kolar and Novak, 1996). When exposed to air it forms calcium carbonate, which works as an alkaline reservoir. Furthermore, $Ca(OH)_2$ has also shown to inhibit fungal growth on paper samples when used as a paper deacidification treatment (Valentin, 1986).

The presence of an alkaline compound in this formulation would also aid to the prevention of chemical degradation of clotrimazole in acidic environment (Hashem et al., 2011).

Since clotrimazole is poorly water soluble, and $Ca(OH)_2$ is insoluble in alcohols, a suspension of $Ca(OH)_2$ nanoparticles (NPs) in isopropanol was tested. This suspension has already been extensively studied and has proven to have good deacidification properties (Giorgi et al., 2002; Sequeira et al., 2006). The smaller size of $Ca(OH)_2$ NPs in comparison to the commercial

micrometric powder form allows for a better penetration in the paper's fibre net. Also, since the specific surface area of a solid exponentially increases with the decrease of its volume, these nanoparticles have a much superior surface area of the alkaline compound available to react, ensuing in a superior capacity of neutralization.

Given the fact that isopropanol is the solvent that provides the best kinetic stability of $Ca(OH)_2$ NPs (Giorgi et al., 2002), and is also the solvent commonly used in CLT antimycotic spray formulations (Bayer, 2015), this solvent was chosen instead of the ethanol solution used in the previous chapter.

The effectiveness of CLT was firstly evaluated by determining the minimum concentration for fungal inhibition on five of the most commonly found fungal species on paper collections: *A. niger*, *P. chrysogenum*, *P. corylophilum*, *Ch. globosum* and *Cl. cladosporioides* (Di Bonaventura et al., 2003; Zielińska-Jankiewicz et al., 2008; Mesquita et al., 2009; Bergadi et al., 2014). This minimum concentration was afterwards tested individually and mixed with calcium hydroxide nanoparticles as a preventive and curative antifungal treatment for paper samples.

The short and long term effects of the developed formulation on the chemical and physical characteristics of treated papers where evaluated as described in Chapter 7, section 7.2.4.

To our knowledge, the use of CLT as an antifungal treatment for paper is presented here for the first time.

7C.2 Materials and Methods

Paper samples, fungal species, inoculum preparation and treatment methods were as described in Chapter 7 - Introduction.

7C.2.1. Tested formulations

Antifungal formulations were prepared by diluting clotrimazole (CLT, CAS No. 23593-75-1, Alfa Aesar, USA) in isopropanol (IPA, CAS Number 67-63-0, ≥99.8%, Sigma-Aldrich) in the following w/v concentrations: 0.001%; 0.01%; 0.05%; 0.1%; 0.5%; 1%; 2.5%.

The formulation presenting the best antifungal results vs minimum concentration was then tested mixed with a dispersion of calcium hydroxide NPs. The mixture was prepared by diluting the respective CLT quantity in the NPs dispersion in isopropanol, achieving the selected concentration.

7C.2.1.1. Synthesis of $Ca(OH)_2$ nanoparticles

The synthesis of $Ca(OH)_2$ was performed according to Ambrosi et al. (2001). Sodium hydroxide (NaOH, CAS No. 1310-73-2Akzo Nobel, Netherlands), calcium chloride dihydrate, ($CaCl_2$,$2H_2O$, CAS No. 10035-04-8, Sigma Aldrich, USA) and isopropanol (Valente & Ribeiro Lda, Portugal) were used without further purification.

Two separate aqueous solutions containing 0.8M of NaOH and 0.4 M of $CaCl_2$, respectively, were heated and maintained at 90°C ± 5°C. The NaOH solution was then rapidly added into the $CaCl_2$ solution. The $Ca(OH)_2$ precipitate was left to settle under a nitrogen atmosphere to avoid carbonatation and the supernatant discarded. The remaining suspension was washed repeatedly with distilled water to remove the NaCl by-product. Each washing consisted of the addition of distilled water followed by 10 minutes sonication and centrifugation at 13000xg for 30 minutes to separate the solid and liquid phases. The removal of chlorine was controlled using Mohr

method (Skoog et al., 1996). The paste resulting from the last centrifugation was concentrated at 40 °C under moderate vacuum using a Büchi Glass oven B-580 C, until a weight ratio $Ca(OH)_2/H_2O$ of 0.8 (Giorgi et al., 2002) was achieved. The paste was then dispersed in isopropyl alcohol, achieving a $Ca(OH)_2$ concentration of 4.3 gL^{-1} (Giorgi et al., 2002).

Before mixing with the antifungal solution, the NPs suspension was diluted in isopropanol to obtain a 0.86 gL^{-1} $Ca(OH)_2$ concentration. This was the concentration that according to a previous study (Sequeira et al., 2006) raised the pH of an early 20th century paper to c. 8.30 (within the safe range of 8-9), avoiding alkaline deterioration (Banik, 1996).

The size of the obtained particles was analysed by Field Emission Gun Scanning Electron Microscope (FEG-SEM, JEOL 7001F) and Dynamic Light Scattering (DLS, Horiba Scientific, nano partica SZ-100).

7C.3 Results and discussion

7C.3.1 Preventive antifungal treatment of clotrimazole on paper: determination of minimum inhibitory concentration

Clotrimazole was first tested individually at varying concentrations to determine its minimum inhibitory concentration on paper. The results regarding fungal growth inhibition by CLT are presented in Table 7.7. These percentages concern 15 days of incubation and not the maximum period, 30 days, since at 15 days the control WT samples (used as references in the calculations) were totally colonized, whilst at 30 days the fungal colonization already widely exceeded the paper limits.

Table 7.7: Calculated fungal growth inhibition (%) at 15 days of incubation for the different treatments on paper samples. Mixed inoculum (5) is the mixture of all 5 species, mixed inoculum (4) is the mixture of all species except *Ch. globosum*. Each value is an average of three replicates ± SD. In each column, values followed by the same letters are not significantly different by the Fisher's LSD test at α=0.05.

	Fungal growth inhibition (%)				
	A. niger	*Ch. globosum*	*Cl. cladosporioides*	*P. chrysogenum*	*P. corylophilum*
Solv (IPA)	0 ± 0 a	0 ± 0 a	0 ± 3 a	-1 ± 0 a	-3 ± 2 a
CLT 0.001%	3 ± 4 a	0 ± 0 a	2 ± 4 a	0 ± 1 a	100 ± 0 b
CLT 0.01%	100 ± 0 b	0 ± 0 a	57 ± 5 b	75 ± 9 b	100 ± 0 b
CLT 0.05%	100 ± 0 b	0 ± 0 a	68 ± 4 c	97 ± 3 c	100 ± 0 b
CLT 0.1%	100 ± 0 b	0 ± 0 a	74 ± 2 c	92 ± 6 c	100 ± 0 b
CLT 0.5%	100 ± 0 b	0 ± 0 a	72 ± 4 c	89 ± 5 c	100 ± 0 b
CLT 1%	94 ± 2 c	0 ± 0 a	72 ± 8 c	91 ± 4 c	100 ± 0 b
CLT 2.5%	100 ± 0 b	0 ± 0 a	67 ± 4 c	91 ± 2 c	100 ± 0 b

There were substantial variations of susceptibility to CLT according to fungal species, namely between *Ch. globosum* and *P. corylophilum*: while *Ch. globosum* showed high tolerance to this antifungal compound at all tested concentrations, *P. corylophilum* was completely inhibited by CLT concentrations as low as 0.001% for up to 30 days of incubation (data not shown). The susceptibility of the tested fungal species to CLT followed the ascending order: *Ch. globosum* < *Cl. cladosporioides* < *P. chrysogenum* < *A. niger* < *P. corylophilum*.

CLT acts by inhibiting ergosterol synthesis, however sterols other than ergosterol can be produced by some species (Taniwaki et al., 2006). This may be the reason why some species show higher tolerance to this antifungal.

As shown in Table 7.7, even the highest tested CLT concentration (2.5%) was not able to totally inhibit fungal growth in samples inoculated with *Ch. globosum*, *Cl. cladosporioides* or *P. chrysogenum*. Since this concentration already leaves the paper somewhat sticky and water resistant (observed during inoculation where the inoculum drop remained at the surface of the paper for a while), no higher concentrations were tested.

For CLT concentrations comprised between 0.05% and 2.5%, the fungal inhibition results are not significantly different for all the tested species, except for *A. niger*, where 1% concentration resulted in a significantly lower fungal inhibition. This difference for *A. niger* at an intermediate concentration, not following any ascending or descending order according to concentration, points out to an isolated occurrence of clotrimazole tolerant spore(s). The selected CLT concentration for the subsequent preventive and curative tests, was therefore 0.05% due to its best lower concentration/higher inhibition relationship.

When comparing the obtained results with *P. chrysogenum* against the ones obtained on the previous Chapter 7B, where 85% ethanol was used as solvent, no significant differences were obtained (p>0.05). The use of isopropanol was therefore continued on the subsequent experiments.

7C.3.2 Preventive antifungal effect of CLT with $Ca(OH)_2$ NPs

7C.3.2.1 Characterization of synthesized $Ca(OH)_2$ NPs

The synthesised $Ca(OH)_2$ nanoparticles were characterized by FTIR spectrometry, XRD, SEM, and DLS.

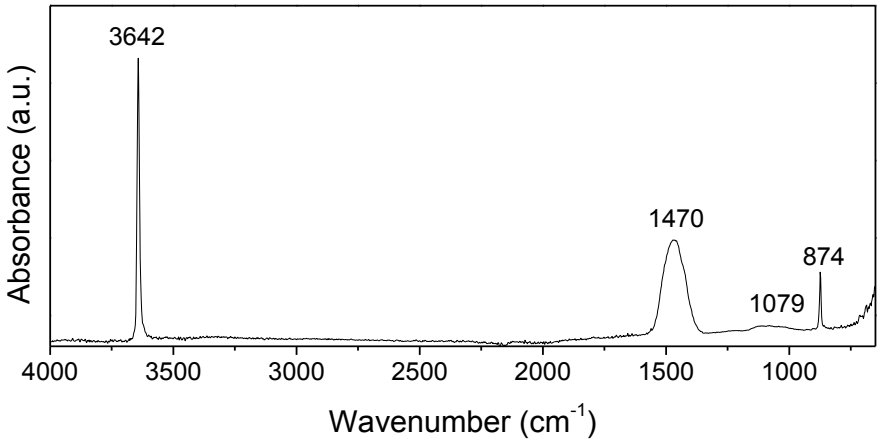


Figure 7.28: Infrared spectrum of synthesized $Ca(OH)_2$ nanoparticles after air exposure.

The infrared spectrum in Figure 7.28 reveals the characteristic band of OH stretch from $Ca(OH)_2$ at 3642 cm^{-1} and also calcium carbonate bands at 1470 cm^{-1} and 874 cm^{-1} (Derrick et al., 1999; Coates, 2000) as a result of $Ca(OH)_2$ carbonatation by air exposure (Giorgi et al., 2002).

The X-ray diffractogram in Figure 7.29 also shows peaks of $Ca(OH)_2$ and $CaCO_3$ (Taglieri et al., 2013), confirming the formation of $Ca(OH)_2$ crystals during the performed synthesis and also its partial carbonatation.

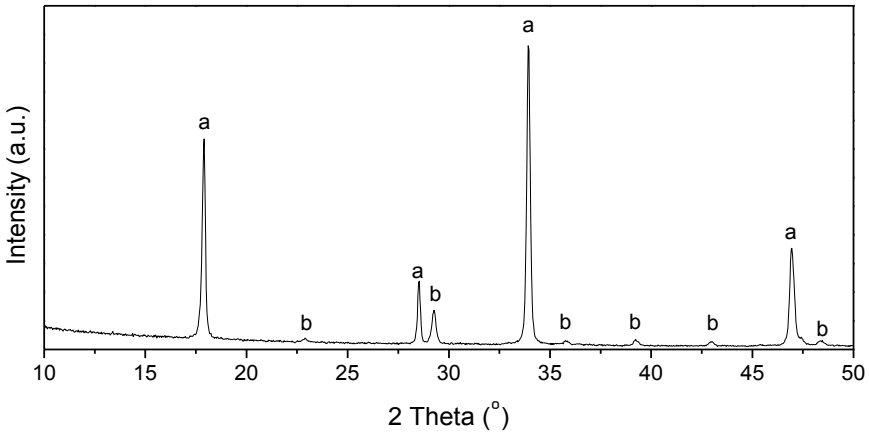


Figure 7.29: X-ray diffractogram of synthesized $Ca(OH)_2$ nanoparticles after air exposure. Bands attributed to $Ca(OH)_2$ are marked with "a" and bands attributed to $CaCO_3$ are marked with "b".

An example of the synthesized $Ca(OH)_2$ NPs is presented in Figure 7.30 A. The particle size distributions were estimated by DLS from the prepared $Ca(OH)_2$ NPs suspension in isopropanol at different dilutions. The inverse of the obtained hydrodynamic diameters was plotted against the respective $Ca(OH)_2$ concentration (Figure 7.30 B).

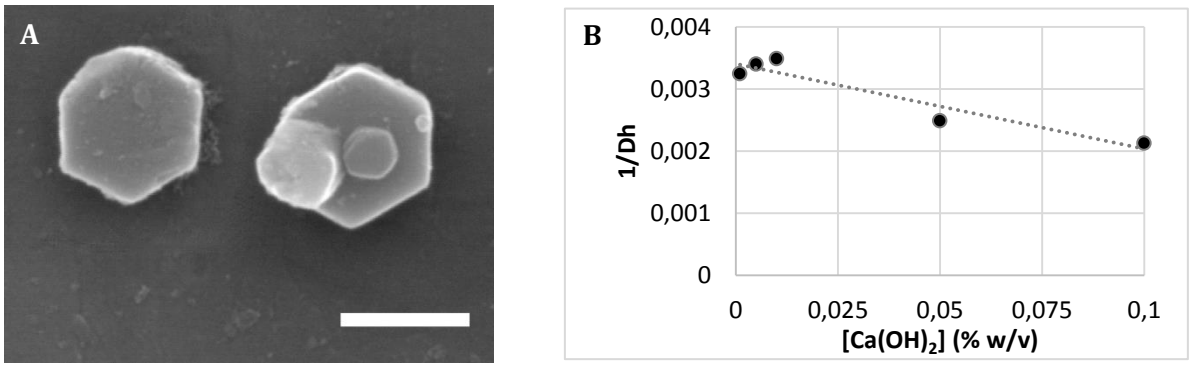


Figure 7.30: (A) SEM image of synthesized $Ca(OH)_2$ particles, scale bar = 500nm. (B) Variation of the inverse of the hydrodynamic diameter with the concentration of $Ca(OH)_2$ in isopropanol.

According to the linear regression in Figure 7.30 B, to an infinite dilution the average particle diameter would be 294±11nm (the inverse of the y-intercept). The Dynamic Virial Coefficient (α), calculated by dividing the slope by the y-intercept, is negative (-4.02±0.59), indicating the predominance of attractive interactions between the particles (Laia et al., 1998).

7C.3.2.2 Antifungal results

Results on fungal growth inhibition caused by $Ca(OH)_2$ NPs and the antifungal – deacidificant formulation 0.05% CLT+ $Ca(OH)_2$ NPs are presented in Table 7.8.

To simulate a paper contamination by several species, a mixture of five fungal species was created (Mixed inoculum 5). Given the high tolerance of *Ch. globosum* to clotrimazole, observed in the previous experiment, and to avoid a full colonization by this fungus, hampering the study

of the reaction obtained with the remaining species, another mixed inoculum was also tested, from which *Ch. globosum* was absent (Mixed inoculum 4).

Table 7.8: Calculated fungal growth inhibition (%) at 4, 8, 15 and 30 days of incubation for the different treatments on paper samples. Mixed inoculum (5) is the mixture of all 5 species, mixed inoculum (4) is the mixture of all species except *Ch. globosum*. Each value is an average of three replicates ± SD.

		Fungal growth inhibition (%)						
	Incubation (days)	*A. niger*	*Ch. globosum*	*Cl. cladosporioides*	*P. chrysogenum*	*P. corylophilum*	Mixed inoculum 5	Mixed inoculum 4
Solv (IPA)	4	-16 ± 10	-24 ± 8	59 ± 39	2 ± 11	-12 ± 11	22 ± 7	52 ± 4
	8	-1 ±11	0 ± 0	14 ± 22	-4 ± 6	-7 ± 7	17 ± 2	0 ± 11
	15	0 ± 0	0 ± 0	0 ± 3	-1 ± 0	-3 ± 2	-3 ± 4	-3 ± 2
	30	0 ± 0	0 ± 0	-1 ± 3	-1 ± 0	2 ± 4	-1 ± 3	-3 ± 1
CLT 0.05%	4	100 ± 0	65 ± 6	93 ± 3	100 ± 0	100 ± 0	94 ± 10	100 ± 0
	8	100 ± 0	0 ± 0	75 ± 5	100 ± 0	100 ± 0	37 ± 25	77 ± 6
	15	100 ± 0	0 ± 0	68 ± 4	97 ± 3	100 ± 0	-6 ± 0	66 ± 5
	30	100 ± 0	0 ± 0	11 ± 5	76 ± 22	100 ± 0	-3 ± 0	43 ± 3
$Ca(OH)_2$ NPs	4	-13 ± 9	0 ± 17	21 ± 8	-14 ± 11	-38 ± 18	18 ± 7	-21 ± 19
	8	-5 ± 10	0 ± 0	4 ± 2	0 ± 4	-2 ± 6	11 ± 3	-8 ± 17
	15	2 ± 5	0 ± 0	-2 ± 1	1 ± 6	7 ± 2	-2 ± 2	3 ± 8
	30	3 ± 5	0 ± 0	-1 ± 0	1 ± 7	2 ± 2	0 ± 1	2 ± 1
CLT+NPs	4	100 ± 0	34 ± 3	78 ± 0	92 ± 7	100 ± 0	67 ± 2	83 ± 4
	8	93 ± 5	2 ± 4	59 ± 3	88 ± 7	100 ± 0	-22 ± 5	67 ± 7
	15	51 ± 4	0 ± 0	42 ± 2	72 ± 9	100 ± 0	-6 ± 0	48 ± 7
	30	0 ± 1	0 ± 0	11 ± 1	14 ± 3	100 ± 0	-3 ± 0	0 ± 5

According to Table 7.8, the inhibition values obtained with $Ca(OH)_2$ NPs are close to zero for all the tested fungal species, indicating a null preventive antifungal effect for this deacidifying compound.

When mixed with CLT, the NPs decreased the antifungal efficacy of the imidazole derivative. As shown in Table 7.8, at 15 days of incubation, there is a decline in the 20-50% range of fungal growth inhibition for *A. niger*, *Cl. cladosporioides*, *P. chrysogenum* and Mixed inoculum 4, when compared with 0.05% CLT alone. For *Ch. globosum* and *P. corylophilum* the results remain the same, 0% and 100%, respectively.

This decline in antifungal activity from CLT when mixed with the NPs was not expected, as according to the literature, clotrimazole in solution is stable in alkaline environments (at pH values as high as 10-13), and only hydrolyses in acidic medium to (2-chlorophenyl)-diphenyl methanol and imidazole (Florey, 1982; Tesic and Trifkovic, 2014). Therefore it is highly unlikely that this phenomenon was caused by a degradation of CLT caused by calcium hydroxide.

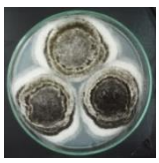
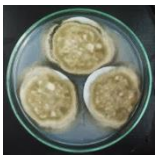
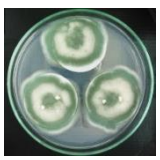
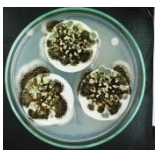
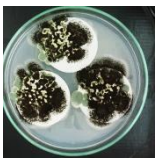
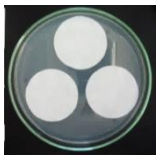
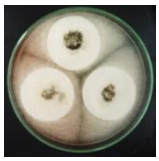
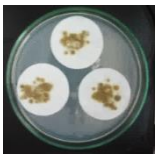
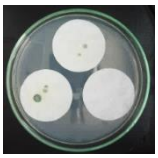
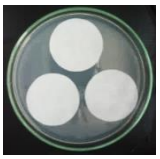
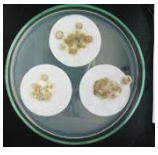
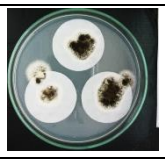
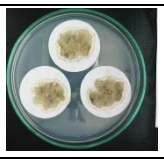
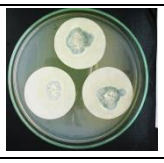
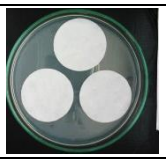
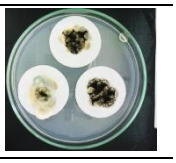
According to the literature, the antifungal activity of CLT decreases below pH 7 (Danby et al., 2012), however to the best of our knowledge, no studies were performed regarding the activity of CLT at such alkaline values as the ones obtained with the $Ca(OH)_2$ NPs treatment (pH~10, according to Figure 7.6).

Beggs (1992) tested the antifungal activity of miconazole, another azole antifungal, from pH 5 to 8, and the maximum activity was achieved at pH 7, diminishing at lower as well as at higher pH values. The author concluded that miconazole had to be at its unprotonated form to be active against fungi. At lower pH, the inactive protonated form was mostly present in solution, whilst at higher pH values there was a rapid decrease in drug solubility, resulting in clustering into colloidal particles diminishing the interaction with the microbial cell (Beggs, 1992). This clustering phenomenon could be also occurring with CLT at alkaline pH, however further research would be necessary to determine the specific cause, which was not within the aims of the present study.

This phenomenon occured in a paper with an original pH close to neutral, however when applied to acidic papers, $Ca(OH)_2$ NPs could in fact improve the efficacy of CLT by preventing its decomposition at low pH values.

On the samples inoculated with Mixed inoculum 5 and treated with CLT or CLT+NPs, only the two fungal species with higher tolerance to CLT were able to grow – *Ch. globosum* and *Cl. cladosporioides* (Table 7.9).

Table 7.9: Images of fungal growth resulting from the preventive treatment of paper samples with CLT (0.05%) and CLT+NPs in comparison with control untreated samples (WT), at 15 days of incubation.

	A. niger	*Ch. globosum*	*Cl. cladosporioides*	*P. chrysogenum*	*P. corylophilum*	Mixed inoculum (5)	Mixed inoculum (4)
WT							
CLT 0.05%							
CLT+NPs							

On the samples inoculated with Mixed inoculum 4 and treated with CLT alone, only *Cl. cladosporioides* developed. However, on the samples treated with CLT+NPs, besides *Cl. cladosporioides*, also *P. chrysogenum* and *A. niger* were able to grow (Table 7.9). This illustrates once again the loss of antifungal efficacy of CLT when mixed with the NPs.

When CLT is absent from the treatments, *Cl. cladosporioides* is never able to compete with the other species, and is overgrown by *Aspergillus* and *Penicillium*. This is observed on the WT samples inoculated with either Mixed inoculum 4 or Mixed inoculum 5 (Table 7.9).

7C.3.3 Curative treatment effectiveness on previously contaminated paper

The fungal growth results obtained from the simulation of a curative treatment, where paper samples were firstly colonized by fungi and subsequently treated, are shown in Figure 7.31.

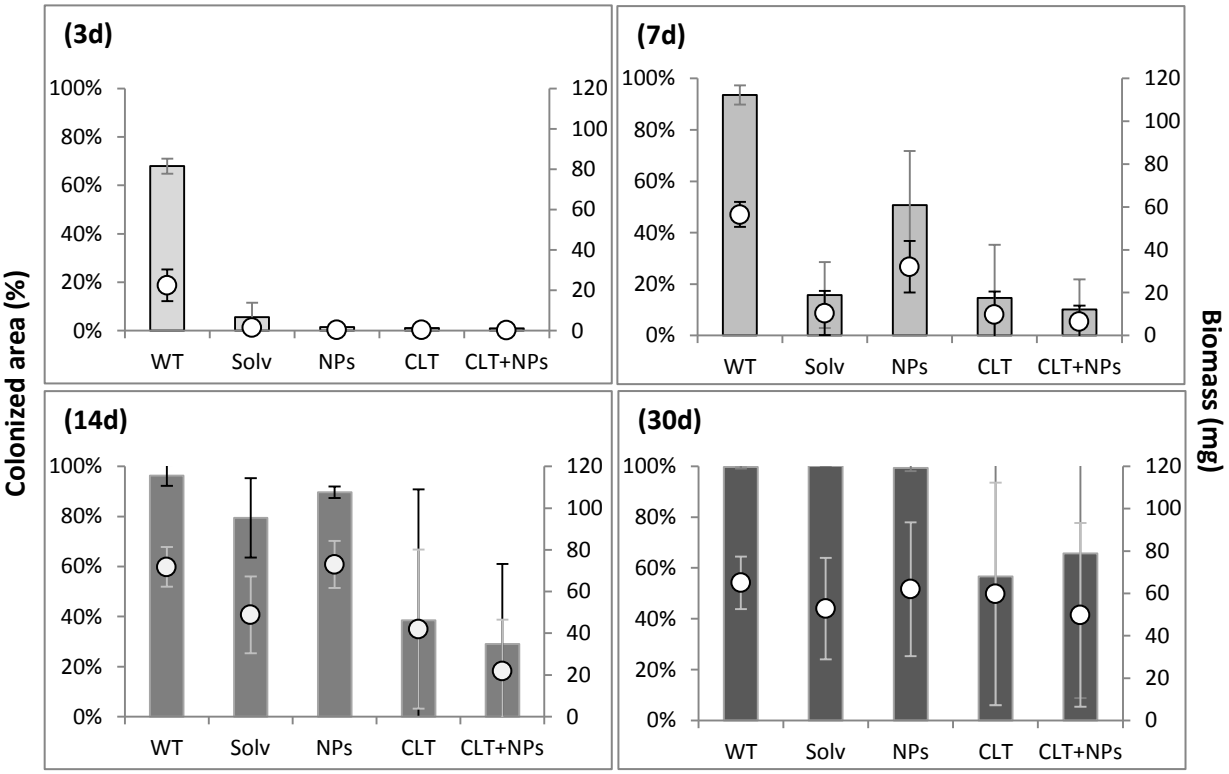


Figure 7.31: Results from the curative treatment on paper inoculated by a mixed inoculum with *A. niger*, *P. chrysogenum*, *P. corylophilum*, *Ch. globosum* and *Cl. cladosporioides*, at 3, 7, 14 and 30 days of re-incubation. Colonized area (represented in columns) and biomass values (represented in white circles) are the average of 3 replicates ± SD.

All treatments exhibited antifungal properties when compared to untreated controls (WT), particularly in the first periods of incubation (Figure 7.31). Even the solvent (IPA) alone had a negative impact on the initial fungal development, being significantly different (p<0.05) from the WT controls at 3 and 7 days of incubation. As previously observed with ethanol (Chapter 6), isopropanol also presents a harmful effect on the fungal mycelium.

The best antifungal activity was obtained with CLT and CLT+NPs. In one third of the samples treated with both formulations, fungal growth was null even after 30 days of incubation (Figure 7.31 and Table 7.3). CLT samples were not significantly different from CLT+NPs samples at any incubation period. Thus, contrarily to what was observed with the preventive treatment, in the curative treatment the presence of NPs did not have a negative impact on the antifungal efficacy of CLT.

The species colonizing the treated samples were *Penicillium* species, *A. niger* and *Ch. globosum*. Since the preventive treatment with CLT totally inhibited *A. niger* and this species was able to grow after the curative treatment with the same compound, this could be a matter of the quantity of CLT absorbed by the paper with the different treatment methods (immersion in preventive treatment versus pipetting in the curative treatment). To investigate this hypothesis,

a curative treatment applied by immersion for 60 seconds was also tested and the results are presented in Figure 7.32.

When comparing fungal growth measurements between the two treatment variants - pipetting and immersion - there are no statistically significant differences (p>0.05). However, a few alterations occurred, namely on the species developing on the treated samples and on the number of samples from where fungal growth was completely absent (Table 7.10).

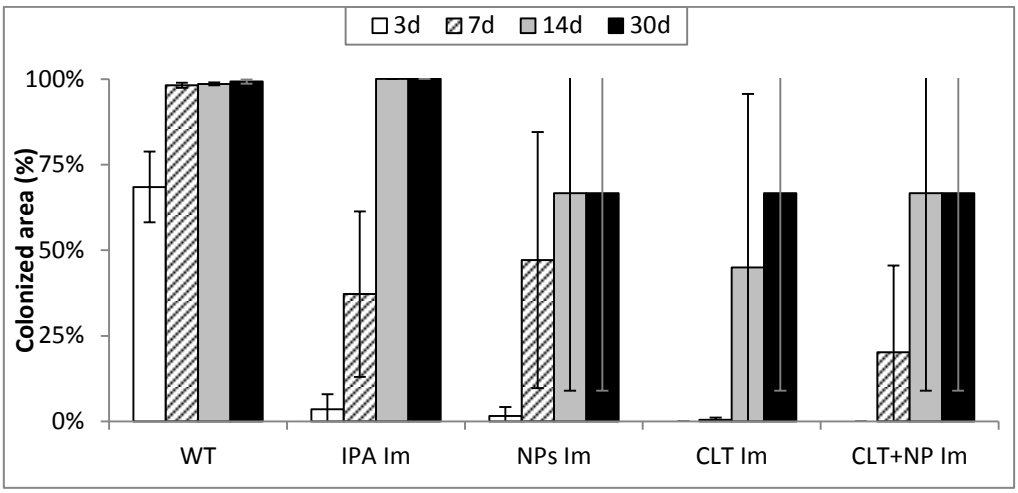
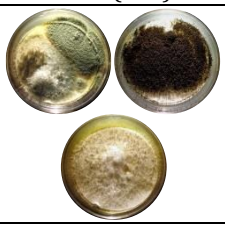
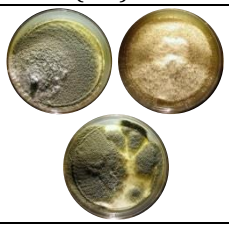
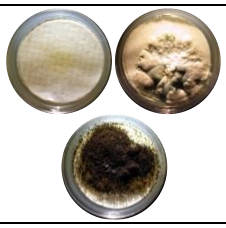
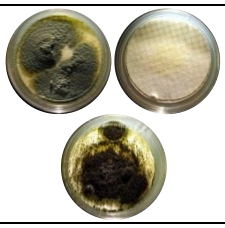
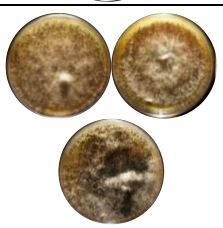
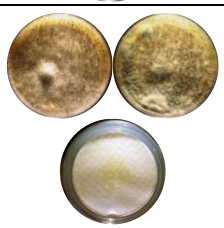
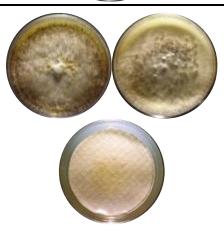
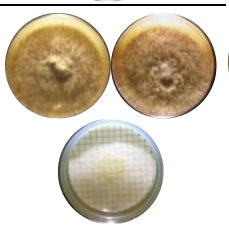


Figure 7.32: Results from the curative immersion treatment on paper inoculated by a mixed inoculum with *A. niger*, *P. chrysogenum*, *P. corylophilum*, *Ch. globosum* and *Cl. cladosporioides*, at 3, 7, 14 and 30 days of re-incubation. Each value is the average of 3 replicates ± SD.

With the immersion treatment, there was a higher efficacy against *A. niger* (total inhibition) and *Penicillium* (only developing in a few samples). *Ch. globosum* was the dominant species colonizing the samples treated by immersion (Table 7.10), as occurred with the preventive treatment. The total inhibition of *A. niger* occurred not only in CLT treated samples but also on Solv and NPs treated samples, meaning that a longer contact with the solvent aids in fungal elimination.

Table 7.10: Pictures taken with raking light of fungal growth on paper samples resulting from the two types of curative treatment tested (pipetting and immersion), at 30 days of incubation.

	Solv (IPA)	$Ca(OH)_2$ NPs	CLT	CLT+ NPs	WT
Pipetting treatment					
Immersion treatment					

While with the localized treatment, only CLT and CLT+NPs samples showed total fungal inhibition in part of the samples, with the immersion treatment NPs samples also presented this result (Table 7.10).

Generally, the immersion treatment improved the antifungal performance of the applied treatments, with a predominance of more resilient species and a slightly higher number of samples completely free of fungal colonization.

Cl. cladosporioides was totally inhibited by both curative treatments, as it did not grow in any of the samples (although its viability was confirmed in control plates), and was also antagonized in the WT samples, like in the preventive treatment when a mixed inoculum was tested.

7C.3.4 Evaluation of physical, chemical and optical alterations on treated paper samples

Alterations in pH, colour, mechanical resistance and molecular changes on treated paper samples were evaluated to assess any positive or negative impact of the applied treatments on the preservation properties of paper, in the short and long term.

7C.3.4.1 pH results

Calcium hydroxide was added to clotrimazole to form a multipurpose formulation that could simultaneously prevent further fungal development and neutralize already excreted acidic metabolites. The results presented in Figure 7.33 (a) show that treatment with $Ca(OH)_2$ nanoparticles (NPs and CLT+NPs) successfully raised the pH of pristine paper.

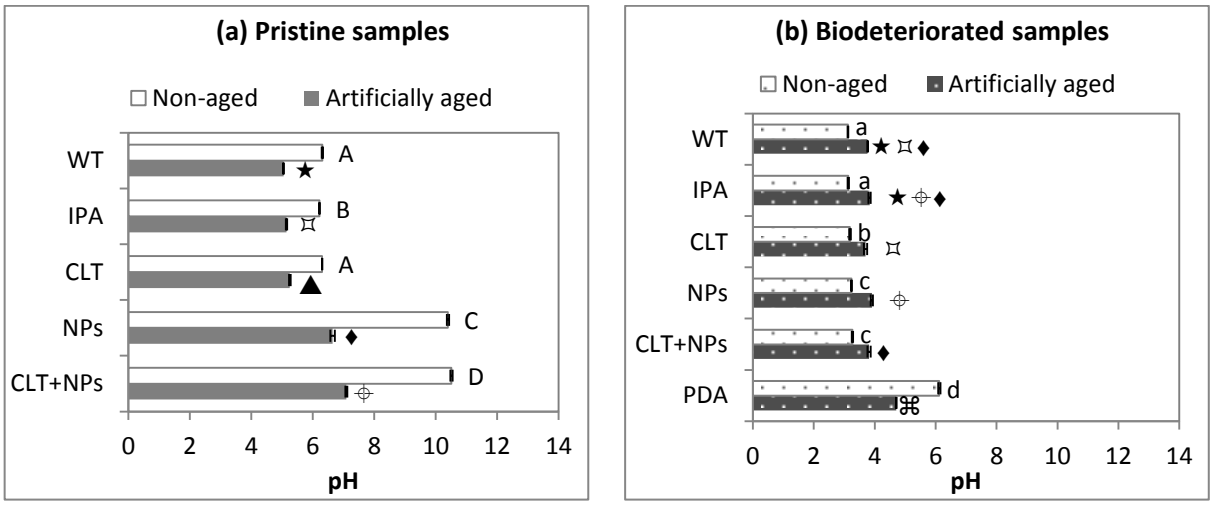


Figure 7.33: Results from pH measurements on pristine (a) and biodeteriorated (b) samples, non-aged and artificially aged. Each value is the average of 3 replicates ± SD. In each graph: bars followed the same letters (Non-aged) or symbols (Artificially aged) are not significantly different by Fisher's LSD test at α=0.05.

As concluded in a previous study (Sequeira et al., 2006), the final pH of paper samples treated with $Ca(OH)_2$ nanoparticles varies with the initial pH value of the paper and the concentration of the applied dispersion. The applied concentration was chosen as the amount needed to raise the pH of a 20th century acidic paper (~pH 5) to circa pH 8.5 (Sequeira et al., 2006), to better simulate a real case scenario. However, in the present study, as the paper originally had a pH

close to neutral values, with the deacidification treatment the pH rose to values above 10. These values may be considered already too high as they may favour oxidation reactions and cause cellulose depolymerization (Malesic et al., 2002; Baty et al., 2010). Nonetheless, with ageing, these values decrease to around neutral pH, likely due to carbonatation of $Ca(OH)_2$ and consumption of the alkaline compound by reaction with acidic groups.

The CLT treatment did not cause any significant alteration of pH in non-aged samples. However, with ageing, CLT protected the paper from acidification to some extent, showing a slightly higher pH value than untreated samples. This may be due to the radical scavenging properties of clotrimazole (Iannelli et al., 2011), which could have inhibited oxidation reactions occurring in the cellulose polymer yielding carboxylic acids. The paper treated with CLT + NPs also showed higher pH than NPs alone, possibly due to the same radical scavenging mechanism.

On biodeteriorated samples Figure 7.33 (b), the treatment with NPs caused a slight increase in pH, but was not sufficient to reach neutral/alkaline values.

After artificial ageing, biodeteriorated samples treated with NPs or CLT+NPs show similar pH values to samples treated with IPA or WT controls. Therefore the treatment with NPs did not have a significant deacidification efficacy on the acidic metabolites excreted by *A. niger*.

7C.3.4.2 Colorimetric results

The overall colour alterations (ΔE) for pristine samples are presented in Figure 7.34 and the differences in the individual colour coordinates L*, a* and b* in Table 7.11. The actual coordinates and pictures of the samples are presented in Appendix V.

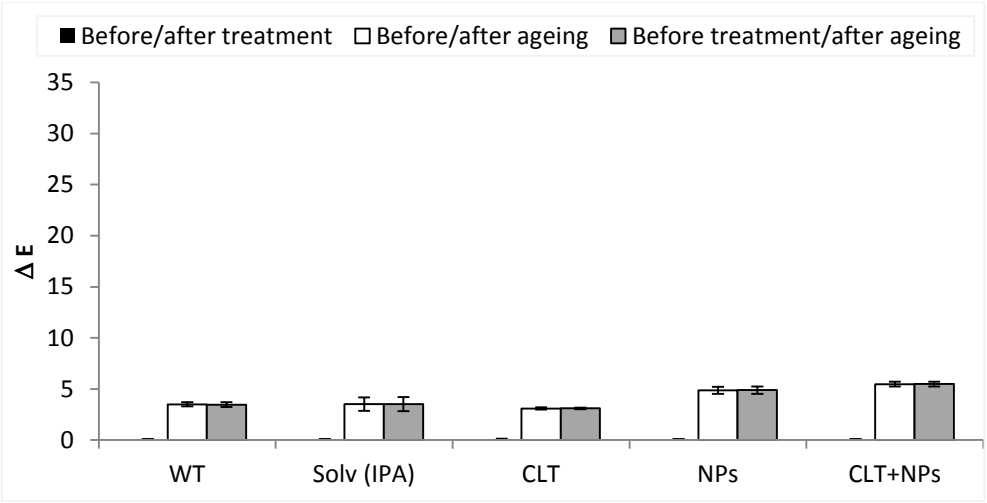


Figure 7.34: Calculated ΔE values for pristine samples. Each value is the average of 3 replicates ± SD.

In pristine samples, none of the treatments caused statistically significant colour alterations (ΔE) (Figure 7.34). After artificial ageing, both NPs and CLT+NPs treatments caused a statistically significant increase in ΔE values, when compared with WT samples (p<0.05). Even tough, the differences between WT samples and samples treated with NPs and CLT+NPs are

small (1<ΔE<2), and can only be noticed by an experienced observer (Mokrzycki and Tatol, 2011).

Table 7.11: Colorimetric alterations calculated for pristine Whatman #1 paper before and after treatments and after artificial ageing. Each value is the average of 3 replicates ± SD. In each column, values followed by the same letters are not significantly different by the Fisher's LSD test at α=0.05.

	Pristine samples					
	After treatment – Before treatment			After artificial ageing - Before Treatment		
	ΔL*	Δa*	Δb*	ΔL*	Δa*	Δb*
WT	0.03 ±0.04 a	0.00 ±0.01 a	-0.01 ±0.04 a	-1.68 ±0.09 a	0.36 ±0.03 a	3.02 ±0.23 a
Solv	0.03 ±0.00 a	0.01 ±0.00 a	0.02 ±0.04 a	-1.86 ±0.29 a	0.36 ±0.08 a	2.97 ±0.62 a
CLT	-0.07 ±0.06 a	0.01 ±0.00 a	-0.03 ±0.02 a	-1.66 ±0.07 a	0.31 ±0.02 a	2.61 ±0.06 a
NPs	-0.01 ±0.02 a	0.00 ±0.00 a	0.02 ±0.06 a	-2.60 ±0.22 b	0.53 ±0.06 b	4.11 ±0.30 b
CLT+ NPs	-0.01 ±0.03 a	0.01 ±0.00 a	0.01 ±0.04 a	-2.93 ±0.15 b	0.63 ±0.08 b	4.59 ±0.22 b

Looking into Table 7.11, it can be seen that with ageing, NPs and CLT+NPs caused a decrease in lightness, an increase in magenta and an increase in yellow coordinates (lower L*, higher a* and higher b*, respectively). Stefanis and Panayiotou (2007) also observed yellowing of paper samples treated with $Ca(OH)_2$ NPs after artificial ageing. These changes were probably caused by oxidation reactions and formation of chromophores in such alkaline samples since oxidation is the dominant degradation pathway in alkaline paper (Strlic and Kolar, 2005:121).

On biodeteriorated samples, the overall colour alterations (ΔE), either with treatment or with ageing, are much higher than on pristine samples. Also, the chromatic heterogeneity caused by fungal staining is revealed by the higher standard deviations (Figure 7.35).

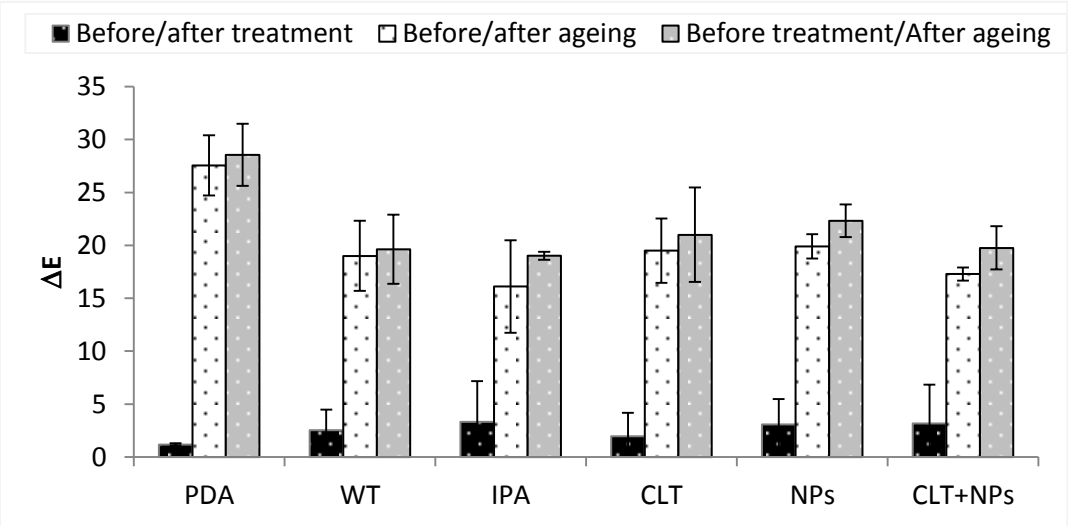


Figure 7.35: Calculated ΔE values for biodeteriorated samples. Each value is the average of 3 replicates ± SD.

None of the treatments caused statistically significant ΔE on biodeteriorated samples, either before or after artificial ageing (Figure 7.35).

Table 7.12: Colorimetric alterations calculated for Whatman #1 paper colonized by *A. niger* before and after treatments and after artificial ageing. Each value is the average of 3 replicates ± SD. In each column, values followed by the same letters are not significantly different by the Fisher's LSD test at α=0.05.

	Biodeteriorated samples					
	After treatment – Before treatment			After artificial ageing - Before Treatment		
	ΔL*	Δa*	Δb*	ΔL*	Δa*	Δb*
PDA	-0.47 ±0.05 a	-0.08 ±0.01 a,c	1.09 ±0.07 a,b	-21.69 ±1.25 a	5.86 ±0.50 a	17.64 ±1.05 a
WT	-0.71 ±3.15 a	0.27 ±0.24 b	0.04 ±1.61 b	-15.09 ±2.13 b	6.15 ±0.32 a	10.91 ±2.82 b
Solv	-2.14 ±0.31 a	0.11 ±0.21 b,a	2.55 ±0.27 a	-15.58 ±0.70 b,c	6.32 ±0.54 a,b	8.81 ±1.17 b
CLT	-0.93 ±0.45 a	0.19 ±0.22 a,c,d	1.72 ±0.64 a	-17.00 ±1.25 b,c	6.95 ±0.87a,b	10.09 ±1.55 b
NPs	-1.69 ±0.24 a	-0.29 ±0.14 c,d	2.57 ±0.19 a	-18.13 ±0.69 c	7.45 ±0.17 b	10.64 ±1.06 b
CLT+ NPs	-1.59 ±1.50 a	-0.44 ±0.11 d	2.58 ±1.09 a	-14.85 ±1.63 b	6.13 ±0.73 a	11.49 ±2.03 b

Regarding the individual colour coordinates, all treatments caused an immediate increase in the b* coordinate (yellowness), and are all identical to the solvent alone (Table 7.12), likely due to dissolution and spreading of coloured fungal debris onto the measured areas.

After artificial ageing, none of the treated biodeteriorated samples presented significantly different colour coordinates from WT or Solv controls, (Table 7.12). These results indicate that the performed treatments did not protect the paper from discoloration caused by fungal metabolites.

The high yellowing and darkening on the PDA controls after ageing is noteworthy, and suggests that the presence of culture medium could have had a great influence on the discoloration of biodeteriorated samples.

7C.3.4.3 Folding endurance results

Table 7.13 summarizes the folding endurance (FE) results on both pristine and biodeteriorated samples.

All pristine non-aged samples show identical FE values (Table 7.13 – first column), indicating that in theshort term none of the treatments altered these mechanical properties of paper. However, with artificial ageing, the tested deacidificant and antifungal treatments (CLT, NPs and

CLT+NPs) prevented the loss of folding endurance on paper (Table 7.13 – second column). The best results were obtained with the CLT+NPS mixture.

Table 7.13: Folding endurance results from pristine and biodeteriorated samples. Each value is an average of ten replicates ± SD. In each column, values followed by the same letters are not significantly different by the Fisher's LSD test at α=0.05.

	Folding endurance			
	Pristine		Biodeteriorated	
	Non-aged	Aged	Non-aged	Aged
WT	2.58 ± 0.18 a	1.82 ± 0.09 a	1.97 ± 0.14 a	0.35 ± 0.33 a
Solv	2.52 ± 0.16 a	1.91 ± 0.11 a b	1.88 ± 0.14 a	0.69 ± 0.35 b
CLT	2.55 ± 0.19 a	1.99 ± 0.18 b	1.83 ± 0.16 a	0.61 ± 0.29 a b
NPs	2.54 ± 0.22 a	2.17 ± 0.17 c	1.82 ± 0.16 a	0.57 ± 0.33 a b
CLT+NPs	2.49 ± 0.17 a	2.24 ± 0.14 c	1.85 ± 0.22 a	0.76 ± 0.26 b
PDA			2.77 ± 0.23 b	2.12 ± 0.17 c

All biodeteriorated non-aged samples show analogous FE results among them (Table 7.13 – third column). Only the non-inoculated control (PDA) presents significantly higher FE values, once more demonstrating the deleterious effect caused on paper by the fungus itself.

With artificial ageing, the FE of all biodeteriorated samples decreases considerably when compared to the non-inoculated controls, reaching values close to zero (Table 7.13 – fourth column). It is important to note that this deterioration is caused only by the fungal remains, as at the environmental conditions at which the artificial ageing was conducted no fungal growth was possible.

All treatments protected the biodeteriorated paper from FE loss with ageing, as all treated samples presented a statistically significant higher FE than WT controls (Table 7.13 – fourth column). However, no significant differences were detected among treatments, which indicate that the prevention of FE loss with ageing may be due to the influence of the solvent. As detected by colourimetric analyses, after treatment, all biodeteriorated samples exhibited a higher yellowing than WT samples, probably due to the dissolution and spreading of coloured fungal debris. This dissolution could have lowered the concentration of degrading compounds, consequently diminishing their deteriorating effect on the paper.

7C.3.4.4 ATR FTIR and XRD results

The samples treated with CLT alone (Figure 7.36 c) exhibit the typical infrared spectrum of cellulose already discussed in Chapter 7A (section 7A.3.3.4) (Garside and Wyeth, 2003; Cocca et al., 2011), similar to untreated controls (Figure 7.36 b), and without any of the characteristic bands of clotrimazole reference (Figure 7.36 a). The low concentration of the applied compound (0.05%) is probably below the detection limits of this technique. IPA treated samples show identical spectra to WT samples and are not shown.

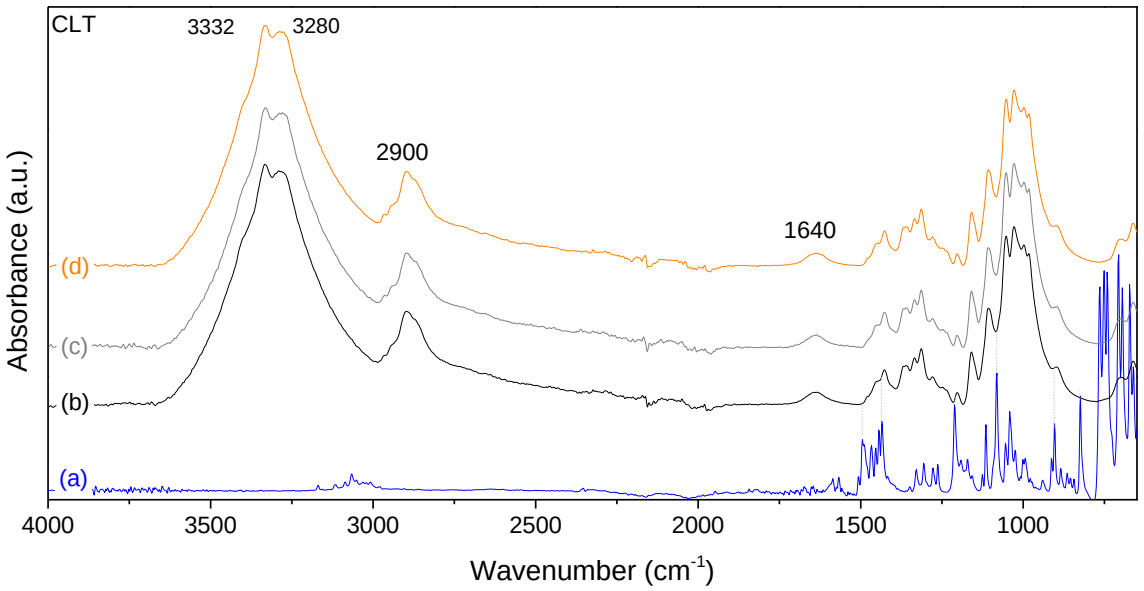


Figure 7.36: Infrared spectra from (a)CLT reference (Alfa Aesar, USA), untreated control sample (b), and paper sample treated with CLT before (c) and after artificial ageing (d).

Although the colorimetric analyses presented above revealed some discoloration on aged samples, which could be attributed to oxidation and formation of chromophores, in the infrared spectra the formation of carbonyl groups could not be detected (Figure 7.36 d). As explained in Chapter 7A, this lack of detection of carbonyls could be due to the low concentration of these groups in the cellulose polymer, below the detection limit of this technique, or due to a masking by the adsorbed water band of cellulose at 1640 cm^{-1}.

FTIR analysis of non-aged pristine samples treated with NPs and CLT+NPs are identical (Figure 7.37 a and b). In addition to cellulose absorption bands, NPs and CLT+NPs samples reveal the characteristic band of $Ca(OH)_2$ at 3640 cm^{-1}.

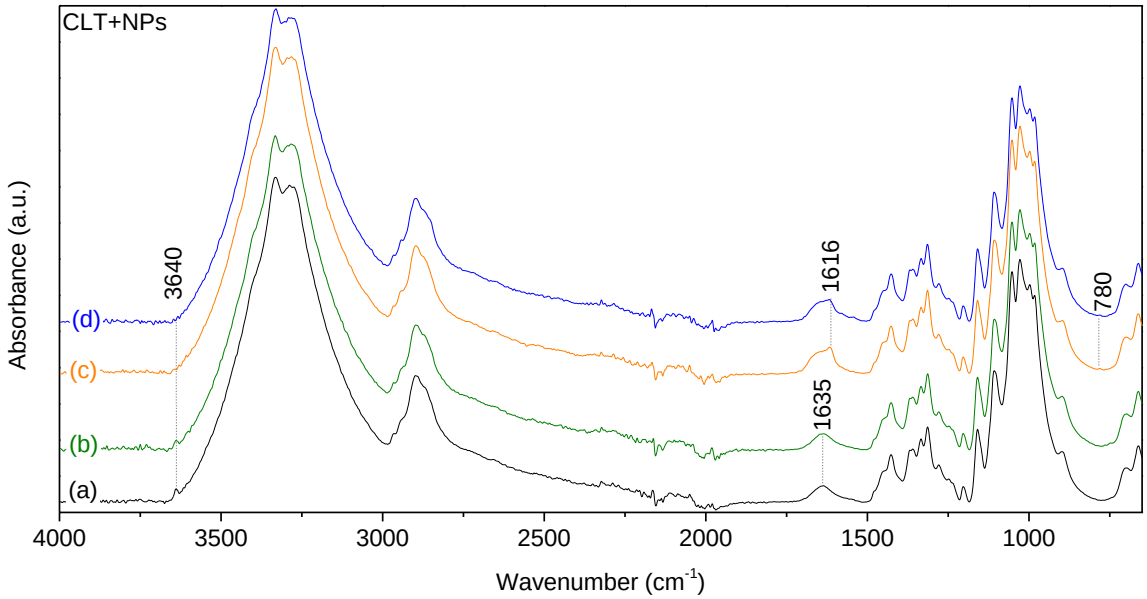


Figure 7.37 : Infrared spectra of pristine samples treated with $Ca(OH)_2$ NPs before (a) and after artificial ageing (c) and CLT+ NPs before (b) and after artificial ageing (d).

After artificial ageing, the band at 3640 cm^{-1} almost disappears (Figure 7.37 (c) and (d)), probably due to the carbonatation and/or reaction with acidic groups (which accounts for the previously detected decrease in pH). $CaCO_3$, the resulting product of $Ca(OH)_2$ carbonatation by air exposure, was not detected in the infrared spectra, probably once more due to a low concentration. As with the WT, IPA and CLT samples, no carbonyl groups were detected in the aged NPs and CLT+NPs samples.

Nevertheless, with ageing, the same phenomenon detected on calcium propionate treated samples on Chapter 7A (section 7A.3.3.4) was observed on the samples treated with $Ca(OH)_2$ - the appearance of small bands at 1616 cm^{-1} and 780 cm^{-1}, which can be attributed to calcium oxalate (Pinzari et al., 2010).

In biodeteriorated samples, the characteristic bands of chitin from the fungus were observed in the infrared spectra, as discussed in Chapter 7A (section 7A.3.3.4). The infrared spectra from biodeteriorated IPA and CLT samples are identical to the WT samples and are not shown here.

Biodeteriorated CLT+NPs samples were similar to NPs samples, and besides cellulose and chitin, exhibited the calcium hydroxide band at 3640 cm^{-1} (Figure 7.38 b) and a small band at 780 cm^{-1} assigned to calcium oxalate. With ageing, the hydroxide band at 3640 cm^{-1} decreased and the bands assigned to calcium oxalate (Figure 7.38 a) increased (Figure 7.38 c) This indicates a consumption of $Ca(OH)_2$ with ageing, with one of the resulting products being calcium oxalate.

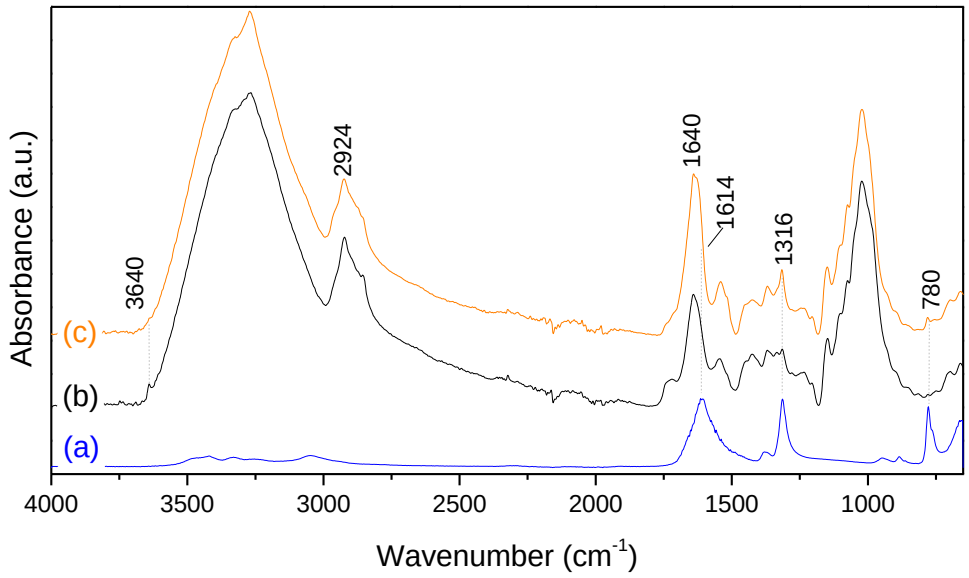


Figure 7.38: Infrared spectra of reference calcium oxalate (a), and biodeteriorated paper samples treated with CLT + $Ca(OH)_2$ NPs, before (b) and after (c) artificial ageing.

Although the presence of calcium oxalate on aged NPs pristine samples detected by FTIR was not detected by XRD (Figure 7.39 c), the biodeteriorated NPs samples did reveal the presence of calcium oxalate (Figure 7.39 b). This is confirmed by the peaks at 14.8°, 24.3° and 30.0°, as in the reference shown in Figure 7.39 (a). As discussed in Chapter 7A, calcium oxalate was detected in all biodeteriorated samples, not only in the ones treated with calcium compounds. Therefore, there is the possibility that the cristaline form of calcium oxalate detected in biodeteriorated samples treated with NPs could have already been formed before treatment, during the fungal colonization period.

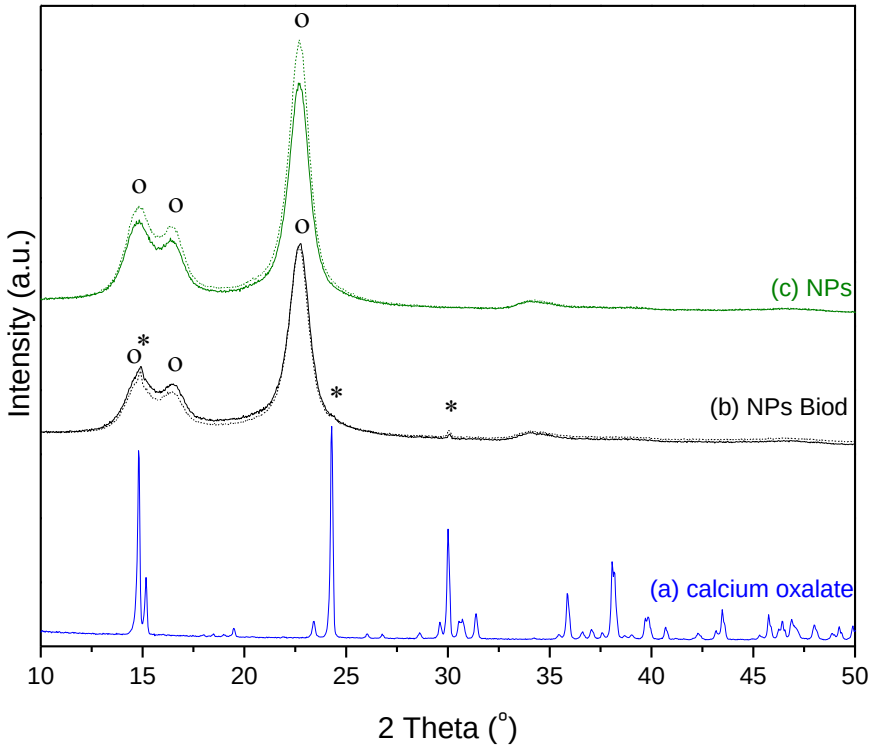


Figure 7.39: X-ray diffractograms obtained from calcium oxalate reference (a) (≥98.0%, Fluka), biodeteriorated paper treated with $Ca(OH)_2$ NPs (b); and pristine samples also treated with $Ca(OH)_2$ NPs (c). Solid lines represent non-aged samples and dotted lines represent artificial aged samples. Peaks attributed to cellulose are marked with "o" and peaks attributed to calcium oxalate are marked with "*".

7C.4 Conclusions

This study was aimed at evaluating the potential of clotrimazole and $Ca(OH)_2$ nanoparticles to prevent and treat fungal biodeterioration on paper.

CLT showed antifungal activity against all tested species and its effectiveness varied according to fungal species. The best results were achieved against *P. corylophilum*, which was totally inhibited with CLT concentrations as low as 0.001% (w/v). CLT at 0.05% was the solution achieving the best lower concentration/higher inhibition relationship.

The addition of $Ca(OH)_2$ NPs generally decreased the preventive antifungal activity of CLT, possibly due to a disturbance in the antifungal mode of action of CLT caused by the pH raise. However, when applied in acidic papers, $Ca(OH)_2$ will be consumed and the final pH of the paper will not be so high, which probably will diminish the negative effect on CLT activity. Also, since CLT decomposes in acidic environment, $Ca(OH)_2$ NPs could help prevent this decomposition in a long term basis.

When applied as a curative treatment, $Ca(OH)_2$ NPs did not diminish the antifungal effect of CLT, as both CLT alone and CLT+NPs samples had identical fungal growth results. This indicates a damaging effect on the fungal structures caused by the direct contact with the NPs alkaline solution, which does not occur in the case of a preventive treatment. With the immersion treatment, CLT and NPs totally inhibited four of the five tested fungal species. *Chaetomium globosum* was the only species able to grow after treatment and only in part of the tested samples.

Regarding the effects these compounds had on the characteristics of treated paper, the treatment with CLT+NPs on pristine samples raised the paper's pH to values slightly above 10. When subjected to artificial ageing, the pH droped to neutral values, but the effects of alkaline degradation were visible through a slight darkening and yellowing of paper. This alkaline content, on the other hand, protected the paper from acidification and loss of mechanical properties at a long term. CLT alone, although in a small concentration revealed a slight protective effect against paper acidification and colour alteration with ageing, which could be attributed to its antioxidant properties.

On biodeteriorated samples the deleterious effects of *A. niger* were very intense, confirmed by the strong acidification of the paper, discoloration and total loss of folding endurance. The excretion of oxalic acid was detected by the formation of calcium oxalate primarily on the samples treated with calcium.

The protective effect of CLT and NPs treatments on biodeteriorated samples was less evident than in pristine samples, due to the strong deteriorating power of *A. niger*. Nevertheless, a slight increase in pH was measured in samples treated with NPs. Washing the paper prior to treatment could help the accomplishment of better results, as it would remove the majority of the acidic and oxidative compounds left by the fungi.

7.3. General conclusions from Chapter 7

In the three subchapters of Chapter 7, different antifungal formulations were evaluated. When comparing the capacity of each antifungal to prevent fungal development, parabens (Chapter 7A) achieved the best results, by completely inhibiting the development of all tested species, with the exception of *Penicillium corylophilum*. Interestingly, on Chapter 7C, where a formulation containing clotrimazole was tested, *Penicillium corylophilum* was the most susceptible fungal species, being totally inhibited even with the lowest concentration of clotrimazole, 0.001%. The remaining fungal species (*A. niger*, *Cl. cladosporioides, Ch. globosum*, *P. chrysogenum*) showed various levels of tolerance to clotrimazole, with *Chaetomium globosum* showing the greatest tolerance.

Parabens and clotrimazole have different antifungal mechanisms. While parabens act by inhibiting the proton motive force across the microbial cell membrane (Russell, 2003), clotrimazole acts by inhibiting ergosterol biosynthesis in the cell membrane (Kleyi et al., 2012). Accordingly, *P. corylophilum* is probably more sensitive to the inhibition of ergosterol synthesis, and *Chaetomium globosum* possibly produces other sterols than ergosterol (Taniwaki et al., 2006) and can therefore survive this inhibition.

These results point to the possibility of another formulation development, where a small percentage of clotrimazole is added to parabens to inhibit fungal species that, like *P. corylophilum*, are more tolerant to the inhibition of proton motive force and more dependent on ergosterol synthesis to develop.

The study of limonene as an antifungal for paper materials on Chapter 7B was interrupted in the first screening phase against *P. chrysogenum*, since it showed signs of increasing fungal development instead of inhibiting it.

When a curative treatment was simulated and the paper samples were first colonized, then dried, cleaned and treated afterwards, different results were obtained. The curative treatment with the formulation studied in Chapter 7A (parabens + calcium propionate) presented excellent antifungal properties as none of the five inoculated fungal species were able to grow after returning to conducive conditions. The formulation studied in Chapter 7C (Clotrimazole + $Ca(OH)_2$ nanoparticles), also exhibited better antifungal results than on the preventive treatment, with one third of the samples being completely free of fungi.

The difference between the preventive and curative treatments relies mainly on the contact between the fungal structures and the alcoholic solvents used in both formulations. While on the preventive treatment the solvent had already evaporated from the paper samples when inoculation occurred, in the curative treatment the solvent came in direct contact with the fungi already present on the samples. In Chapter 7A, ethanol at 72.15% is used as a solvent, and in Chapter 7C pure isopropanol is used instead. Whereas the contact with the ethanolic solution only enables the development of *Ch. globosum*, the contact with isopropanol is less efficient, with a higher number of species surviving the treatment. The percentage of water in the ethanolic solution, which does not exist in the pure isopropanol, makes a difference in the antifungal performance. As observed before on Chapter 6, pure ethanol was less effective than 70% ethanol.

Regarding the effects of the tested formulations on the chemical and physical stability of treated paper, the parabens + calcium propionate formulation provided a higher protection from long term deterioration on biodeteriorated samples. This protection was mainly due to the contribution of calcium propionate. However, in pristine samples, calcium propionate caused a significant yellowing of the samples. Clotrimazole and calcium hydroxide caused much less yellowing and protected the paper

from long term loss of mechanical resistance. On the other hand the protective effect of this formulation on biodeteriorated samples was negligible.

In general, the formulation containing parabens + calcium propionate had better antifungal activity and protected the paper from the deteriorating effects of fungal metabolites at a long term. However, a much higher quantity of products (antifungal and deacidificant) were applied, the pH after artificial ageing remains higher than desirable, and on pristine samples, this treatment caused a significant yellowing of the samples.

The clotrimazole + $Ca(OH)_2$ nanoparticles formulation, although showing a lower antifungal activity, was effective at very low concentrations (0.05%). Also, calcium hydroxide and its reaction product to air exposure, calcium carbonate, are compounds already present in most historic papers. These characteristics make this formulation less interventive than the previous one. Besides, although clotrimazole + $Ca(OH)_2$ did not significantly protect paper from the deterioration caused by the strongly acidic *A. niger* metabolites, the yellowing caused on pristine samples were much lower than the one caused by calcium propionate.

8

Concluding remarks and future perspectives

The interdisciplinary work described in this thesis was aimed at studying and developing accessible remedial conservation treatments with low toxicity to mitigate one of the main causes of deterioration occurring on paper based documents and works of art - fungal biodeterioration. The experimental approaches displayed throughout the different chapters were developed in accordance with this objective.

The critical literature review presented in Chapter 2 allowed to conclude that calcium propionate, parabens and ethanol, were the currently existing chemical antifungal compounds combining better antifungal properties with minor health effects and less negative impact in paper properties. Among these three, parabens were the ones with best antifungal properties. However, these compounds required further testing before being considered safe for application in cultural heritage materials, and therefore were regarded for additional analysis in the present thesis.

Through the questionnaire presented in Chapter 3, it was possible to determine that the great majority of paper conservators who responded, already experienced active fungal infestations on paper based collections and all of them have already dealt with paper showing signs of fungal deterioration, despite using preventive measures to control fungal biodeterioration in their institutions. Also, the surveyed paper conservators, considered the study of non-toxic/safer antifungals as the area with greatest need for further research, revealing that the currently available products and methods to prevent and suppress fungi were not fulfilling their requirements. These results highlighted the importance of the present work, and helped to aim the research towards the needs of conservation-restoration practitioners, hence improving its future impact and practical use in paper conservation.

Before starting the experiments with antifungal compounds it was necessary to select representative fungal species to test, and determine which method for fungal growth quantification would be more appropriate to paper samples and would give the most reliable results.

Based on a literature review where fungal species were isolated from paper on areas showing signs of fungal deterioration, five fungal species were selected: *Aspergillus niger*, *Chaetomium globosum*, *Cladosporium cladosporioides*, *Penicillium chrysogenum* and *Penicillium corylophilum*.

Three methods for fungal growth quantification on paper were analysed, photochemical quantification of NAGase (an enzyme excreted by fungi during growth), measurement of colonization area by image analysis, and biomass dry weigh determination. The photochemical method revealed several limitations, like the correlation between fluorescence and biomass

differing according to fungal species and stage of growth, and also the possibility of false positive or negative results.

The measurement of colonization area using raking light illumination, proved to be a simple, non-destructive method, with high sensitivity and high correlation with biomass dry weight.

Biomass dry weight determination, although being a destructive method and having less sensitivity than the previous method, was more appropriate for quantification of aged cultures and takes into account the density of fungal colonies, rather than just the extent of colonized area. In view of the positive results achieved with these last two methods, both were used to evaluate the efficacy of antifungal treatments in the subsequent experiments.

In Chapters 6 and 7, different antifungal treatments were evaluated in terms of antifungal efficacy and effects on treated paper. Besides the treatments already selected from the literature review in Chapter 2 (ethanol, and a formulation containing parabens and calcium propionate (PBs+CP)), other two novel antifungal compounds were screened for testing on paper: clotrimazole and limonene. It was concluded that limonene did not have any antifungal activity on the tested fungus (*P. chrysogenum*), while clotrimazole was very effective. Clotrimazole (CLT) was then added to a deacidification agent, calcium hydroxide nanoparticles (NPs) to constitute a multipurpose formulation with antifungal and deacidificant properties.

All the main tested formulations (70% ethanol; parabens+calcium propionate; clotrimazole+calcium hydroxide nanoparticles) revealed a curative antifungal effect, meaning that when applied in paper already colonized by fungi they diminished or totally repressed fungal regrowth when paper returned to ideal fungal growth conditions. The curative antifungal effect was the highest with PBs+CP and the lowest with 70% EtOH. *Ch. globosum* was the species showing the highest level of tolerance to these curative treatments.

Regarding the preventive antifungal effect, where paper samples were previously treated with the antifungal formulations and subsequently inoculated and incubated, 70% ethanol was not successful, since as expected, no alcohol is left in the paper in the longer term. PBs+CP was the most effective in inhibiting the growth of all tested species, except for *P. corylophilum*, for which CLT+NPs had a higher inhibiting effect, achieving a complete inhibition, even at very low concentrations.

When tested on plain Whatman paper, all treatments prevented the acidification of paper with artificial ageing, although only the formulations containing deacidificant agents (CLT+NPs and PBs+CP) maintained the pH of the paper above 7. While all treatments prevented the loss of mechanical resistance, the presence of NPs and CP in the respective formulations resulted in a higher folding endurance outcome than the treatment with 70% EtOH. However, these deacidificant agents caused discoloration of paper when artificially aged. Whereas the discoloration on samples treated with CLT+NPs is hardly discernible from untreated controls (see Chapter 7C), the discoloration caused by PBs+CP is rather evident (Chapter 7A).

Nonetheless, PBs+CP was the only formulation that could prevent paper degradation caused by the metabolites left by *A. niger* at a long term, namely preventing discoloration, loss of folding endurance and acidification.

When comparing all the studied antifungal treatments, taking into account the ethical principles of conservation of cultural heritage (ECCO, 1993), since the treatment with 70% ethanol is the one having the best relation between antifungal activity and minimal intervention, it should be considered first. Although not possessing preventive antifungal properties, there is an advantage in this treatment not leaving any residues, as no unexpected reactions can result from the long term degradation of those residues. However, for a collection at high fungal biodeterioration risk, a more interventive treatment should be considered, like the one with the formulation containing clotrimazole and calcium hydroxide. This formulation, besides having better curative antifungal efficacy than ethanol, also has long term preventive antifungal, allied with a better protective effect against paper deterioration.

The formulation containing parabens with calcium propionate, due to its excellent results in preventing deterioration caused by fungal metabolites at a long term, could be a good option for treating paper seriously affected by fungi. Even tough, its application as a prophylactic treatment is not advisable, due to the obtained discoloration in pristine samples.

Taking into account all the obtained results, it is possible to conclude that the main objective of this thesis was successfully accomplished. Either 70%EtOH, PBs+CP or CLT+NPs are effective antifungal treatments against five of the most common fungal species affecting paper collections, have low toxicity and are easy to obtain and to apply. Except for the discoloration mainly caused by CP, the studied treatments did not cause further deterioration on paper and PBs+CP was very effective in increasing the chemical stability of biodeteriorated paper.

In this study it was the first time, to our knowledge, that paper was first colonized by fungi before being subjected to artificial ageing. The results illustrate how damaging the fungal metabolites of an already inactive fungus can be to cellulose in the long term. These findings reinforce the need for cleaning the affected objects, not only mechanically, but also chemically, if possible.

The application of any of the studied treatments on paper documents or artworks should always be preceded by solubility tests, as common in conservation practice, to guarantee that there will be no undesirable dissolutions. Although in this research the treatments were applied by immersion or by pipetting, in conservation practice they could be applied as more convenient to the characteristics of the paper object, minimizing the risks of dissolution and/or deformation.

Since antimicrobial compounds are, by definition, biologically active, they have the potential to induce toxicological effects in humans. It is, therefore, advisable to avoid skin or eye contact, ingestion or direct inhalation of such products.

When dealing with objects showing fungal development, additional health precautions should always be taken, as the colonizing fungi might be toxigenic or allergenic. It is important that those objects are handled with personal protective equipment like gloves and masks with HEPPA filters, and when possible, inside a vertical laminar flow chamber.

All the reagents used in the tested formulations can be bought in chemical products or conservation products retailers and used in a regular conservation studio/laboratory. Calcium hydroxide nanoparticles can be synthesized in laboratory as described in Chapter 7C, but it requires equipment that is not common in paper conservation laboratories. Alternatively, the

suspension of calcium hydroxide nanoparticles in isopropanol can be purchased from conservation products retailers (http://www.csgi.unifi.it/products/paper.html).

Notwithstanding, the best way to control fungal development is by preventing it. Preventive conservation is always the most economic and safe (for human health and for collections) method to control fungal biodeterioration. Keeping the relative humidity in paper depositories below 60%; using proper ventilation to homogenize RH and temperature, allied with a microparticle filtration system to capture fungal propagules; protecting the objects from dust; and performing regular inspections on depositories, namely opening compact shelves regularly to detect any early infestation; are some of the most important preventive measures to avoid fungal development on paper collections.

However, when fungal infestations still occur, it is advisable to know what the best treatment options are. It is hoped that the discussions and findings of this thesis contribute to aid paper conservators in decision-making effort, and open the way for further research in this challenging field of cultural heritage conservation.

Future perspectives

In the present work, one of the main gaps detected in this field of research, was the lack of sensitive and non-destructive techniques for the quantification of initial, minute fungal growth on a paper matrix. The development of such technique would be of great interest, not only to use in biodeterioration scientific research, but also to detect initial fungal growth on cultural heritage objects.

Further analysis could be performed to complement the study of the effects of the tested formulations on paper preservation at a long term, namely:

- accelerated ageing by exposure to light, to test the effect of photochemical degradation on the tested treatments;
- testing on papers containing lignin or hemicelluloses and additives, like different types of sizing agents and fillers;
- testing on paper with writing and painting media, like iron gall ink, watercolors, pastel, crayons, and pencils.

Another important application of the studied antifungal compounds that would be worth testing would be their addition to adhesives used in paper conservation (e.g. starch paste), to increase their durability in solution and diminish their bioreceptivity after application on paper.

The studied antifungal formulations with long term antifungal efficacy (PBs+CP and CLT+NPs), could also be applied in paper labels, or cardboard boxes and other protective materials containing cultural heritage objects, to help prevent biodeterioration on these external protection barriers.

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Appendix I

Table I.1: Fungal genera and species isolated from paper/book materials, with the respective geographic location and bibliographic references.

Fungal species	Country	References
Acremonium sp. Link	Brazil, Iran, Finland, Portugal	(Hyvärinen et al., 2002; Lourenço et al., 2005; da Silva et al., 2006; Shamsian et al., 2006)
Alternaria sp. Nees	Argentina, Cuba, Iran, Italy, Portugal	(Lourenço et al., 2005; Shamsian et al., 2006; Michaelsen, 2010; Borrego et al., 2012; Pinheiro, 2014)
Alternaria alternata (Fr.) Keissler	India, Portugal	(Das et al., 1997; Mesquita et al., 2009)
Arthrinium sp. Kunze	Portugal	(Pinheiro, 2014)
Arthrinium urticae M.B. Ellis	Brazil	(Corte et al., 2003)
Aspergillus sp. P. Micheli ex Haller	Argentina, Cuba, Finland, Iran, Italy	(Hyvärinen et al., 2002; Corte et al., 2003; Shamsian et al., 2006; Zotti et al., 2007; Borrego et al., 2012; Montanari et al., 2012)
Aspergillus awamori Nakazawa	Poland	(Zerek, 2003)
Aspergillus candidus Link	India, Italy, Nigeria, Portugal	(Das et al., 1997; Zotti et al., 2008; Bankole, 2010; Pinheiro, 2014)
Aspergillus carneus (Tiegh.) Blochwitz	Israel	(Nol and Kenneth, 2001)
Aspergillus flavus Link	Argentina, Cuba , Israel	(Nol and Kenneth, 2001; Borrego et al., 2012)
Aspergillus fumigatus Fresen.	India, Israel, Nigeria, Portugal, Slovakia, USA	(Das et al., 1997; Nol and Kenneth, 2001; Di Bonaventura et al., 2003; Lourenço et al., 2005; Mesquita et al., 2009; Bankole, 2010; Kraková et al., 2012)
Aspergillus glaucus Link.	Portugal	(Pinheiro, 2014)
Aspergillus japonicus Saito	Brazil, France	(da Silva et al., 2006; Rakotonirainy, Heude, et al., 2007)
Aspergillus melleus Yukawa	Italy, Morocco	(Zotti et al., 2011; Bergadi et al., 2014)
Aspergillus nidulans(Eidam) G. Winter	Italy, Portugal	(Lourenço et al., 2005; Mesquita et al., 2009; Michaelsen et al., 2009)
Aspergillus niger Tiegh.	Argentina, Brazil Cuba, India Israel, Italy, Nigeria, Morocco, Portugal, Spain	(Das et al., 1997; Nol and Kenneth, 2001; Adelantado et al., 2005; Lourenço et al., 2005; da Silva et al., 2006; Michaelsen et al., 2009; Bankole, 2010; Borrego et al., 2012; Bergadi et al., 2014)
Aspergillus ochraceus Wilhelm	Poland, Portugal	(Zerek, 2003; Lourenço et al., 2005)
Aspergillus oryzae (Ahlb.) Cohn	France, Morocco	(Rakotonirainy, Heude, et al., 2007; Bergadi et al., 2014)
Aspergillus penicillioides Speg.	Italy, Japan, Portugal	(Arai, 2000; Michaelsen et al., 2010; Pinheiro, 2014)
Aspergillus puniceus Kwon-Chung et Fennell	Portugal	(Lourenço et al., 2005)
Aspergillus sclerotiorum G.A. Huber	Italy	(Zotti et al., 2011)
Aspergillus sydowii (Bainier & Sartory) Thom & Church	Portugal	(Lourenço et al., 2005)
Aspergillus tamarii Kita	Israel	(Nol and Kenneth, 2001)

Fungal species	Country	References
Aspergillus terreus Thom	Israel, Italy, Poland, Portugal	(Fabbri et al., 1997; Ricelli et al., 1999; Nol and Kenneth, 2001; Zerek, 2003; Michaelsen et al., 2010; Pinheiro, 2014)
Aspergillus ustus (Bainier) Thom & Church	France, India, Portugal	(Das et al., 1997; Lourenço et al., 2005; Rakotonirainy, Heude, et al., 2007)
Aspergillus versicolor (Vuill.) Tiraboschi	Brazil, Finland, Italy, Japan, Poland, Portugal	(Hyvärinen et al., 2002; Zerek, 2003; Lourenço et al., 2005; da Silva et al., 2006; Mesquita et al., 2009; Michaelsen et al., 2009, 2010; Pinheiro, 2014; Sato et al., 2014)
Aureobasidium sp. Viala & G. Boyer	Finland	(Hyvärinen et al., 2002)
Aureobasidium pullulans (de Bary) G. Arnaud	Italy, Portugal	(Zotti et al., 2007, 2008; Michaelsen et al., 2010; Pinheiro, 2014)
Bjerkandera adusta (Willd.) P. Karst.	France	(Rakotonirainy, Heude, et al., 2007)
Botrytis sp. P. Micheli ex Haller	Portugal	(Lourenço et al., 2005)
Botrytis cinerea Pers.	Italy, Portugal	(Mesquita et al., 2009; Michaelsen et al., 2009)
Botryotrichum piluliferum Sacc. & Marchal	Poland	(Zerek, 2003)
Candida oleophila Montrocher	Portugal	(Pinheiro, 2014)
Chaetomium sp.Kunze	Finland, Portugal, USA	(Hyvärinen et al., 2002; Di Bonaventura et al., 2003; Lourenço et al., 2005)
Chaetomium barilochense Calviello	USA	(Szczepanowska and Cavaliere, 2000)
Chaetomium brasiliense Bat. & Pontual	USA	(Szczepanowska and Cavaliere, 2000)
Chaetomium bostrychodes Zopf	Portugal	(Pinheiro, 2014)
Chaetomium gracile Udagawa	Italy	(Corte et al., 2003)
Chaetomium cymbiforme Lodha	USA	(Szczepanowska and Cavaliere, 2000)
Chaetomium elatum Kunze	Itally	(Fabbri et al., 1997; Ricelli et al., 1999)
Chaetomium globosum Kunze	Italy, India, France, Portugal USA	(Das et al., 1997; Szczepanowska and Cavaliere, 2000; Corte et al., 2003; Lourenço et al., 2005; Rakotonirainy, Heude, et al., 2007; Mesquita et al., 2009)
Chaetomium indicum Corda	Portugal	(Lourenço et al., 2005)
Chromelosporium carneum (Pers.) Hennebert	Portugal	(Mesquita et al., 2009)
Chrysonilia sp. Arx	Portugal	(Pinheiro, 2014)
Chrysosporium sp. Corda	Portugal	(Lourenço et al., 2005)
Chrysosporium carmichaelli	Portugal	(Pinheiro, 2014)
Cladosporium sp. Link	Argentina, Cuba, Finland, Iran, Italy, Nigeria, Poland, Portugal, USA	(Hyvärinen et al., 2002; Di Bonaventura et al., 2003; Lourenço et al., 2005; Shamsian et al., 2006; Michaelsen et al., 2009, 2010; Bankole, 2010; Borrego et al., 2012; Montanari et al., 2012; Sterflinger and Engel, 2013; Pinheiro, 2014)
Cladosporium cladosporioides (Fresen.) G.A. de Vries	Brazil, Italy, Portugal	(da Silva et al., 2006; Zotti et al., 2008; Mesquita et al., 2009; Michaelsen et al., 2009)
Cladosporium herbarum (Pers.) Link	India, Italy	(Das et al., 1997; Michaelsen et al., 2009)
Cladosporium oxysporum Berk. & M.A. Curtis	USA	(Di Bonaventura et al., 2003)

Fungal species	Country	References
Cladosporium sphaerospermum Penz.	Italy, USA	(Corte et al., 2003; Di Bonaventura et al., 2003; Zotti et al., 2011)
Cordyceps sinensis (Berk.) Sacc.	France	(Rakotonirainy, Heude, et al., 2007)
Coprinus sp.Pers.	Portugal	(Mesquita et al., 2009)
Cunninghamella elegans Lendner	Italy	(Corte et al., 2003)
Curvularia uncinata Bugnic.	India	(Das et al., 1997)
Davidiella tassiana (De Not.) Crous & U. Braun	Poland	(Sterflinger and Engel, 2014)
Debaromyces hansenii (Zopf) Lodder & Kreger-van Rij	Italy	(Michaelsen et al., 2009)
Doratomyces stemonitis (Pers.) F.J. Morton & G. Sm	Italy	(Zotti et al., 2007, 2008)
Epicoccum purpurascens Ehrenb *(*formerly known as *Epicoccum nigrum* Link)	Italy, Portugal	(Corte et al., 2003; Michaelsen et al., 2009; Pinheiro, 2014)
Eurotium sp. Link	Canada, Finland	(Florian and Manning, 2000; Hyvärinen et al., 2002)
Eurotium halophilicum C.M. Chr., Papav. & C.R. Benj. (Anamorph: *Aspergillus halophilicus*)	Italy, Portugal	(Michaelsen et al., 2010; Montanari et al., 2012; Pinheiro, 2014)
Eurotium herbariorum (Weber ex F.H. Wigg.) Link	Japan	(Arai, 2000)
Eurotium pseudoglaucum, (Blochwitz) Malloch & Cain	Italy	(Corte et al., 2003)
Exophiala sp. J.W. Carmich	Finland, Portugal	(Hyvärinen et al., 2002; Pinheiro, 2014)
Fusarium sp. Link	Brazil, Finland, Portugal	(Hyvärinen et al., 2002; da Silva et al., 2006; Pinheiro, 2014)
Fusarium oxysporum Schltdl.	India, Portugal, USA	(Das et al., 1997; Di Bonaventura et al., 2003; Pinheiro, 2014)
Fusicladium sp. Bonord.	Italy	(Corte et al., 2003)
Geomyces sp.Traaen	Japan	(Sato et al., 2014)
Geomyces pannorum (Link) Sigler & J.W. Carmich	Italy	(Zotti et al., 2007, 2008)
Geosmithia putterillii (Thom) Pitt	Italy	(Zotti et al., 2007, 2008)
Gliocladium catenulatum Gilman et Abbott	Poland	(Zerek, 2003)
Gliocladium roseum Bainier	Israel	(Nol and Kenneth, 2001)
Gloeotinia temulenta (Prill. & Delacr.) M. Wilson, Noble & E.G. Gray	France	(Rakotonirainy, Heude, et al., 2007)
Helminthosporium sp. Link	Iran	(Shamsian et al., 2006)
Humicola sp. Traaen	Portugal	(Lourenço et al., 2005)
Humicola grisea Traaen	Portugal	(Pinheiro, 2014)
Hypocrea lixii Pat.	Morocco	(Bergadi et al., 2014)
Kockovaella sp. Nakase, I. Banno & Y. Yamada	USA	(Di Bonaventura et al., 2003)
Madurella mycetomatis (Laveran) Brumpt	USA	(Di Bonaventura et al., 2003)
Memnoniella echinata (Rivolta) Galloway	India	(Das et al., 1997)
Mucor sp. Fresen	Finland, Iran, Portugal	(Hyvärinen et al., 2002; Lourenço et al., 2005; Shamsian et al., 2006)

Fungal species	**Country**	**References**
Mucor racemosus Fresen	Morocco	(Bergadi et al., 2014)
Mucor spinosus Tiegh.	Slovakia	(Kraková et al., 2012)
Mycelia sterilia	Brazil, Italy	(Corte et al., 2003; da Silva et al., 2006; Zotti et al., 2007, 2008)
Myxotrichum deflexum Berk.	Japan	(Sato et al., 2014)
Neurospora sp. Shear & B.O. Dodge	Nigeria	(Bankole, 2010)
Oidiodendron sp. Robak	Finland	(Hyvärinen et al., 2002)
Oidiodendron citrinum G.L. Barron	Italy	(Corte et al., 2003)
Oidiodendron majus G.L. Barron	Italy	(Corte et al., 2003)
Paecilomyces sp. Bainier	Finland, Portugal	(Hyvärinen et al., 2002; Lourenço et al., 2005; Pinheiro, 2014)
Paecilomyces variotii Bainier	Italy, Poland	(Zerek, 2003; Zotti et al., 2008)
Penicillium sp. Link	Argentina, Brazil, Cuba, Finland, Iran, Italy, Japan, Nigeria, Poland, Portugal, USA	(Hyvärinen et al., 2002; Di Bonaventura et al., 2003; Shamsian et al., 2006; da Silva et al., 2006; Mesquita et al., 2009; Michaelsen et al., 2009, 2010; Bankole, 2010; Borrego et al., 2012; Montanari et al., 2012; Sterflinger and Engel, 2013; Sato et al., 2014; Pinheiro, 2014)
Penicillium arenícola Chalabuda	Brazil	(da Silva et al., 2006)
Penicillium bilaiae Chalab.	Portugal	(Lourenço et al., 2005)
Penicillium brevicompactum Dierckx	Italy, Portugal	(Corte et al., 2003; Lourenço et al., 2005)
Penicillium canescens Sopp	Italy, Portugal	(Corte et al., 2003; Mesquita et al., 2009)
Penicillium chrysogenum Thom	Argentina, Cuba, Italy, Japan, Morocco, Poland, Portugal, USA	(Fabbri et al., 1997; Ricelli et al., 1999; Corte et al., 2003; Di Bonaventura et al., 2003; Mesquita et al., 2009; Michaelsen et al., 2009, 2010; Borrego et al., 2012; Bergadi et al., 2014; Sterflinger and Engel, 2014; Sato et al., 2014)
Penicillium citreonigrum Dierckx	Brazil, Japan, Portugal	(Lourenço et al., 2005; da Silva et al., 2006; Rakotonirainy, Heude, et al., 2007)
Penicillium citrinum Thom	Argentina, Brazil, Cuba, France, Italy, Portugal	(Corte et al., 2003; Lourenço et al., 2005; da Silva et al., 2006; Rakotonirainy, Heude, et al., 2007; Borrego et al., 2012)
Penicillium commune Thom	Argentina, Cuba, Italy, Japan, Morocco, Slovakia	(Michaelsen et al., 2009; Borrego et al., 2012; Kraková et al., 2012; Bergadi et al., 2014; Sato et al., 2014)
Penicillium corylophilum Berk. and M. A. Curtis	Portugal	(Lourenço et al., 2005)
Penicillium decumbens Thom	Argentina, Cuba, Italy, Portugal	(Corte et al., 2003; Lourenço et al., 2005; Borrego et al., 2012)
Penicillium expansum Link *(formely known as Penicillium variabile* Sopp)	Italy, Portugal	(Corte et al., 2003; Lourenço et al., 2005)
Penicillium echinulatum Biourge	Japan	(Sato et al., 2014)
Penicillium fellutanum Biourge	Brazil	(da Silva et al., 2006)
Penicillium glabrum (Wehmer) Westling	Portugal	(Lourenço et al., 2005)
Penicillium griseofulvum	Portugal	(Lourenço et al., 2005)

Fungal species	Country	References
Dierckx		
Penicillium islandicum Sopp	India	(Das et al., 1997)
Penicillium ochraceum (Bainier) Thom	Poland	(Zerek, 2003)
Penicillium oxalicum Currie and Thom	Portugal	(Lourenço et al., 2005)
Penicillium paxilli Bainier	Italy	(Zotti et al., 2007, 2008)
Penicillium restrictum Gilman & Abbott	Brazil, Italy	(da Silva et al., 2006; Zotti et al., 2007, 2008)
Penicillium simplicisimum (Oudem.) Thom	Portugal	(Lourenço et al., 2005)
Penicillium solitum Westling	Japan, Portugal	(Lourenço et al., 2005; Sato et al., 2014)
Penicillium spinulosum Thom	Italy, Poland, Portugal	(Zerek, 2003; Lourenço et al., 2005; Zotti et al., 2007, 2008)
Penicillium steckii K.M. Zalessky	Italy	(Zotti et al., 2007, 2008)
Penicillium turbatum Westling	Italy	(Zotti et al., 2007, 2008)
Penicillium vancouverense Houbraken, Frisvad & Samson	Japan	(Sato et al., 2014)
Penicillium verrucosum Dierckx	Poland	(Zerek, 2003)
Penicillium wortmannii Klöcker	India	(Das et al., 1997)
Peziza ostracoderma Korf	Italy	(Corte et al., 2003)
Phialemonium sp W. Gams & McGinnis	Portugal	(Pinheiro, 2014)
Phialophora sp.Medlar	Finland, Portugal	(Hyvärinen et al., 2002; Lourenço et al., 2005)
Phlebia subserialis (Bourdot & Galzin) Donk	Portugal	(Mesquita et al., 2009)
Phlebiopsis gigantea (Fr.) Jülich	Portugal	(Mesquita et al., 2009)
Phoma sp. Sacc.	Portugal	(Lourenço et al., 2005)
Phoma herbarum Westend	Slovakia	(Kraková et al., 2012)
Phoma medicaginis Malbr. & Roum.	Poland	(Sterflinger and Engel, 2014)
Phoma pomorum Thüm.	Italy	(Corte et al., 2003)
Polyporus brumalis (Pers.) Fr.	France	(Rakotonirainy, Heude, et al., 2007)
Rhizopus sp. Ehrenb.	Finland, Iran, Nigeria, Portugal	(Hyvärinen et al., 2002; Lourenço et al., 2005; Shamsian et al., 2006; Bankole, 2010)
Rhizopus arrhizus A. Fisch (previously known as *Rhizopus oryzae* Went & Prins. Geerl.)	Italy	(Michaelsen et al., 2009)
Rhodotorula aurantiaca (Saito) Lodder	Italy	(Michaelsen et al., 2010)
Saccharicola bicolor (D. Hawksworth, W.J. Kaiser & Ndimande) D. Hawksworth & O.E. Eriksson	France	(Rakotonirainy, Heude, et al., 2007)
Scedosporium sp. Sacc. ex Castell. & Chalm.	Portugal	(Pinheiro, 2014)
Schizophyllum commune Fr.	Morocco	(Bergadi et al., 2014)
Scopulariopsis sp.Bainier	Argentina, Cuba, Finland	(Hyvärinen et al., 2002; Borrego et al., 2012)
Scopulariopsis fusca Zach	Portugal	(Pinheiro, 2014)
Scytalidium sp.Pesante	Portugal	(Pinheiro, 2014)
Skeletocutis sp. Kotlába & Pouzar	Portugal	(Mesquita et al., 2009)
Sphaeropsidales	Finland	(Hyvärinen et al., 2002)

Fungal species	**Country**	**References**
Stachybotrys sp. Corda	Finland	(Hyvärinen et al., 2002)
S. chartarum (Ehrenberg) S. Hughes *(Stachybotrys atra* Corda*)*	India, Italy	(Das et al., 1997; Fabbri et al., 1997; Ricelli et al., 1999)
Talaromyces funiculosus (Thom) Samson, Yilmaz, Frisvad and Seifert (formerly known as *Penicillium funiculosum* Thom)	Israel, Poland	(Nol and Kenneth, 2001; Zerek, 2003)
Talaromyces helicus C.R. Benj. *(*formerly known as *Penicillium helicum* Raper & Fennell*)*	Argentina, Cuba, Portugal	(Mesquita et al., 2009; Borrego et al., 2012)
*Talaromyces miniolututeus (*formerly known as *Penicillium minioluteum* Dierckx.)	France	(Rakotonirainy, Heude, et al., 2007)
*Talaromyces pinophilus (*formerly known as *Penicillium pinophilum* Hedgc.)	Italy	(Michaelsen et al., 2009, 2010)
Talaromyces purpurogenus Stoll *(formerly known as Penicillium purpurogenum* Stoll)	Italy	(Zotti et al., 2011)
Thanatephorus cucumeris (A.B. Frank) Donk	Portugal	(Mesquita et al., 2009)
Thielavia hyalocarpa Arx	USA	(Di Bonaventura et al., 2003)
Torula herbarum (Pers.) Link	India	(Das et al., 1997)
Toxicocladosporium irritans Crous & U. Braun	Portugal	(Mesquita et al., 2009)
Trichoderma sp. Pers.	Finland, Iran, Italy, Portugal	(Hyvärinen et al., 2002; Corte et al., 2003; Lourenço et al., 2005; Shamsian et al., 2006; Michaelsen et al., 2009)
Trichoderma citrinoviride Bissett	France	(Rakotonirainy, Heude, et al., 2007)
Trichoderma harzianum Rifai	Poland	(Zerek, 2003)
Trichoderma koningii Oudem.	France	(Rakotonirainy, Heude, et al., 2007)
Trichoderma pseudokoningii Rifai	Italy	(Zotti et al., 2007, 2008)
Trichoderma víride Pers. Ex Gray	Portugal	(Lourenço et al., 2005; Pinheiro, 2014)
Trichosporum sp. Vuill.	Brazil	(da Silva et al., 2006)
Trichurus sp. Clem	Portugal	(Lourenço et al., 2005)
Tritirachium sp. Limber	Finland	(Hyvärinen et al., 2002)
Ulocladium botrytis Preuss	Italy	(Corte et al., 2003)
Ulocladium chartarum (Preuss) E.G. Simmons	France	(Rakotonirainy, Heude, et al., 2007)
Ulocladium cucurbitae (Letendre & Roum.) E.G. Simmons	France	(Rakotonirainy, Heude, et al., 2007)
Wallemia sebi (Fr.)	Italy	(Michaelsen et al., 2010)

Appendix II

The photochemistry behind the method for fungal growth measurement using NAGase activity quantification

In order to better understand the method for fungal growth measurement using NAGase activity quantification it was necessary to comprehend the photochemistry behind it, namely why choosing a 377 nm excitation wavelength, why 4-MUF-NAG does not fluoresce at 446nm while 4-MUF does and why there is a need to raise the pH to 10 before analysis of fluorescence like described in Konkol et al. (2010, 2012).

According to the literature (Seixas de Melo and Fernandes, 2001), the 4-MUF chromophore in solution (liberated from 4-MUF-NAG by NAGase), when excited for fluorescence measurement, will be mostly in its anionic form, since its pKa decreases from 7.48 to 0.74 in the excited state (Figure II-1).

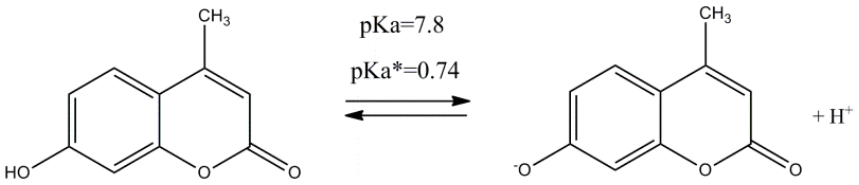


Figure II-1: Neutral and anionic form of 4-MUF, with the respective pKa values in the fundamental (pKa) and excited states (pKa*). Adapted from Moriya (1983).

When 4-MUF-NAG in solution is excited, since 4-MUF is bonded to the NAG group, whatever the pH level, 4-MUF it will always remain neutral. The neutral form of 4-MUF absorbs at a different wavelength than its anionic form (Moriya, 1988), which allows for a distinction between the two forms (Figure II-2). This is the main feature behind the whole NAGase activity detection mechanism.

In order to determine the exact wavenumber at which both 4-MUF forms have a maximum absorbance in the used solvent, the following assay was conducted.

The UV absorbance spectrum of commercial 4-MUF (97%, Sigma-Aldrich, USA) at the maximum concentration that could be achieved through the total enzymatic decomposition of 4-MUF-NAG in each analyzed well (5.5 μM), was collected. 4-MUF-NAG at the same concentration was also analyzed. The spectra of both compounds were collected in two solvents: in Millipore water (Figure II-2 (a)), and in the solvent resulting from the mixture of all solutions added to each sample in the correspondent proportions (ethanol, 50 mM Tris Maleate, 2.5 M Tris-HCL), with pH 10 (Figure II-2 (b)).

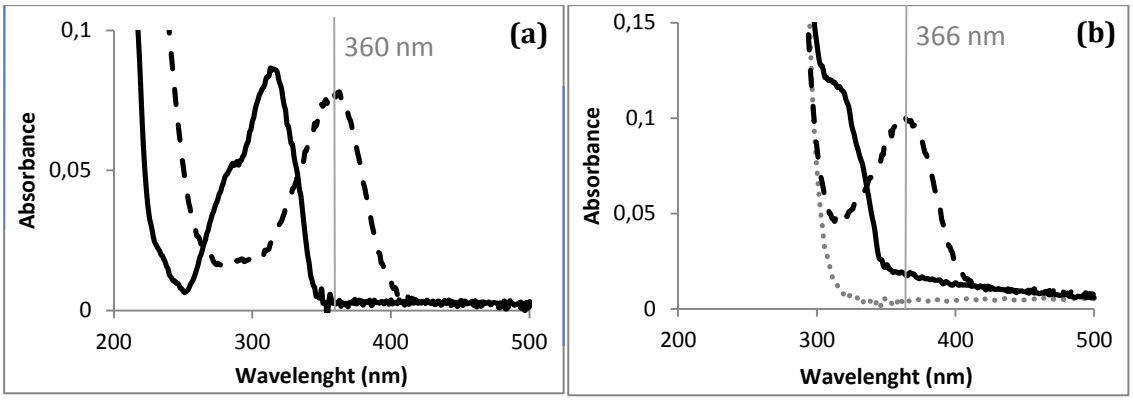


Figure II-2: UV absorbance spectra of 4-MUF (– –) and 4-MUF-NAG (——) in water (a) and in the solvent mixture used in analysis (b). The solvent in (b) is represented as (·····).

In pure water, the maximum absorption 4-MUF is observed at 360 nm (Figure II-2 (a)). In the solvent mixture (Figure II-2 (b)), this peak is slightly shifted to higher wavelengths, being located at 366 nm. The excitation wavelength used to detect the presence of 4-MUF in the NAGase activity experiments was therefore 366 nm, since by exciting at the maximum of absorbance, a higher yield of fluorescence would be achieved, and the contribution of 4-MUF-NAG excitation would be kept to a minimum. This would allow for a better distinction between samples showing NAGase activity. Konkol et al. (2010, 2012) used a 377 nm excitation wavelength. This small difference may be related to the availability of wavenumber selection filters for the plate reader used in those studies.

In Figure II-2 (b) it can be seen that the solvent mixture shows strong absorption from c. 300 nm. This caused a deformation on that spectral region in 4-MUF-NAG and 4-MUF bands.

The same compounds at the same concentrations were then analyzed with a fluorimeter (Fluorolog® Horiba Jobin Yvon) at 366 nm excitation, with 4 nm slit widths, between 380 and 600 nm. In Figure II-3, it is shown that when excited at 366 nm, 4-MUF shows a maximum fluorescence at 448 nm and 4-MUF-NAG shows no fluorescence at all. This is the reason why 448 nm was the wavelength chosen to collect the fluorescence intensity data.

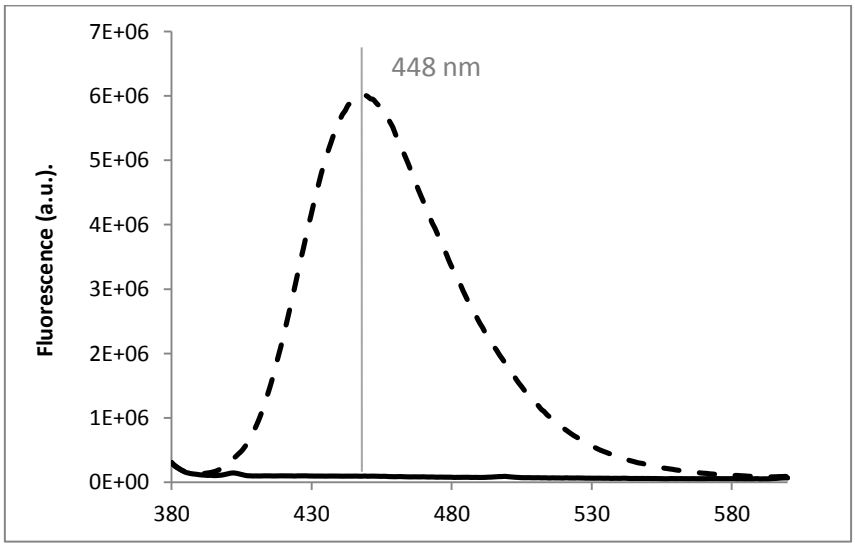


Figure II-3: Fluorescence spectra of 4-MUF (– –) and 4-MUF-NAG (——) in the solvent mixture used in analysis. Excitation wavelength = 366 nm.

The reason why pH 10 was used for fluorescence analysis is related to yields of fluorescence.

4-MUF can have four different photo-excited states: neutral, anionic, tautomeric and cationic (Figure II-4), according to pH (Moriya, 1983; Seixas de Melo and MaCanita, 1993).

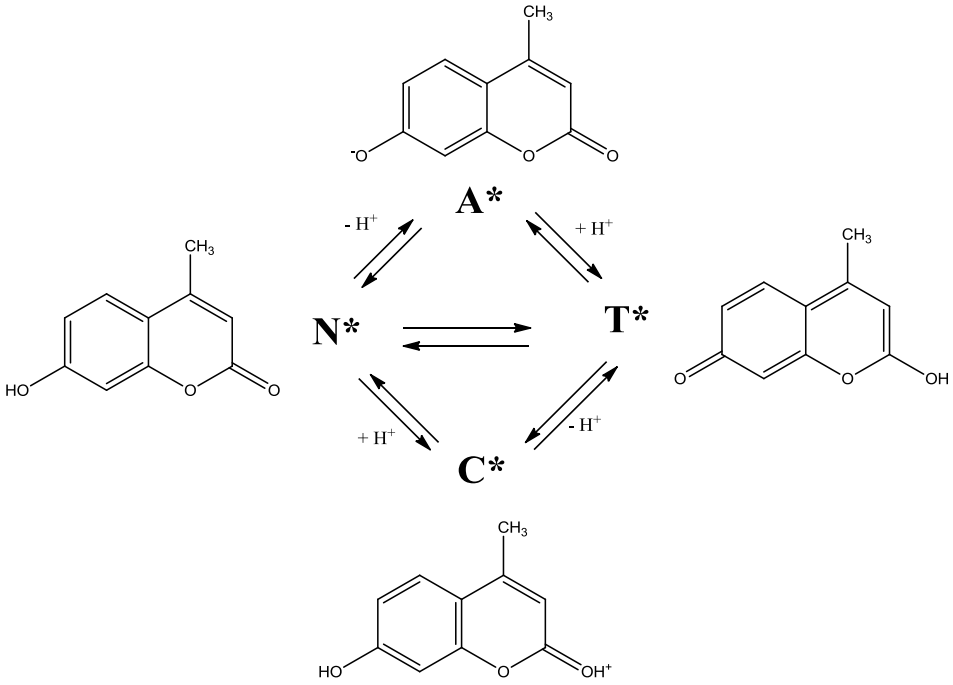


Figure II-4: Reaction scheme of the photo-excited states of 4-MUF, adapted from Moriya (1983).

As presented in Figure II-5 (Moriya, 1983), from pH 10 all the fluorescence is given by the anionic form of 4-MUF (A^-), which is on its maximum of fluorescence yield. Since this anionic form corresponds to the compound the present fungal growth quantification method is aimed at detecting (the liberated 4-MUF from 4-MUF-NAG by NAGase), the higher yield of fluorescence allows for a detection of smaller amounts of compound, and increase the detection limits of analysis.

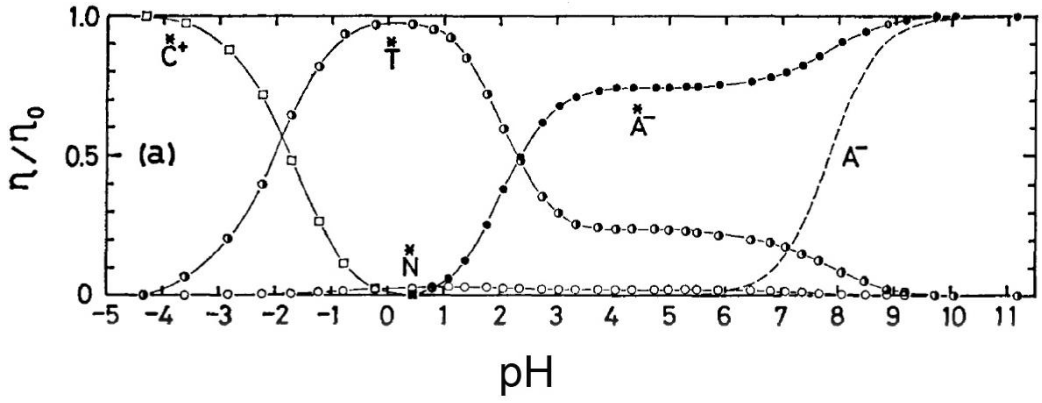
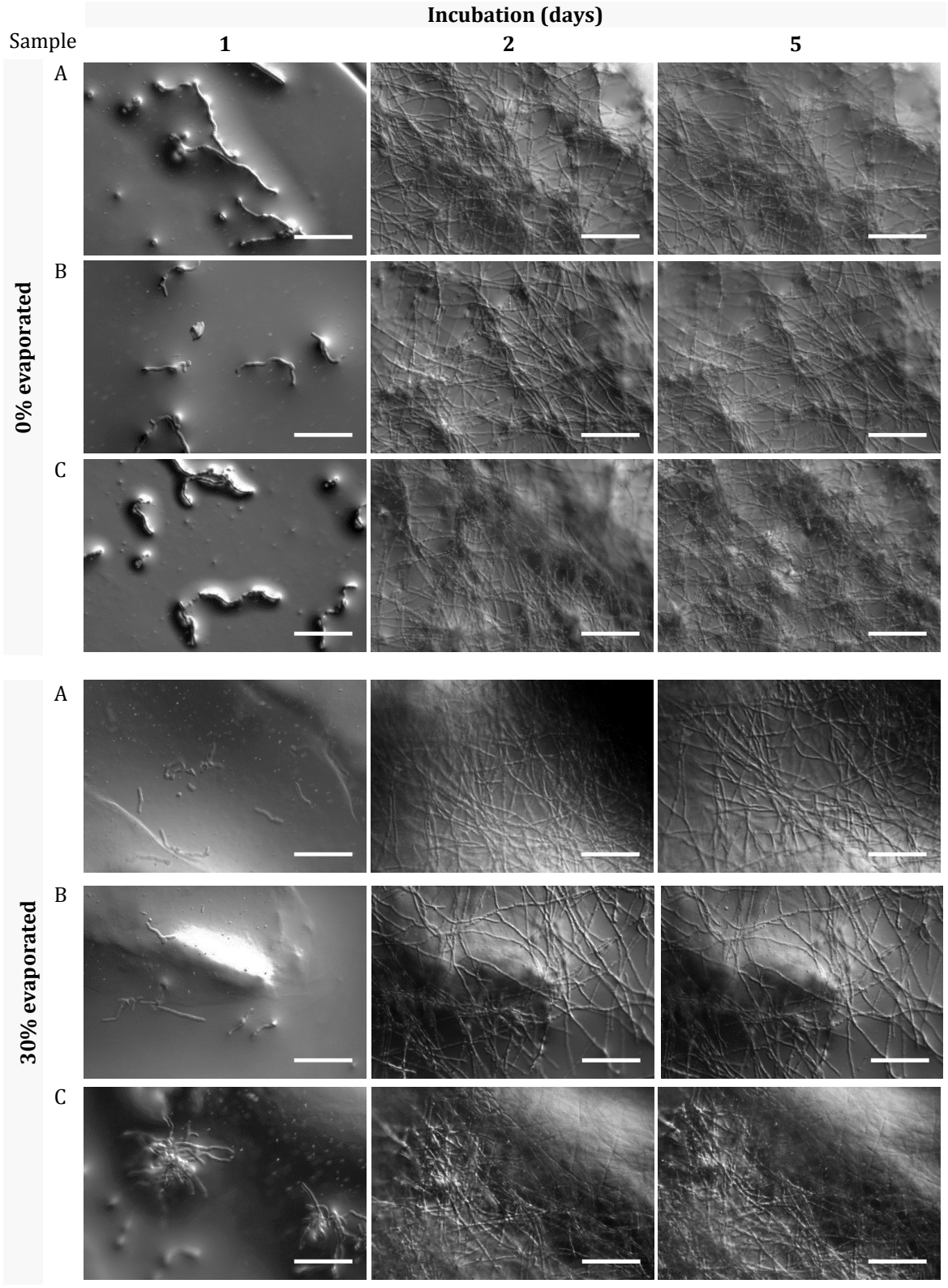
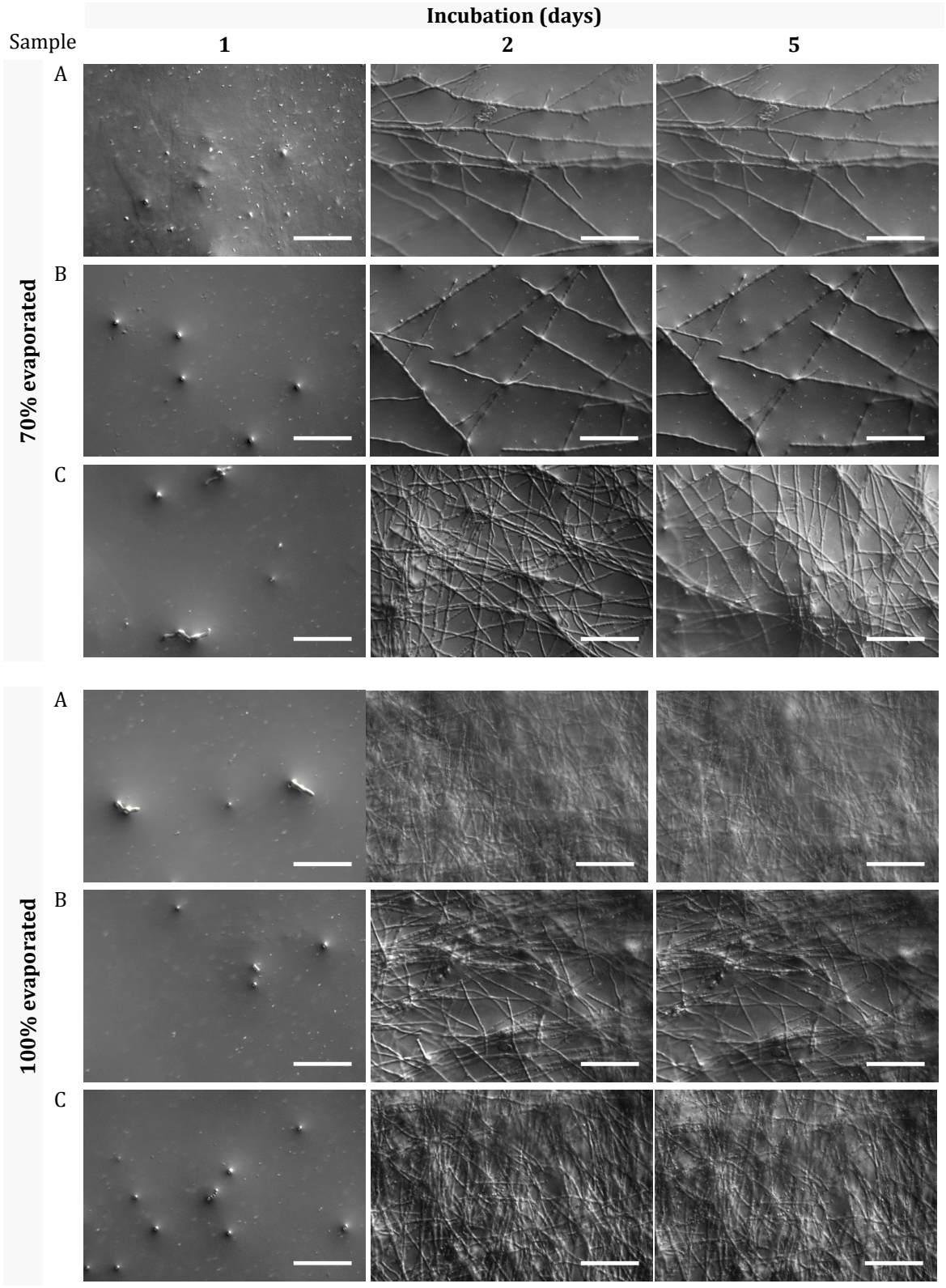


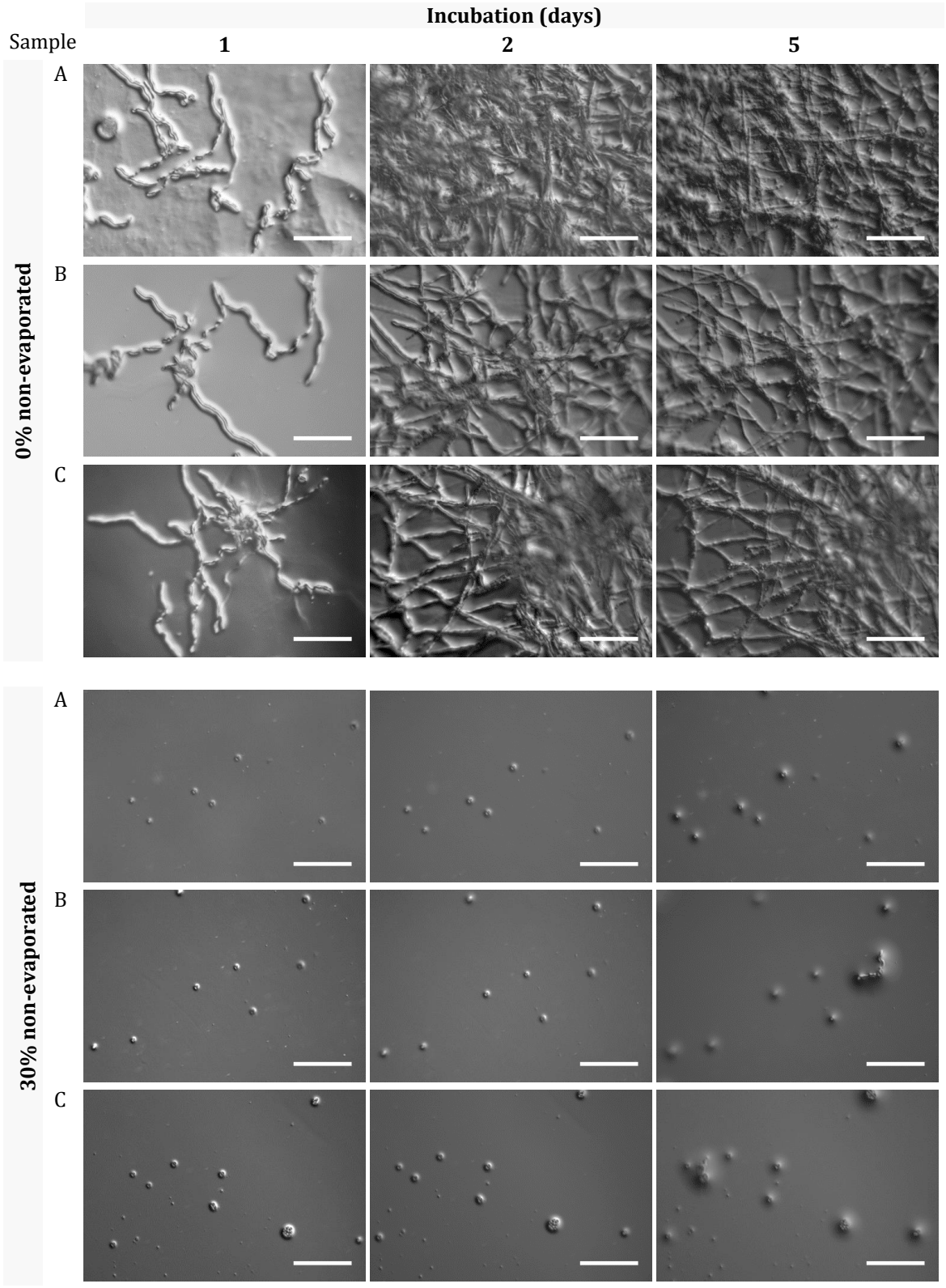
Figure II-5: Relative fluorescence efficiency of the excited species of 4-MUF in aqueous solution (6.3×10^{-5} M); λ_{ex}~334 nm. η/η_0 = relative efficiency of fluorescence, from Moriya (1983).

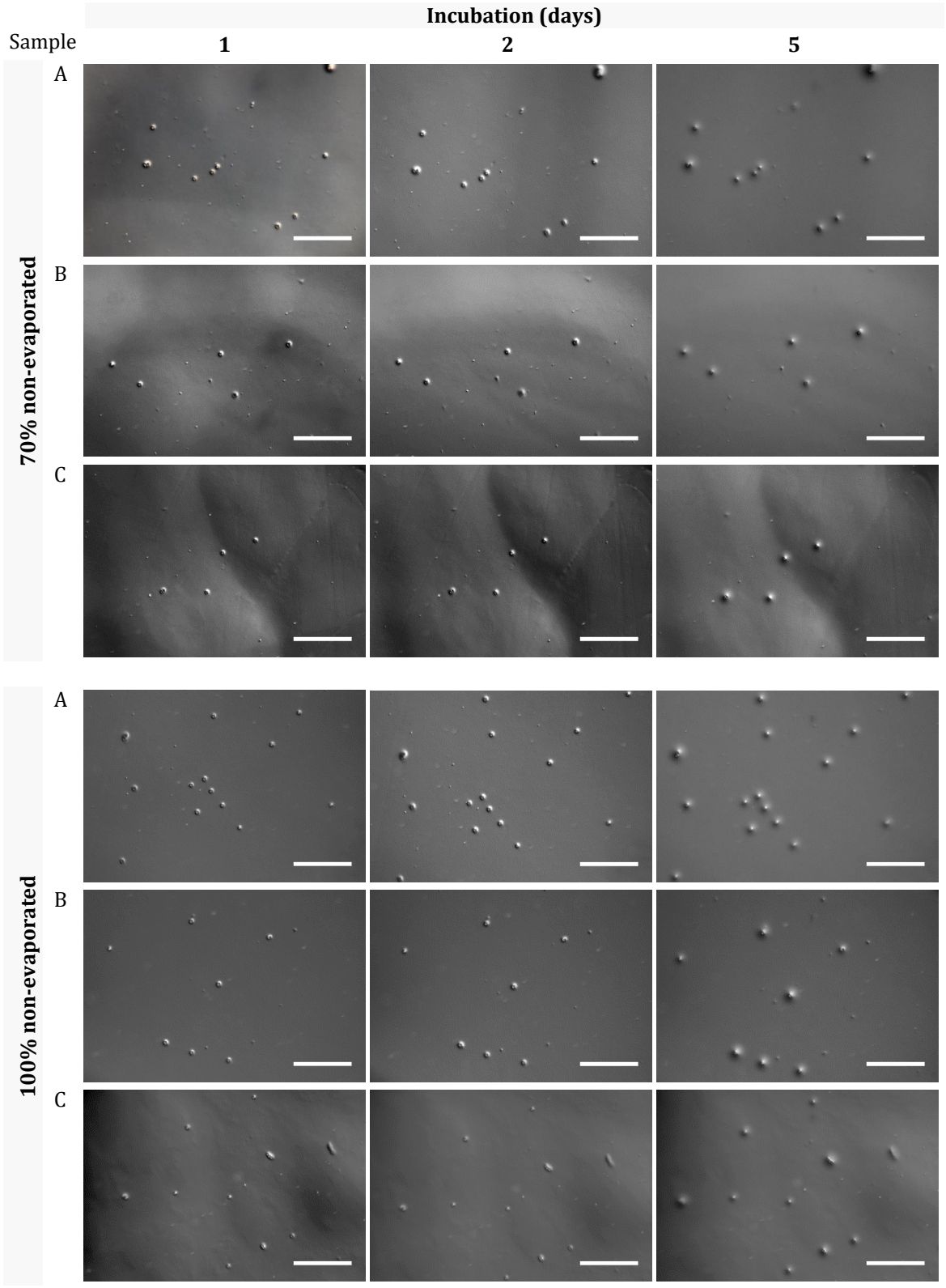
Appendix III

Table III.1: Microscopic observation images of *P. chrysogenum* conidia after treatment with different ethanol concentrations at 1, 2 and 5 days of incubation. Scale bar=50µm.



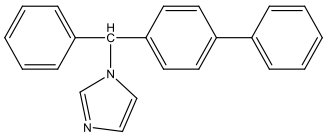
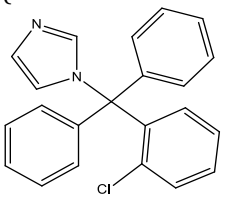

Incubation (days)
Sample
1
2
5
70% evaporated
A
B
C
100% evaporated
A
B
C

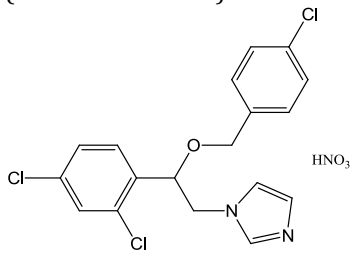
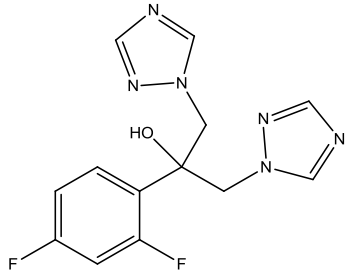

Incubation (days)
Sample
1
2
5
0% non-evaporated
A
B
C
30% non-evaporated
A
B
C

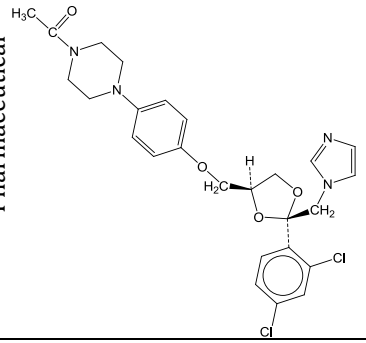
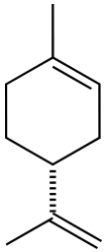

Incubation (days)
Sample
1
2
5
70% non-evaporated
A
B
C
100% non-evaporated
A
B
C

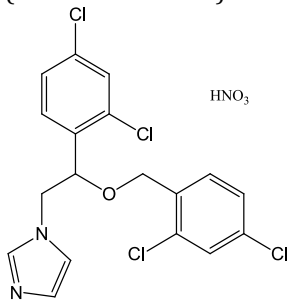
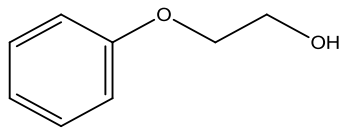
Appendix IV

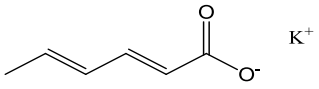
Table IV.1: List of common antifungal compounds used in the pharmaceutical and cosmetics industries.

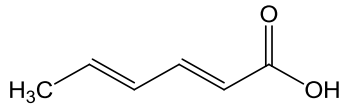
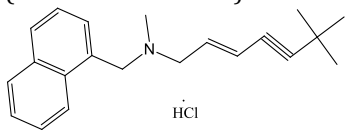
Industry	Chemical compound	Colouration	pH/pKa	Usual concentration	Solubility	Toxicity/health effects	Observations	References
Pharmaceutical	**Bifonazole** (CAS — 60628-96-8) 	White or almost white	pka=5.85	1%	Water: <1mg/l Sparingly soluble in anhydrous ethanol Soluble in alcohols, DMSO	Oral rat LD_{50}: 1463-2854 mg/Kg May cause local reactions including burning and itching	Mainly topical application on skin and nails Less effective than clotrimazole	(Shadomy et al., 1982; Sweetman, 2009; Alagarsamy, 2010; Domańska et al., 2010; Haider and Asif, 2011)
Pharmaceutical	**Clotrimazole** (CAS — 23593-75-1) 	Solid: white Liquid: colourless	Weak base pKa=4.70	1-2%	Water: 0.49 mg/l Soluble in acetone, ethanol, methanol, isopropanol, chloroform	Oral rat LD_{50}: 708 mg/kg Anti-malarial, anti-cancer and neuroprotective activities May cause local reactions including skin irritation and burning sensation	Mainly topical application Corrosion inhibition and radical scavenging properties Stable at alkaline pH Unstable in acidic pH	(Isaev et al., 2002; Prabagar et al., 2007; Obot et al., 2009; Sweetman, 2009; Meloun et al., 2010; Iannelli et al., 2011; Borhade et al., 2012; Furtado et al., 2012)

Industry	Chemical compound	Colouration	pH/pKa	Usual concentration	Solubility	Toxicity/health effects	Observations	References
Pharmaceutical	**Econazole nitrate** (CAS - 24169-02-6) 	White or almost white	pka=4.8	1%	Water: 500mg/l Slightly soluble in alcohol and dichloromethane Sparingly soluble in chloroform and methylene chloride Soluble in DMSO	Oral rat LD_{50}: 668mg/Kg May cause local reactions including skin irritation and burning sensation	Antifungal activity similar to that of ketoconazole Low absorption when applied to the skin	(Raab, 1980; Sweetman, 2009; Alagarsamy, 2010)
Pharmaceutical	**Fluconazole** (CAS — 86386-73-4) 	White or almost white	pKa = 11.01±0.29; 2.94 ± 0.10; 2.56 ± 0.12	0.5%-1%	Slightly soluble in water Soluble in methanol and acetone Sparingly soluble in isopropanol Very slightly soluble in toluene	Oral rat LD_{50}: 1271mg/kg Adverse effects on the gastrointestinal tract Headache, dizziness, leucopenia, thrombocytopenia, hyperlipidaemias, and raised liver enzyme values May have a teratogenic effect – not recommended in pregnancy	Narrow spectrum of activity, targeting mainly yeast and dimorphic fungi, with no activity against moulds Its large molecular weight and strong hydrophilic property hampers its use as a topical application	(Lattif and Swindell, 2009; Sweetman, 2009; Alagarsamy, 2010; Yim et al., 2010; Corrêa et al., 2012)

Industry	Chemical compound	Colouration	pH/pKa	Usual concentration	Solubility	Toxicity/health effects	Observations	References
Pharmaceutical	**Ketoconazole** (CAS — 65277-42-1) 	White or almost white	Weak base pka=6.51; 2.94 pH values of formulations approach pH 5-6	2%	Practically insoluble in water, soluble in methylene chloride and in methanol Sparingly soluble in alcohol Freely soluble in dichloromethane	Oral rat LD_{50}: 166 mg/kg Gastrointestinal disturbances Risk of hepatitis Allergic reactions Skin irritation, dermatitis and burning sensation	Wide spectrum antimicrobial activity Used topically and orally May undergo degradation including oxidation and hydrolysis, especially in aqueous media	(Skiba et al., 2000; Sweetman, 2009; Alagarsamy, 2010)
Cosmetics	**Limonene** (CAS - 138-86-3) 	Colourless liquid		0.5-2%	Water: 13.8mg/l Soluble in acetone, dimethyl sulfoxide and ethanol	Oral rat LD_{50}: 4400-5300 mg/Kg Classified skin sensitizer *d*-Limonene is not classifiable as to its carcinogenicity to humans (IARC-Group 3) May reduce breast cancer risk	Strong inhibitory effect of cellulase activity Antioxidant properties (radical scavenging) Broad fungitoxic spectrum High aflatoxin inhibitory efficacy May form hydroperoxides by autoxidation	(Karlberg et al., 1994; Filipsson et al., 1998; Crowell, 1999; IARC, 1999a; Shaw et al., 2006; Chee et al., 2009; Singh et al., 2010; Yazar et al., 2011; Marei et al., 2012)

Industry	Chemical compound	Colouration	pH/pKa	Usual concentration	Solubility	Toxicity/health effects	Observations	References
Pharmaceutical	**Miconazole nitrate** (CAS - 22832-87-7) 	White or almost white	pKa=6.5	2%	Very slightly soluble in water Sparingly soluble in methanol Slightly soluble in alcohol	Oral rat LD_{50}: 920 mg/kg 500 mg/kg Skin irritation and sensitivity reactions, contact dermatitis Fetotoxic affect in animals – not recommended during pregnancy	Used topically and orally Non-protonated neutral drug molecules must be in solution or in extremely small aggregates to elicit direct lethal action against *Candida albicans*.	(Raab, 1980; Beggs, 1992; Sweetman, 2009; Alagarsamy, 2010)
Cosmetics and pharmaceutical	**Phenoxyethanol** (CAS - 122-99-6) 	Colourless to off white oily liquid	Optimum pH: 4-5	0.5-2%	Slightly soluble in water (26.7g/l) Soluble in: alcohol, ether and alkaline solutions	Oral rat LD_{50}: 1260-1900 mg/Kg Can depress the central nervous system and cause vomiting and diarrhoea Has shown cytotoxic and neurotoxic effects	Stable in acid solutions Its antimicrobial activity is drastically reduced in the presence of cellulose derivatives (MC, CMC, HPMC) Its antimicrobial activity may be reduced by interaction with non-ionic surfactants	(Liebert, 1990; Kurup et al., 1995; Paulus, 2004; Sweetman, 2009; Regulska et al., 2010; SUBSPORT, 2013)

Industry	Chemical compound	Colouration	pH/pKa	Usual concentration	Solubility	Toxicity/health effects	Observations	References
Cosmetics	**Potassium sorbate** (CAS — 590-00-1) 	White or almost white	pka= 4.8	<0.2%	Water: 582g/l Ethanol: 20 g/l Acetone: 1g/l	Oral rat LD_{50}: > 7360 Sorbates can be irritant and have caused contact dermatitis	In aqueous solutions is unstable and degrades through oxidation More effective than calcium propionate Relatively ineffective above a pH of about 6 Causes loss of tensile strength and flexibility in films of cellulose derivatives (CMC)	(Suhr and Nielsen, 2004; Stopforth et al., 2005; Sweetman, 2009; Sayanjali et al., 2011; ECHA, 2013)

Industry	Chemical compound	Colouration	pH/pKa	Usual concentration	Solubility	Toxicity/health effects	Observations	References
Cosmetics	**Sorbic acid** (CAS — 22500-92-1) 	Colourless/ white	Weak acid pka= 4.8	0.01–0.2%	Water: 1.5g/l Ethanol: 129–148 g/l Acetone: 92g/l Isopropanol: 84g/l Toluene:19g/l	Oral rat LD_{50}: 7360-10500 mg/Kg Can irritate mucous membranes 0.2%at concentrations Some of its degradation products are toxic	More effective than propionic acid Inhibits both moulds and yeasts Can be inactivated by oxidation and by non-ionic surfactant and plastics Has antioxidant properties Most effective at pH4 or less Relatively ineffective above a pH of about 6 Antimicrobial activity tends to be microbistatic unstable in aqueous solutions	(Budavari, 1996; Paulus, 2004; Suhr and Nielsen, 2004; Stopforth et al., 2005; McDonnell, 2007; Shibamoto and Bjeldanes, 2009; Sweetman, 2009; Reda, 2011; Lopes et al., 2012; Fraise et al., 2013)
Pharmaceutical	**Terbinafine hydrochloride** (CAS — 78628-80-5) 	Solid: white to off-white	pKa= 4.19	1%	Water: 3 g/l Ethanol: 45g/l DMSO: 30 g/l Slightly soluble in acetone Soluble in anhydrous ethanol and methanol	Oral rat LD_{50}: 4000mg/Kg Has been associated with liver failure May cause serious skin reactions	Used topically and orally Broad spectrum activity against yeast, moulds	(Budavari, 1996; Davies, 2006; Sweetman, 2009; Meloun et al., 2010)

Appendix V

Table V.1: Chromatic coordinates L*a*b* of Whatman #1 paper after each different treatment and after artificial ageing.

	Pristine samples					
	After treatment			After artificial ageing		
	L*	a*	b*	L*	a*	b*
WT	97.37 ± 0.04	-0.07 ± 0.01	1.22 ± 0.06	95.69 ± 0.09	0.29 ± 0.03	4.23 ± 0.23
EtOH 70%	97.35 ± 0.02	-0,06 ± 0.00	1.17 ± 0.06	95.92 ± 0.24	0.20 ± 0.06	3.93 ± 0.40
PBs	97.30 ± 0.05	-0,05 ± 0.00	1.14 ± 0.05	95.60 ± 0.03	0.27 ± 0.02	4.33 ± 0.05
CP	97.25 ± 0.01	-0,08 ± 0.00	1.28 ± 0.01	92.02 ± 0.91	1.08 ± 0.33	12.18 ± 1.43
PBs+CP	97.29 ± 0.06	-0,07 ± 0.01	1.33 ± 0.01	90.83 ± 0.39	1.65 ± 0.07	12.74 ± 0.85
IPA	97.39 ± 0.00	-0.06 ± 0.00	1.23 ± 0.04	95.50 ± 0.29	0.29 ± 0.08	4.17 ± 0.62
CLT	97.30 ± 0.06	-0.07 ± 0.00	1.19 ± 0.02	95.71 ± 0.07	0.24 ± 0.02	3.82 ± 0.06
$Ca(OH)_2$ NPs	97.36 ± 0.02	-0.07 ± 0.00	1.24 ± 0.06	94.77 ± 0.22	0.46 ± 0.06	5.31 ± 0.30
CLT+NPs	97.36 ± 0.03	-0.07 ± 0.00	1.23 ± 0.04	94.44 ± 0.15	0.56 ± 0.08	5.80 ± 0.22

Table V.2: Pictures of 3 replicate Whatman #1 paper samples (7x4 cm) after each different treatment and after artificial ageing.

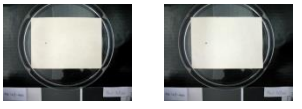
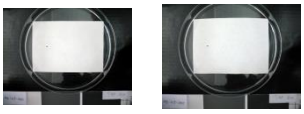
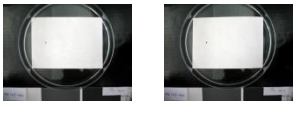
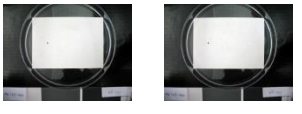
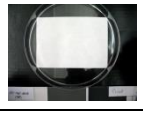
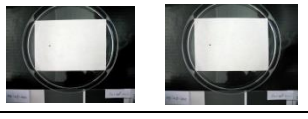
	After treatment			After artificial ageing		
WT						
EtOH 70%						
PBs						
CP						
PBs+CP						
IPA						
CLT						
$Ca(OH)_2$ NPs						
CLT+NPs						

Table V.3: Chromatic coordinates L*a*b* of Whatman #1 paper previously colonized by *A. niger* and vacuum cleaned, after each different treatment and after artificial ageing.

	Biodeteriorated samples					
	After treatment			After artificial ageing		
	L*	a*	b*	L*	a*	b*
PDA controls	96.90 ± 0.07	-0.15 ± 0.01	2.31 ± 0.10	75.68 ± 2.17	5.79 ± 0.87	18.85 ± 1.81
WT	86.66 ± 4.20	-0.06 ± 0.19	9.38 ± 2.11	72.28± 1.97	5.92 ± 0.19	20.25 ± 0.90
EtOH 70%	82.61 ± 3.75	-0.93 ±0.15	11.60 ± 2.47	67.92 ± 2.09	6.19 ± 0.18	19.55 ± 1.02
PBs	80.63 ± 1.23	-0.95 ±0.40	13.55 ± 2.35	69.21 ± 4.12	6.35 ± 0.08	20.01 ± 1.97
CP	76.88 ± 3.63	0.22 ±0.44	14.26 ± 0.19	75.87 ± 2.20	4.47 ± 0.61	24.25 ± 1.11
PBs+CP	72.04 ± 7.82	0.94 ±0.41	14.42 ± 1.06	72.75 ± 5.14	5.31 ± 1.07	23.02 ± 0.65
IPA	82.31 ± 2.47	-0.19 ± 0.25	13.22 ± 2.04	68.87 ± 2.81	6.02 ± 0.57	19.48 ± 0.86
CLT	86.20 ± 1.70	-0.61 ± 0.14	12.47 ± 1.60	70.14 ± 3.26	6.54 ± 0.78	20.85 ± 1.78
$Ca(OH)_2$ NPs	84.84 ± 3.87	-0.80 ± 0.10	13.40 ± 1.22	68.39 ± 4.05	7.07 ± 0.38	21.47 ± 0.16
CLT+NPs	86.65 ± 1.21	-0.15 ±0.01	11.22 ± 0.57	73.40 ± 0.60	5.77 ± 0.61	20.12 ± 2.22

Table V.4 Pictures of 3 replicate Whatman #1 paper samples (7x4 cm) previously colonized by *A. niger* and vacuum cleaned, after each different treatment and after artificial ageing.