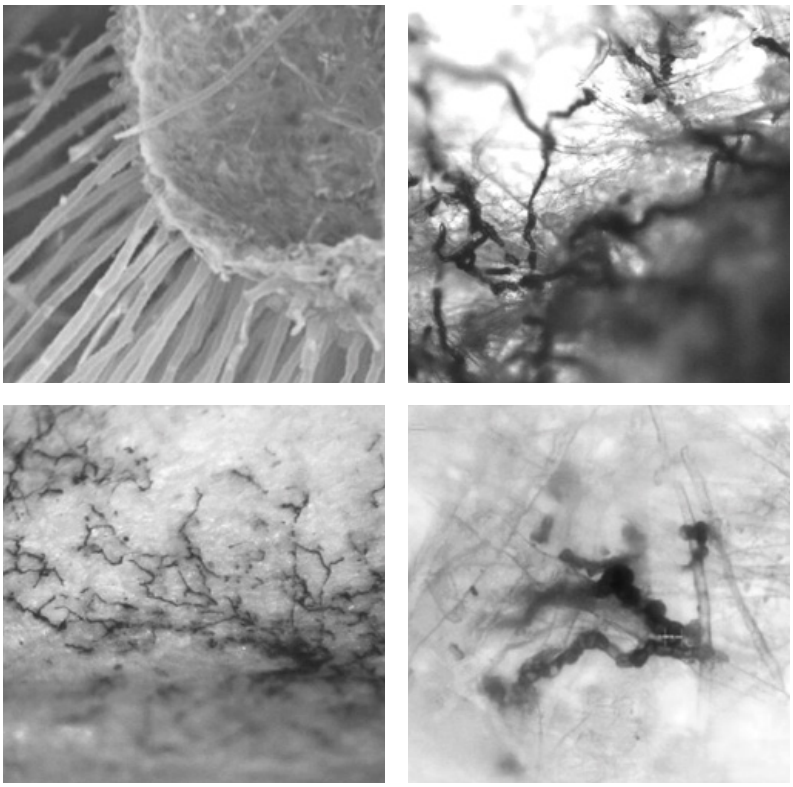
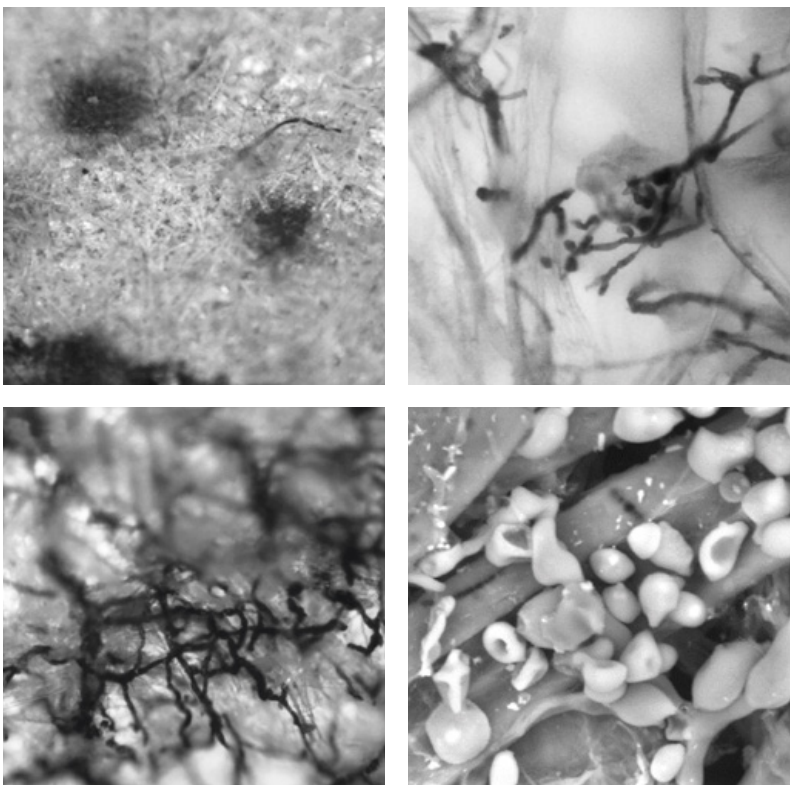


Biodeterioration of Cultural Heritage

Dynamic Interfaces Between Fungi, Fungal Pigments and Paper



Hanna M. Szczepanowska

Biodeterioration of Cultural Heritage: Dynamic Interfaces between Fungi, Fungal Pigments and Paper

Biodeterioration of Cultural Heritage: Dynamic Interfaces between Fungi, Fungal Pigments and Paper

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Dedication

This book is dedicated to Rafal Szczepanowski, who contributed over the years with creative visualizations of intricate fungal structures.

Table of Contents

About the Author

Hanna Szczepanowska, Ph.D. from the University of Lyon, France, is a material scientist specialized in the analysis of cultural heritage material. She currently works as a Physical Scientist at the Bureau of Engraving and Printing, Washington DC, and is an adjunct Professor at West Virginia University. During her four-year employment by the National Heritage Board in Singapore, she established a research laboratory and analytical research program for the collection of Southeast Asian artifacts. She had previously worked for 14 years at the Smithsonian Institution, and was also an adjunct professor at George Washington University, Washington DC. Hanna has a Master's Degree in the conservation of paper and parchment from the University of Nicolaus Copernicus in Torun, Poland. Hanna has published a number of articles on various aspects of bio-degradation of paper and recently on the chemical analysis of natural lacquer and biogenic silica. She has also authored a handbook, Conservation of Cultural Heritage: Key Principles and Approaches, Routledge 2012.

Preface

The surface of paper in cultural heritage objects is of great importance as it conveys the aesthetic intent of artists or messages of historic value. The surface of the microbial cell is of paramount importance for cell survival. What happens at the interfaces of paper–microbial cell surfaces?

A glimpse into the complexities of interactions between paper and living systems, such as fungi, which lead to paper degradation and staining, is the focus of this book. Considering the multitude of events occurring at the interfaces and gamut of colorful pigmentation produced by fungi, only Dematiaceous fungi, which produce black pigments in their spores, vegetative and reproductive structures and metabolic secretions, were taken into consideration in this study.

Interfacial interactions between paper fibers and fungal cells surfaces play a crucial role in the formation of stains and degradation of paper; therefore, a brief characteristic of each player, the paper and fungal cells was provided as a backdrop to their complex interactions. Two model papers, a watercolor paper and laboratory-grade paper on which stains were induced in a bio-simulation experiment under controlled conditions, were compared with papers on which stains occurred naturally in artworks, such as prints on Western and Oriental papers. This close examination aimed to elucidate why it is so difficult, nearly impossible, to reverse the stains on paper that are induced by fungi.

The proposed analytical instruments used in the examination of fungi that grow on paper and produce stains were evaluated for their efficiency in providing data towards the design of remediation and handling strategies. The analytical instruments were used to evaluate paper topography, fibers' morphology, the chemical composition of fillers and additives, and their impact on fungal growth and the production of stains. Fungal structures were examined with X-ray microtomography on a synchrotron beam, in addition to the standard optical microscopy and electron scanning microscopic techniques used in the study of biological specimens. The application of surface metrology tools, such as confocal laser scanning microscopy and white light optical profilometers, indicated the interdisciplinary nature of this study in the hope that it will generate ideas for future investigations. A note of caution regarding the mycotoxins that are produced by fungi and may affect those who handle infested cultural heritage material concludes this study.

This book is an expansion of doctoral theses presented at the University of Lyon, Department of Tribology and Dynamic Systems, led by Prof. Dr. Thomas Mathia. The author expresses gratitude for his leadership. The doctoral jury who reviewed the content comprised microbiologists and engineers, to whom I am indebted for their comments. This jury included Prof. Dr. D. Mazuyer, University of Lyon, Prof. Dr. Jean-Francs Bloch, School of Paper and Industrial Graphics, Grenoble, Dr. M. Mecklenburg, Smithsonian Institution, Washington DC, Dr. P. Lanteri, University of

Claude Bernard, Lyon, and two microbiologists, Dr. R. Cavaliere, Gettysburg College, Gettysburg PA and Dr. S. Lafont, Bio Merieux, Marcy l'Etoile, France. Special thanks are due to the CEO of Altimet, Serge Carras, for his generosity in facilitating access to the suite of surface metrology instruments.

Acknowledgments

The author acknowledges the editorial suggestions of Dr. Ralph Cavaliere in the early developmental stage of this manuscript.

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1. Introduction

Biodeterioration in the context of cultural heritage refers to the degradation processes induced by microorganisms that occur in various substrates upon which historic and artistic works of our cultural heritage were created. Paper, a biological, cellular material that has served for centuries as a carrier of human thought, and a base upon which artworks were rendered, is a particularly bio-susceptible material. The complex interactions between paper and diverse microbial communities involve, among others, physico-chemical aspects of microbial adhesion, extracellular material secretions by microbes, the impact of surface topography on which microorganisms grow, and elements of the environment such as light, temperature and relative humidity. These interactions and their role in the formation of fungi-induced stains are the subject of this book.

Biodeterioration is one of the most challenging forms of deterioration to study and prevent because it involves living organisms, fungi and bacteria as well as the unpredicted ways in which they respond to changes in the environment and modifications of materials upon which they grow. Moreover, interactions of fungi and bacteria with surfaces are of great importance in the study of biofilms in medicine, agriculture, and industry. Attachment to surfaces and the formation of biofilms are associated with pathogenesis in animals and plants and are therefore studied extensively in the respected fields. Although the biodeterioration of cultural heritage has been studied over the past several decades, very few have focused on exploring the interfaces of microorganisms and cultural heritage material. This deficiency was noted by Orio Ciferri, Chairman of the Scientific Committee, during the 1999 International Conference on Microbiology and Conservation (ICMC) in Florence, which was exclusively devoted to the relationships between microorganisms and cultural heritage: " ... although enough data are now available demonstrating a direct link between colonization by microbes and defacement of cultural artifacts, a frustrating ignorance concerning the mechanisms of such interactions continues to exist" [...] one could say that very often the causative agent has not been identified and very little is known concerning the microbe-host interactions" (Ciferri et al. 2000).

A multitude of events occur at interfaces between substrates and fungi, from the moment of spores' first contact with a surface, to their germination, and during subsequent growth and response to the environment. These interactions are explored by the author. The multiscale and multisensory analysis of interfaces between black-pigmented fungi and paper substrate are discussed and an analytical protocol is proposed and evaluated for its practical efficiency. Two scenarios of black fungal pigments formations were analyzed: one that occurred naturally on the original artworks (three cases studies are presented) and the other one induced in biosimulation on two known papers, in a controlled environment, and induced by a known organism, *Aspergillus niger*.

The synergy of interactions between water, light, and temperature in the environment, biological clocks of the microorganisms and characteristics of the materials' surface and bulk require a multidisciplinary approach to the study of biodeterioration. Awareness of this inherent, enormous complexity of the subject guided the selection of the study area, narrowing it to the formation of dark-pigmented fungal stains on cellulose paper substrates. Paper is a heterogeneous cellular material produced from fibers and additives, the impact of which has been briefly discussed with respect to their promotion or inhibition of fungal growth. The interaction of these two entities, the microorganisms and paper, opens the door to a little-known and -studied area of science.

Surfaces of objects are of great importance in the study and preservation of cultural heritage artifacts. The artworks are created to convey aesthetic and artistic intent through their three-dimensional form and surface finish. Surface decorations, deposits, or debris reveal objects' history and the cultures that created them. Thus, surfaces of cultural objects are an important source of information about our past aesthetics and technology. The accuracy of information about these artworks and artifacts relies on the quality of data that we extract from their analysis. Paper characteristics such as the surface topography and structure, morphology of fungi and patterns of their pigmented biomass deposition, as well as fungal growth were examined with an array of analytical instruments and methods: transmitted light microscopy, scanning electron microscopy, confocal laser scanning microscopy, white light confocal profilometry, and X-ray microtomography. Qualitative and quantitative data are supplemented with surface metrological instruments used in traceology, a branch of tribology (the science of measuring surfaces). Surface metrology offers a promising set of methods and instruments contributing to a better understanding of the surface topography of living systems and tracing changes induced by the biodeterioration of surfaces.

This work deals, in depth, with the theoretical background of biodeterioration of cultural heritage with a focus on investigating the interfaces of black fungal stains on paper. The theoretical part defines the terms used in the text laying the foundation for the understanding of materials used in the experiments and choices that were made in their selection. The experimental work is inserted among the theoretical portions where appropriate.

The Fungi Imperfecti (Class Deuteromycetes), which are the most prevalent type of fungi on cultural, paper-based materials, are discussed in detail; a critical bibliographical review of the conservation literature dealing with biodeterioration is included. Some parallels are drawn between the biodeterioration of cultural heritage material and selected aspects of fungal pathogenicity in agriculture and the field of medicine, such as microbial adhesion to surfaces and discussion on the hydrophobicity of microbial cells.

The reported experimental work deals with the influence of light on fungal pigmentation and how that pigmentation modifies the paper surface and paper

matrix. The data were collected by culturing the common black fungus, *Aspergillus niger*, on two types of paper (Whatman 4 and Winsor & Newton, watercolor paper), in dark and light conditions. The analysis of selected artworks exemplified different types of papers and fungal biodeterioration, such as staining of the paper surface by pigmented aerial mycelium, staining of the paper matrix with colored deposits, and by the secretion of bio-pigments. Correlations are made between the substrate's composition, the environment, and the resulting microbial interfacial interactions.

The handling strategies of fungi-infested objects are proposed as a practical outcome of this study. The removal of fungal deposits is proposed as one of the forms of cleaning fungal residues; the application of enzymes for dissolving fungal adhesives is proposed as another option. Recommendations for further studies and research are outlined, based on identifying the areas which would most benefit the advancement of cultural heritage preservation. The ultimate goal of the conservator is to develop a preservation strategy for either preventing or limiting the degree of bio-deterioration of cultural heritage material that is caused by fungi. This work is the first attempt to support that goal through a better understanding of the interfacial forces of fungi and their secretions and substrate, resulting in stains on paper.

1.1. Definitions

Two diverse entities, fungi and heterogenous cellular material, paper, participate in the dynamic processes occurring at their interface. The interactions of those two entities occur in varied environments and change over time in concert with the life cycles of fungi. The effects of these interactions, leading to biodeterioration of paper substrates, are explored in the context of cultural heritage objects. Each entity, paper, fungi, cultural heritage and biodeterioration represents an enormous area of study; therefore, abbreviated definitions are offered here for clarity in further discussions.

Cultural heritage is the legacy of humanity passed on from previous generations. It encompasses tangible, physical artifacts, and intangible ones, which are attributed to a group or society, inherited from past generations and bestowed for the benefit of future generations. Tangible heritage includes buildings, monuments, landscapes, books, works of art, artifacts, and smaller objects such as artworks and cultural masterpieces, often collected in museums and art galleries. Intangible heritage refers to non-physical cultural expressions such as folklore, traditions, language, and music.

Cultural heritage, as it is unique and irreplaceable, needs to be preserved. Conservation is one of the forms of preservation, aiming to stabilize and restore these artifacts. "Preservation is action taken to retard or prevent deterioration of, or damage to, cultural properties by control of their environment and/or treatment of their structure in order to maintain them as close as possible to the original, unchanging state" (International Committee of Museums, ICOM 2011).

Fungi is a term that encompasses thousands of species of heterotrophic organisms, meaning consumers, but not producers, of their own food. The classes of fungi that are associated with biodeterioration are Ascomycetes (so-called sac fungi),

Basidiomycetes (club fungi—to which most mushrooms belong) and Deuteromycetes (so-called Fungi Imperfecti). The terms 'Fungi Imperfecti' and 'Deuteromycetes' are not taxonomic classifications (Volk 2013; Sutton 2014); however, in most of the bibliographic references related to cultural heritage, they are frequently used. This nomenclature has thus also been adapted in this manuscript. This book will focus on a few selected species from each taxonomic class that are the most prevalent in the bio-deterioration of paper-based, cultural material. Although all three classes are known to cause deterioration, the Fungi Imperfecti are the most common. Their designation "imperfect fungi" is derived from the fact that they either lack a sexual stage in their reproductive cycle or may possess one that has not yet been discovered. The hyphal growth of imperfect fungi is capable of inflicting massive destruction to paper-based artifacts. Many fungi produce colored bio-pigments, resulting in stains; black pigments, the focus of this book, are discussed in greater detail.

Paper has been used for centuries; its invention is attributed to a 105 AD Chinese scripter (Hunter 1978), yet its properties are still tirelessly studied, rediscovered, engineered and manipulated. Paper consists of fibers intertwined with airy pores between them and additives to bulk and surface (sizing and fillers). All components of paper fibers, pores and additives play a role in biodeterioration, especially with respect to the movement of water and transfer of nutrients.

1.1.1. Biodeterioration, a Definition

Biodeterioration and biodegradation are often used interchangeably, referring to processes of material alteration resulting from microbial activities. Even though these processes have exist for as long as the living world, the word 'biodeterioration' has been in use for only about 60 years (Allsopp et al. 2004). Furthermore, no formal definition has been established, and the processes of biodeterioration are not completely understood.

The definition of biodeterioration proposed by Hueck (1965) and Allsopp et al. (2004) applicable to cultural heritage refers to "any undesirable change in the properties of a material caused by the vital activities of organisms". Biodegradation, on the other hand, emphasizes the positive aspect of the process that refers to the decaying abilities of micro-organisms to render a waste material more useful (Cavaliere 2003). Especially in the last decade, the fungal biodegradation abilities have been viewed as a hopeful potential method for degrading otherwise difficult-to-breakdown plastics (Hyde et al. 2019).

Classifying biodeterioration is not simple yet has been attempted in many areas of science. Some researchers, such as Allsopp et al. (2004), classify biodeterioration in general as "mechanical" or "aesthetic". By "mechanical", the earlier mentioned authors understand distortion of materials by the growth, or movement, of microorganisms but not the use of the substrate as their food source. In support of that description, some authors cite examples of the expansion of microbial masses causing rock cracking and the erosion of underground pipes. By "aesthetic biodeterioration"

(fouling or soiling), the same authors understand the "presence of an organism or its dead body, excreta, or metabolic products" on any substrate. Although these two above distinctions are not entirely clear, because in both cases aesthetic change in the material occurs and, in both cases, the substrate on which microorganisms grow is structurally affected, both signal alterations of materials.

Another classification of biodeterioration proposed by the same researchers (Allsopp et al. 2004) is "assimilatory" and "dissimilatory". The utilization of the substrate as a food source is the basis of the first type. Chemical damage caused by the secretion of waste products such as pigments or acidic compounds is the basis of the second type. Again, one may argue that the utilization of the substrate as a food source involves enzymatic breakdown, which in turn causes the secretion of products of metabolism and pigments; thus, the division is not clear. Furthermore, other researchers point out that pigments are not necessarily 'waste products. They consider pigmentation as a protective mechanism shielding the fungal structure against oxidative stress and harmful light radiation (Malik et al. 2012; Gmoser et al. 2017).

Other researchers studying cultural materials and their biodeterioration focus on genetic DNA sequencing rather than the classification of the deterioration processes. A separate chapter is dedicated to the review of biodeterioration research in conservation.

In this study, a broad descriptive definition of biodeterioration was accepted, as (1) alterations of the surface and (2) alterations of the matrix, with the intention to encompass all forms of microbial interactions with paper-based materials.

The objects of cultural heritage are composed of all imaginable types of materials that support equally diverse microbial communities (Figure 1.1). Fungi do not discriminate among nutritional food sources, so cultural materials, like any other, are not exempt from microbial biodegradation. Outdoor monuments, underwater archaeological sites, and underground hypogeum can all be infested by microorganisms; a mural painting in a tomb of the Egyptian desert was even overgrown with fungi (Szczepanowska and Cavaliere 2004). Underwater sites and hypogeum provide an acceptable environment for bacteria and algae. Outdoor monuments can be disfigured by lichens. Although forms of biomass differ just as the cultural materials vary, the underlying common denominator is the alteration of the surface first and then the degradation of the materials' structure.

Following the above-suggested definition, the biodeterioration of paper can be discussed in two main categories, as a phenomenon occurring on the surface and in bulk (matrix). Each category can be subdivided into physical and chemical types of biodeterioration, although all are interdependent.

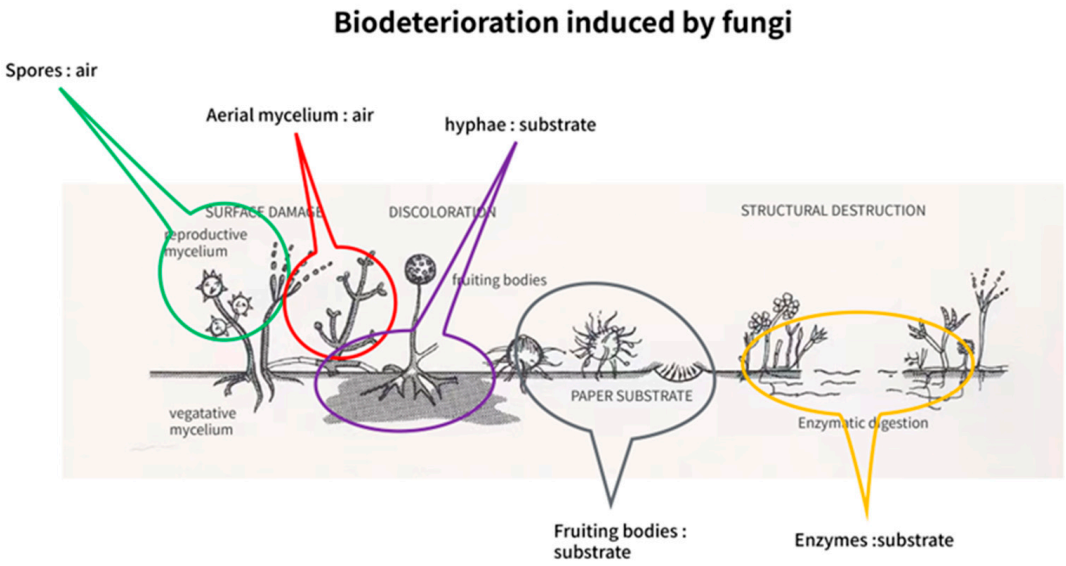

Figure 1.1. Diagrammatic illustration of bio-pigmentation of paper substrate induced by fungi. Source: Diagram expanded based on (Szczepanowska 1986, p. 31). For examples of the biodeterioration recorded on the actual artworks see Figures 1.2–1.13.

An overview of the interactions of pigmented fungal structures and paper following the above diagrammatic illustration is shown as follows:

- Pigmented spores in aerial mycelium and fruiting structures (Figures 1.2 and 1.3)
- Pigmentation secreted into substrate (Figure 1.4). B. Stains caused by deposits of pigmented cells in the substrate matrix (Figures 1.5–1.7)
- Pigmented cells and fruiting bodies embedded into substrate (Figures 1.8 and 1.9). B: Biogenic reconfiguration of paper filler (Figure 1.10) 4: Enzymatic digestion of material (Figures 1.11–1.13).

Pigmented spores in aerial mycelium

Figure 1.2. The 19th-century Japanese woodblock print is covered with mycelium and fruiting structures. The image is obscured by mycelium and fruiting bodies growing vertically. Although the growth occurred on the surface, the bulk of the substrate was also affected by the secretion of pigmented products of metabolism that cause stains (see Figure 1.4). The artwork served as Case Study No. 2. Digital camera image with a Leica D-Lux, scale 1:1. Source: Figure by author.

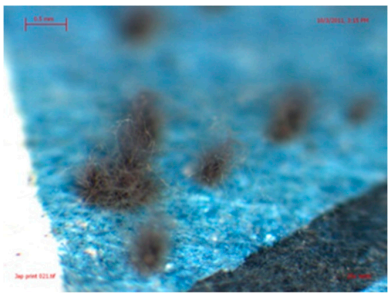

Figure 1.3. Aerial growth of perithecia, characteristic fruiting structures of *Chaetomium* sp, which utilize cellulose as a nutrient source. Enzymatic secretions produced by these species chemically degrade cellulose into simpler units to make it suitable as a nutrient. Detail of Figure 1.2. Digital, stereo-micrograph, scale 500 μm. Source: Figure by author.

Pigmentation secreted into substrate

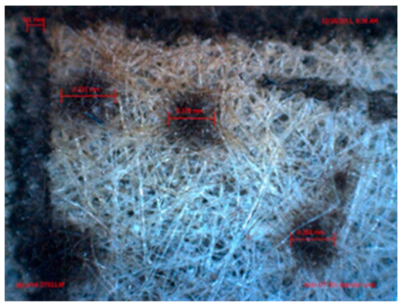

Figure 1.4. A detail of the 19th-century Japanese woodblock print shows staining caused by fungi. The stains were caused by pigmented metabolic products secreted into the substrate. Detail of Figure 1.2 stereo-micrograph, scale 100 μm. Source: Figure by author.

Staining caused by deposits of pigmented cells in the substrate matrix

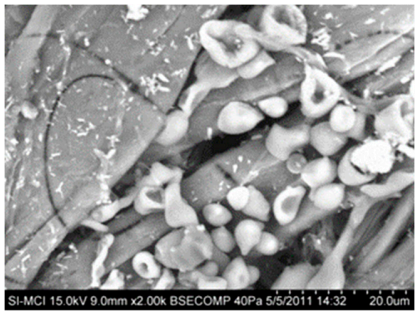

Figure 1.5. Fungal cells attached to the surface of paper fibers. The black fungal species produced cells with features pointed towards meristematic fungi. Scanning electron microscopy with variable pressure (SEM-VP) was utilized to characterize both fungi and the surface of paper. SEM micrograph, scale 20 μm. Source: Figure by author.

Figure 1.6. The morphology of the surface with fungal deposits revealed spatial distribution of fungal cells in stains on a 17th century Study Sheet, No. 1. The fungal cells visible here as small, spherical particles on the surface of paper, nested along fibers, were characterized with confocal laser scanning microscopy (CLSM). Confocal laser scanning micrograph, scale 10 μm. Source: Figure by author.

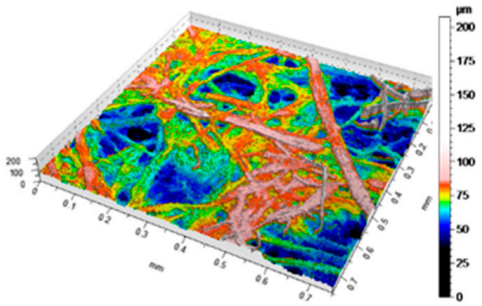

Figure 1.7. A 3D topography of fungal residue on the paper surface of the 17th century Study Sheet. The fungal cells were characterized with white light confocal profilometer and visualized as 'orange' residue along paper fibers. A map, a 3D topography of paper in stain area, generated based on profilometry data; 17c Study Case No 1. Source: Figure by author.

Pigmented cells and fruiting bodies embedded into substrate; biodeterioration of paper, bulk

Figure 1.8. Black-pigmented fungal cells are partially anchored to the paper fibers. The dark mycelium often grew through the substrate's matrix, causing dark stains. Transmitted light microscopy was used to investigate interactions between microorganisms and the substrate. Transmitted light micrograph of areas with fungal stains; scale 10 µm. Source: Figure by author.

Figure 1.9. Bio-deposits in the paper matrix are shown on a cross-section of paper with stains; black fungal cells are near the surface and just below the surface. A sample of the 1920 Engraving, Study Case No. 3, embedded in resin was examined using transmitted light microscopy followed by observations in SEM-VP. Transmitted light micrograph of a cross-section of a fungal stain on paper shows the extent of microbial penetration; scale 50 µm. Source: Figure by author.

Biogenic reconfiguration of paper additives

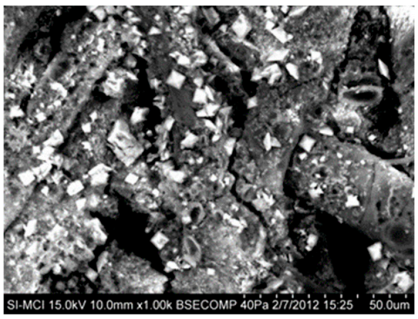

Figure 1.10. Biogenic re-mineralization of calcium-carbonate to calcium oxalate by enzymatic activities of fungi. The chemical alteration of calcium carbonate, an alkaline filler in paper, was caused by oxalic acid produced by fungi, leading to the formation of calcium oxalate. The crystals of calcium oxalate were formed in the paper matrix. Scanning electron microscopy in variable pressure with energy-dispersive X-ray spectroscopy (SEM-EDS) was utilized in the analysis. Biogenic re-mineralization shows well-formed calcium oxalate crystals in SEM micrograph; scale 50 μm. Source: Figure by author.

Enzymatic digestion of material

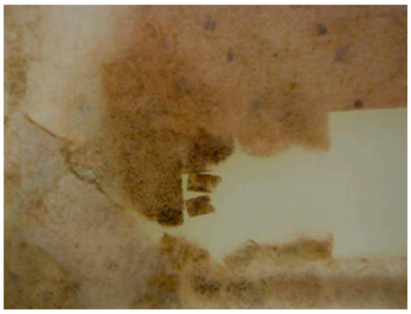

Figure 1.11. The macro-image of paper digested by cellulolytic fungi shows discoloration and stains in areas of severely weakened paper. Sample from Study Case No 1.; Leica D-lux digital camera, scale 1:1. Source: Figure by author.

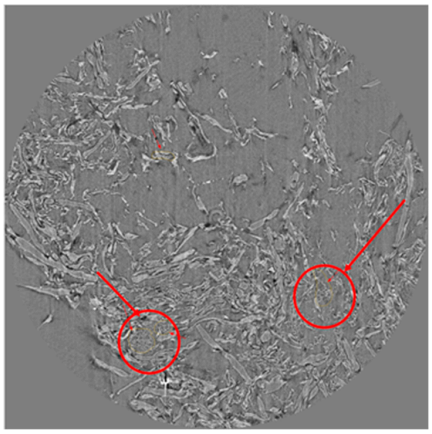

Figure 1.12. The aerial parts of the perithecia are visible with an unaided eye on fig. 1.11; and their full extension into the substrate matrix can be evaluated using X-ray microtomography and producing the 3D visualization. The red arrows indicate spherical fruiting structures nested among paper fibers. A 'slice', one of the tomographs generated on X-ray microtomography at the European Synchrotron Facility (ERSF), Grenoble; 17th century Study Case No 1. Source: Figure by author.

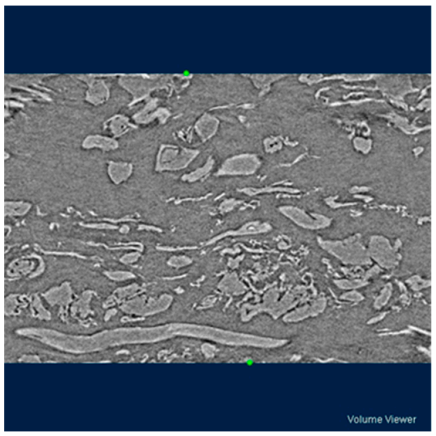

Figure 1.13. A cross-section of paper with fungal deposits, extracted from one of the slices of X-ray micro-tomography, shows fungal inclusions in the paper matrix. In addition to the analysis of fungal structures in the paper matrix, the characteristics of the paper fibers were revealed. Cross-section generated by X-ray microtomography on synchrotron at ERSF. Source: Figure by author.

2. Elements Involved in Bio-Deterioration

2.1. Fungi, an Overview in the Context of Cultural Heritage

Fungi are microorganisms that are essential to many of our life functions. The evidence of fungi origin indicates that they likely first appeared one billion years ago.[1] Fungi degrade and decompose most organic materials to obtain nutrients. This distinctive nutritional strategy is one of the defining characteristics of the entire fungal kingdom. These organisms secrete acids and enzymes into the surrounding environment that break down polymeric molecules into simpler ones that are then absorbed back into the fungal cell. As decomposers and recyclers, fungi are a vital link in the food web and ecosystem as a whole. In the ecosystem, different substrates are attacked at different rates by a consortium of organisms from various kingdoms. In addition, fungi are extremely important for the existence of human life. Some species of *Penicillium* and *Aspergillus* produce antibiotics such as penicillin and griseofulvin, which are widely used in medicine. Moreover, fungi play an important role in wine and cheese production, both of which enhance our dietary choices and tantalize our palette. Mushrooms, too, are culinary delicacies and play a significant role as a food source in many cultures. The many positive attributes of fungi make them an important component of our life (Cavaliere 2003; Gmoser et al. 2017; Hyde et al. 2019).

Fungi make up a kingdom of heterotrophic, single-celled, uninucleate, multinucleate, or multicellular organisms that include yeasts, molds, and mushrooms. Recent DNA evidence suggests that fungi are more closely related to animals than to plants. Because of that fact, fungi are used as experimental organisms in attempts to solve many human problems, particularly cell divisions, growth patterns and metabolic pathways (Hyde et al. 2019). Because fungi are heterotrophic and do not photosynthesize, they are capable of dwelling in light as well as in dark habitats. In addition, the ability to produce and secrete enzymes allows them to invade substrates with their massive numbers of absorptive filaments. These exoenzymes are the most important reason why fungi are so successful; by secreting enzymes from the tips of their hyphae, they are capable of dissolving the substrate upon which they are growing into simple, absorbable molecules. Small proteins, called hydrophobins, that are secreted by hyphae and spores are unique to filamentous fungi and facilitate their attachments to surfaces, a crucial step in starting a new life cycle (van der Mei et al. 1998; Wessels 1997; Wösten et al. 1994; Wösten and Scholtmeijer 2015; Inoue et al. 2016). Saprophytic fungi are those that gain nutrients from dead organisms, and those feeding off living organisms are termed parasitic fungi.

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[1] Encyclopedia Britannica, https://www.britannica.com/science/fungus/Form-and-function-of-lichens (accessed on 20 July 2020).

Fungi reproduce by sexual and asexual means. The asexual phase is characteristic of all fungi and is referred to as the anamorphic stage; the sexual state is known as the telemorphic stage. Structures produced via sexual reproduction serve as criteria for the division of fungi into four major phyla: Chydridiomycota, Zygomycota, Ascomycota and Basidiomycota. An additional group, the Deuteromycetes (or Fungi Imperfecti), for which there is no known sexual state, are affiliated with at least three phyla, most of which belong to Ascomycetes or Basidiomycetes, which lack a sexual state (Alexopoulos et al. 1996; Samson 2016). Once a sexual form of reproduction is found, the fungus is moved into an appropriate taxonomic group based on the morphology of the taxonomic form. Deuteromycetes, therefore, is not a valid taxonomic name but a loose assemblage of organisms that do not reproduce via sexual modes. The advent of the phylogenetic analysis of fungi using DNA sequencing allows the description and naming of fungi that previously had no established association and taxonomic placement (Redhead and Rossman 2007).

Many fungi encountered on cellular, heterogeneous material of cultural importance belong to the Deuteromycetes, referred to in bibliographic sources as anamorphic fungi, asexual fungi, anamorphs, or conidial fungi. This 'unnatural' group comprises around 2600 genera and 15,000 species (Cannon and Sutton 2004). The other name frequently used by researchers is soil fungi, because many of them live in soil, playing an important ecological role in decomposing plant and animal remains. The Deuteromycetes include approximately 30,000 widely distributed species (O'Connor 2007). However, to avoid confusion, in 2013, a new regulation was proposed to be implemented by the Department of Agriculture that aimed to classify fungi based on their genetic makeup. The revised classification was made available on 27 January 2019, in open access form.[2] This supersedes the modifications of the nomenclature proposed in July 2018.[3] Although the most recent term was proposed, "anamorphic fungi", for 'Fungi Imperfecti' and 'Deuteromycota' (Samson 2016), the latter one will be used throughout this text in alignment with references in publications dealing with the deterioration of cellulose-based cultural heritage collections. At the time of writing (early 2020), a Google search listed 25,000 hits classified as Fungi Imperfecti/Deuteromycota, indicating that this nomenclature is still acknowledged. The slow acceptance of the new nomenclature systems over the years has been also acknowledged by other researchers (Powers-Fletcher et al. 2016).

Fungi identified on cellulose-based materials have well-developed, branching, multicellular, filamentous mycelia. The term 'mycelium' refers to the total mass of hyphae (Figure 2.1). A hypha is a branching, tubular structure from 2 to10 μm in

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[2] https://imafungus.biomedcentral.com/articles/10.1186/s43008-019-0019-1 (accessed on 12 February 2023).

[3] The modifications can be tracked on: http://www.iapt-taxon.org/nomen/main.php (accessed on 12 February 2023).

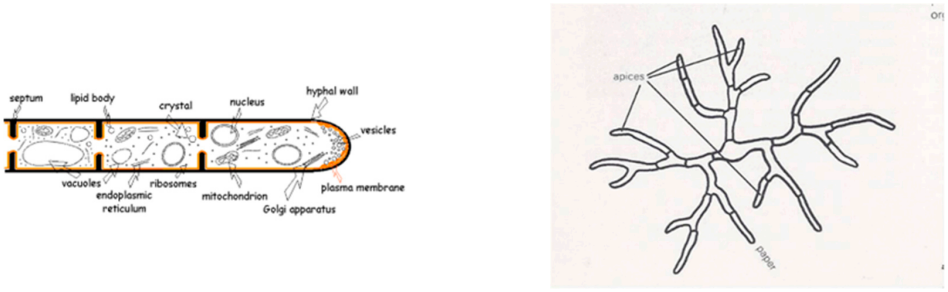
diameter and is usually divided into cell-like units by cross walls, called septa. The portion of the mycelium that anchors the fungus and absorbs nutrients is called the vegetative mycelium; the portion that produces asexual reproductive spores and grows on the surface is termed the aerial mycelium, as illustrated in Figure 1.1. The appearance of the hyphae and mycelium, along with the forms of spores/conidia, provide the main criteria for identifying and classifying fungi.

In their quest to find the most suitable surface to attach to and begin anew their live cycle, fungi indiscriminately populate and thrive on all types of materials that, unfortunately, include objects of cultural value. To understand the many forms of fungal interactions with substrates, their growth and expansion, propagation, pigment formation, and some environmental and nutritional requirements are detailed in the subsequent chapters.

2.1.1. Mechanism of Fungal Growth

A new life cycle of a fungus begins with spore germination. For that to occur, a spore must attach to a suitable, digestible substrate under optimal environmental conditions (Hoch et al. 1987; Brand et al. 2007; Brand and Gow 2009) in the presence of water. Those two conditions, a suitable surface and water, are essential and involve physical and chemical forces aiding adhesion and subsequent growth; the complex attachment mechanisms are elaborated in Chapter 4, Paper and Fungi Interfaces. Once the spore germinates, the fundamental unit of the fungal organism, the hypha (pl. hyphae), forms. The hypha contains all the typical eukaryotic cytoplasmic components confined within a rigid wall (Burnett 2003; Read and Roca 2013; Samson 2016).

Hypha is polar and, for over a century, it was believed that it grew only at one end, forming a new wall that is tubular with the help of high, internal hydrostatic pressure (turgor). The classic model of radial colony growing at its periphery where apical extension of each hyphal filament forms branches was introduced in 1931 by Buller (Read and Roca 2013). In 2007, research expanded the pattern of hyphae growth by the inclusion of cell division (Christensen et al. 2008). Combining those two modes of growth, we could say that the fungal colony expands by hyphal branching and extends into the surrounding environment (Figure 2.1). A constant ratio that is maintained between the total length of hyphae and the number of tips is known as the “hyphal growth unit” (Wessels 1997). Hyphae are designed to transport water, nutrients, and cytoplasmic components through pores in septa. In the absence of nutrients, hyphal growth can be sustained by the transport of water and nutrients contained in the cytoplasm, until a patch of nutrient is found. Hyphae can expand over large areas that contain only isolated patches of nutrients.



(**a**) (**b**)

Figure 2.1. Schematic illustration of a hyphal tip (**a**) and radial growth of hyphae (**b**). (**a**) Hyphae are highly polar, when growing at the tip, a new wall is formed to keep the hyphal tubular shape. Enzymes are secreted at the apex (Wessels 1997). Source: Diagram adapted from www.Fungionline.org.uk, used with permission. (**b**) Hyphae regularly branch and give rise to a mycelium that forms a colony. A constant ratio between the total length of hyphae and the number of tips is known as the 'hyphal growth unit' Source: Diagram reprinted from (Szczepanowska 1986, p.32).

The emergent growth of hyphae, extending into the air as felt-like mats of mycelium, may be a manifestation of hyphal adaptation to a lack of nutrients (Figure 2.2). The crossing of the solid–air interface is aided by hydrophobins, small proteins secreted by fungi (Wessels 1997, 2000; Inoue et al. 2016; Wösten and Scholtmeijer 2015; Urbar-Ulloa et al. 2019). As mentioned earlier, hydrophobins also aid in spores' adhesion to surfaces.

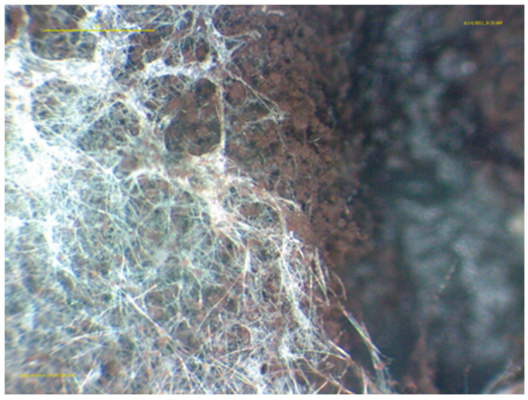


Figure 2.2. Mycelium of *Aspergillus niger* growing in nutrient-starved culture. White, filamentous growth bears dark brown or black fruiting structures. Stereo-surface analysis microscope, magnification 12×. Scale: 500 µm. Source: Figure by author.

Aerial hyphae bear fruiting structures that break at their apices, dispersing spores (Figure 2.3). This is part of the vegetative, asexual stage of the reproduction of fungi and is found to be the mode of dispersion in *Aspergillus* sp. (Figures 2.3 and 2.4) and *Penicillium* sp. alike (Figures 2.5 and 2.6). Once a suitable surface and

environment are available, spores land, attach to the surface, and the continuation of their life cycle ensues; details on the modes of attachment are discussed in the following chapters.

In addition to many spores being pigmented, colored colonies cause staining on materials on which they grow by the secretion of pigmented, secondary metabolites; one such example could be Rhizopus (Figure 2.7). A review of various forms of staining caused by fungi on artworks is illustrated in Figures 1.2–1.11 and discussed in detail in Chapter 3.

In addition to the physical forces of hyphae expansion, fungi secrete exoenzymes to degrade the substrate in advance of invading it (Money and Howard 1996). The enzymes are secreted at the apex and break down the solid organic substrates, degrading the constituent polymers from within. This leads to the complete degradation of the paper matrix. Examples of such enzymatic digestion of paper are evident on the 17th century Study Sheet (Figure 1.11) and in Chapters 5 and 6. Furthermore, the secreted enzymes are often pigmented as well and are therefore one of the means of stain formation in substrates. During the life cycle of the fungus, the colony undergoes several phases—penetration into and digestion of the substrate, spore production and dispersal (the asexual phase), and the sexual reproductive phase with the production of sexual spores (Burnett 2003). At each stage, depending on the species, pigmentation is produced by fungi; some examples of colored mycelium and fruiting structures are illustrated on the actual artworks infested by fungi and discussed in detail in Chapter 6.

2.1.2. Methods of Propagation, an Overview

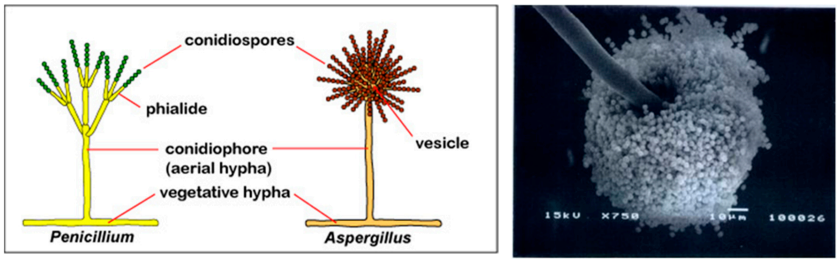
Considering an enormous number of fungi species and corresponding equally large variations in modes of propagation, many are discussed in monographs dedicated to specific species. In this publication, focused on black fungal pigments, only representatives of two groups are reviewed, which known to produce bio-pigments: Fungi Imperfecti and Meristematic Fungi. The latter is also referred to as microcolonial fungi, or black yeast (Sterflinger and Krumbein 1995; Sterflinger and Krumbein 1997; Sterflinger 1998; Sterflinger et al. 1999; Sterflinger 2006; de Leo et al. 2003, 2019). The species in both groups are the main producers of black stains on cultural heritage material via their dark pigmented propagules, dark mycelium and fruiting bodies as well as black secretions into substrate.

Fungi Imperfecti propagate by the production of asexual spores (conidia) or the division of the hyphae. Most fungi are capable of asexual reproduction, meaning that the living systems give rise to new systems, similar to themselves, without the fusion of cells, which is observed in the sexual mode of propagation. Conidiospores, sporangiospores, and pycnidiospores are some of the most common forms of asexually produced spores (conidia) found on cultural heritage objects. Their morphological features can aid in the identification of species. Conidiospores are borne externally in chains on aerial hyphae called conidiophores (Figures 2.3–2.7).

Sporangiospores are borne internally within a terminal sac (sporangium). Pycnidiospores are spores produced in a fruiting structure, globose or flask-shaped, called a pycnidium (Figures 2.10–2.16). The conidiophores inside the pycnidium are usually very short. The same methods of forming pycnidia were observed in the formation of acervuli, which makes the differentiation of species more challenging.[4]

Condidospores

Among the Fungi Imperfecti, various types of conidiogenesis can be observed. A common type of conidia production occurs through the phialides, resulting in masses of conidia in the form of chains or conglomerates, as exemplified by *Aspergillus* sp. and *Penicillium* sp. (Figures 2.3–2.6), or in so-called 'slimy heads', such as in *Fusarium* (Abdel-Azeem et al. 2016; Samson 2016). Other fungi are characterized by thallic, blastic, or sporoconidia, such as in Cladosporium or Alternaria (Samson 2016), all of which were identified in cases of cultural heritage infestation (Kowalik 1980; Szczepanowska and Moomaw 1994). Alternaria in particular is characterized by dark brown conidia and dark hyphae. In addition to the phialides, conidia can grow from different types of conidiogenous cells, for example, by emerging directly from hyphae, as aggregated hyphal structures (Samson 2016).



(a) (b)

Figure 2.3. (**a**) A schematic illustration of conidiospores growing on conidiophores. Source: Reprinted from https://www.ndvsu.org/images/StudyMaterials/Micro/2_reproduction_in_fungi.pdf, not to scale. (**b**) *Aspergillus niger* with conidiospores growing on a vesicle. After the spores are dispersed, a well-defined vesicle is visible, as seen in Figure 2.4; transmitted light, 400×; scale bar: 10 µm. Source: Reprinted from (Szczepanowska and Cavaliere 2003).

[4] www.faculty.ucr.edu/~legneref/fungi/deuteromycota.htm (accessed 20 March 2020).

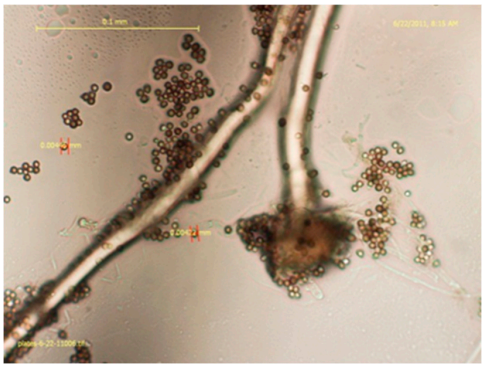


Figure 2.4. Conidiophore of *Aspergillus niger* with dispersed spores and vesicle. Transmitted light micrograph, scale 100 µm. Source: Figure by author.

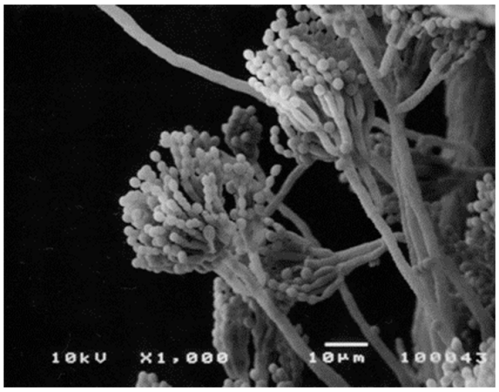


Figure 2.5. Conidiophores and conidia on phialides. The conidiospores of *Penicillium chrysogenum*, usually grey, green, or blue, are produced in chains on finger-like projections called phialides growing from the conidiophore. SEM micrograph, scale bar: 10 µm. Source: Reprinted from (Szczepanowska and Cavaliere 2003, p. 141).

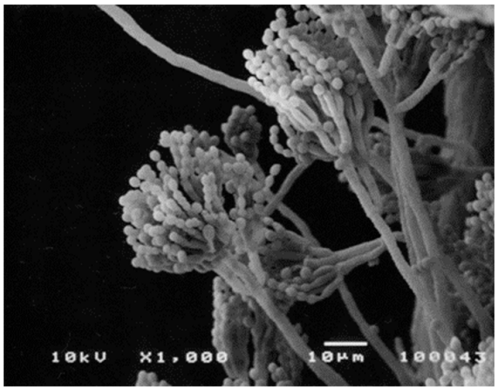
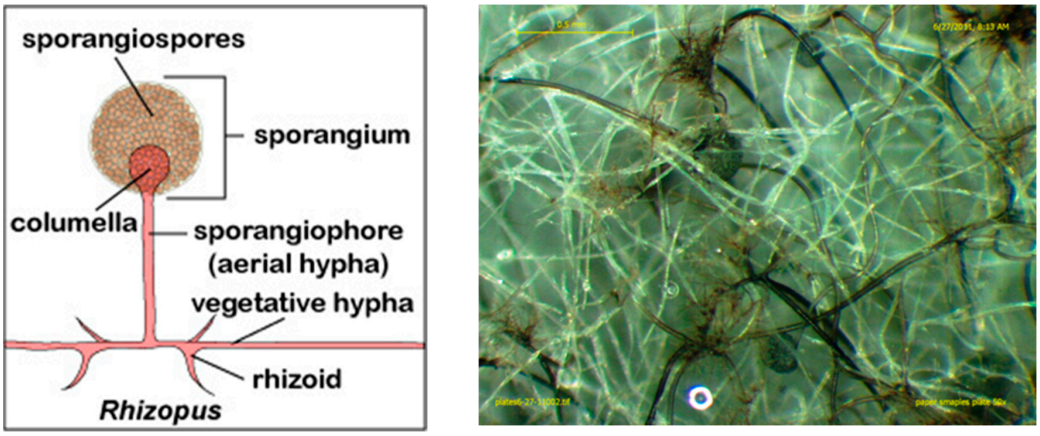


Figure 2.6. Chains of conidiospores of *Penicillium chrysogenum.* SEM micrograph, scale bar: 50 µm. Source: Reprinted from (Szczepanowska and Cavaliere 2003, Figure 8b, p. 141).

Sporangiospores

Sporangiospores, another form of propagules, are produced within a sac, or sporangium, on an aerial hypha called a sporangiophore. The sporangiophore

terminates with a dome-shaped terminus called a columella that extends into the sporangium.



(**a**) (**b**)

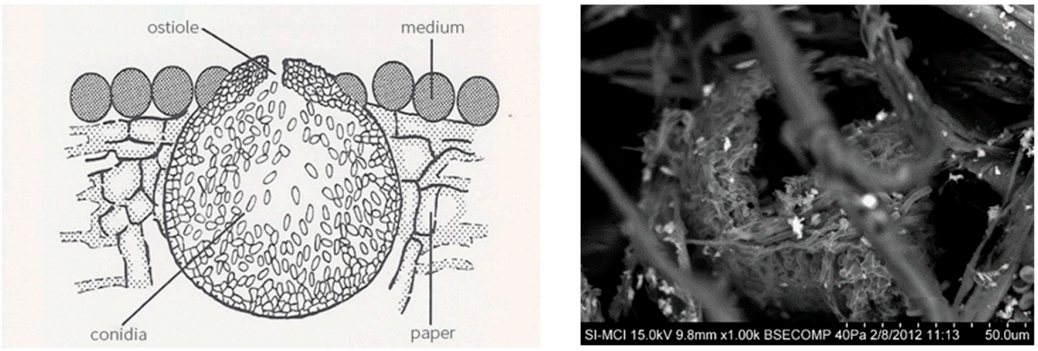
Figure 2.7. (**a**) A schematic illustration of sporangiospores within the sporangium. Source: Reprinted from https://www.ndvsu.org/images/StudyMaterials/Micro/2_reproduction_in_fungi.pdf, not to scale). (**b**) *Rhizopus stolonifer* with dark sporangiophores and rhizoids. One of the functions of rhizoids is to attach the organism to a substrate. Their pigmented filaments cause staining of substrates. Optical microscopy of a specimen on culture plate, 50×; scale bar: 500 µm. Source: Figure by author.

Rhizopus is an example of a fungus that produces sporangiospores. The sporangiospores, typically dark brown or black, are produced within the sporangium (Figure 2.7). The anchoring structures, which attach the fungus to a surface, called rhizoids, are produced on the vegetative hyphae. The rhizoids of the studied fungus also showed black pigmentation within their cells, in addition to the dark pigmentation in the fruiting structures (Figure 2.7b). *Rhizopus* has been identified by various researchers as growing on paper-based cultural material (Kowalik 1980; Zyska 1997).

Sporangiospores are disseminated by air, water, or animals, and once landing on a substrate in a suitable environment, germinate and produce new hyphae. As dispersal units, spores may travel great distances and may be long-lived. Under certain conditions related to the substrate and environment, conidia may further develop into yeast-like cells (Alexopoulos et al. 1996; Isola et al. 2016). However, the viability of conidia is reduced rapidly in dry conditions.

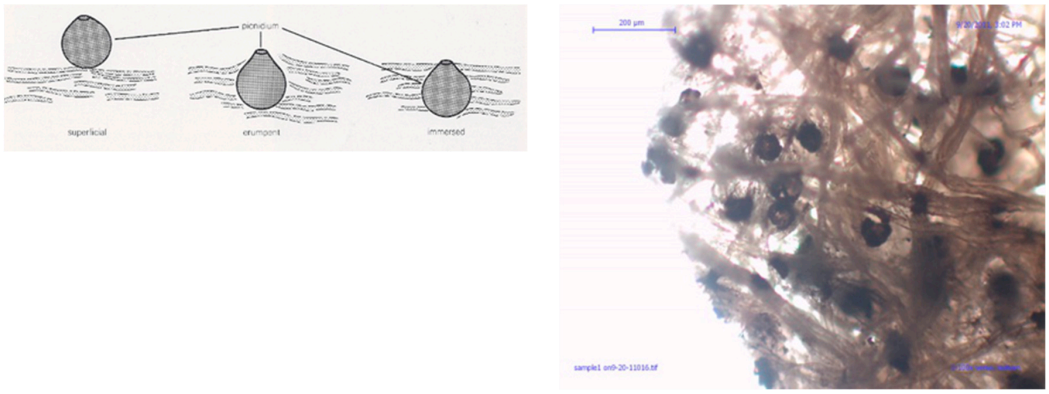
Pycnidiospores

Pycnidiospores (or pycniospores) form within the fungal tissue itself (a pycnidium), which is spherical or shaped like a bulging flask. The spores are released through a small opening at the apex, the ostiole. These fruiting structures can be located on the surface of the substrate or completely embedded in it (Figures 2.8 and 2.9).



(**a**) (**b**)

Figure 2.8. (**a**) A diagram of a pycnidium partially embedded in the paper structure. Source: Adapted from (Szczepanowska 1986, p. 34). (**b**) A pycnidium in the paper matrix, partially embedded; 17th century Study Sheet, No 1. SEM micrograph, 1000×, scale 50 µm. Source: Figure by author.



(**a**) (**b**)

Figure 2.9. (**a**). The positions of pycnidia, as growing on the surface, show partially embedded and nearly completely embedded fruiting structures in the paper matrix. Source: Reprinted from (Szczepanowska 1986, p. 33). (**b**) The pycnidium structure embedded in paper matrix seen on the transmitted light micrograph, 17th century Study Sheet; 400×, scale 200 µm. Source: Figure by author.

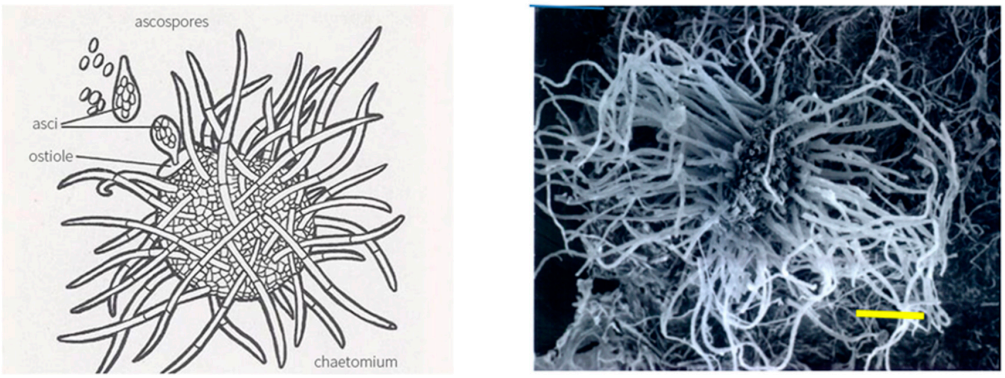
Some external structures of pycnidia may resemble perithecia, and only by crushing them can one determine their nature under higher magnification. In pycnidia, spores are produced via asexual mode; in perithecia, ascospores are produced via sexual mode. One of the earliest descriptions of pycnidia formation (Kempton 1919) refers to three different types resulting from the division, branching, and interweaving of hyphae cells. Recent publications (Abdel-Azeem 2020) elaborate on other morphological features of pycnidia and perithecia that may assist in the characterization and identification of the genus Chaetomium. An example of Chaetomium was documented in detail on the 17th-century Study Sheet (Figures 2.9 and 6.10b, 6.11 and 6.16).

Germination is a complex and vital episode in the life of the fungi. Molecular water is an essential component for this fascinating process to begin. Once favorable conditions are created, the spore germinates, starting anew the life cycle.

Ascospores

Ascospores are produced during sexual reproduction. These types of propagules are encased within spherical or flask-shaped structures (ascocarps) formed by the aggregation of hyphae. The ascospores are produced within ascus (plural asci), with a sac-like structure, each one usually containing eight ascospores. The various types of ascocarps are cleistothecia, perithecia and apothecia. Ascocarps' shape and appearance are major criteria used in separating the Ascomycota into classes. An example of such a mode of reproduction is found in *Chaetomium* species.

Chaetomium species require special attention because they are one of the most common cellulolytic fungi found on paper (Figure 2.10). Cellulolytic fungi are so named because they require cellulose for their healthy growth and fructification. This translates into the digestion of cellulose-based paper artifacts and artworks as their main nutritional component. *Chaetomium* is one of the largest genera of saprophytic Ascomycetes (Ingold and Hudson 2003; Sutton et al. 2009; Abdel-Azeem 2020). Since their first characterization in 1817, over 400 species have been described (von Arx et al. 1986; Abdel-Azeem 2020). The identification of *Chaetomium* species relies on the shape and decoration of perithecial hairs (seate) (Figure 2.11), ascus morphology, ascospore shape, germ pores, colony color, and exudates (Abdel-Azeem 2020). The Chaetomium documented on Study Cases No. 1 and No. 2 were attached to substrates by rhizoidal hyphae. The ascospores developed in asci were brown or grey olive when mature but were not black, which was in agreement with von Arx et al. (1986). The optimal temperature range for Chaetomium growth is considered 25–35 °C, while 45 °C is already too high; *Chaetomium* sp development can thus be regulated by adjusting ambient temperatures.



(**a**) (**b**)

Figure 2.10. (**a**) A schematic drawing of an ascocarp (perithecium) of Chaetomium, showing seta (perithecial hairs) and asci being released via the ostiole. Each ascus contains eight ascospores. Source: Reprinted from (Szczepanowska 1986, p. 34). (**b**) *Chaetomium globosum*, perithecium with seta, perithecial hairs, growing in all directions. The species was growing on deteriorated paper pamphlets. Scale 20 μm. Source: Reprinted from (Szczepanowska and Cavaliere 2000, p. 248).

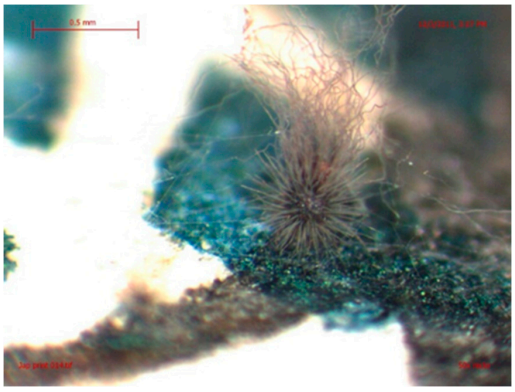


Figure 2.11. A single perithecium of *Chaetomium globosum* growing on the surface of the Japanese woodblock print, Case Study No 2. Perithecial hairs extend from the walls of the perithecium. Attachment of a single perithecium on the substrate can be attributed to electrical charges between the surface of the substrate and the electron-dense hyphae and perithecial hairs. Stereo-micrograph, scale bar: 0.5 mm. Source: © Szczepanowska 2011.

The characteristics of the perithecial walls and structural details of ascospores are used in species identification, some of which are discussed here, in the context of biodeterioration. The perithecium is composed of densely intermingled and branched hyphae; the outer interhyphal wall is slightly more electron dense than the inner layer. The cells shaping the outer regions of the perithecial surface are filled with electron-dense particles, resembling glycogen (glycogen is the analogue of starch, a glucose polymer in plants, and is sometimes referred to as 'animal starch').

Actively growing hyphal tips, surrounded only by a single wall layer, are present on the entire perithecial surface. The electrons fill the outer cells of growing hyphae throughout the development of the perithecium until its maturity (Froeyen 1980). The cells of the terminal perithecial hairs are bound by a thin wall, which is made up of outer electron-dense melanized zones (Ellis 1981). Therefore, it can be deduced that the attachment of a single perithecium on the substrate can be attributed to the electrical charges between the surface of the substrate and the electron-dense hyphae and perithecial hairs.

2.1.3. Aspergillus Niger

The microorganism *Aspergillus niger* was selected for closer examination for two reasons: first, it is known for its production of black pigmentation, which was used in this study, to produce black stains, and second, it is one of the commercially most important microorganisms has therefore been extensively researched. The genus of *Aspergillus* is one of the oldest genera; it was named and described in 1729 (Bennett 2010).

Currently, the *Aspergillus* genus contains 339 accepted species (Abdel-Azeem et al. 2016) and is one of the microbial communities found in soil. Species of *Aspergillus* propagate asexually via spores that drift on air currents, dispersing

themselves both short and long distances, depending on the environmental conditions. When the spores come in contact with a solid or liquid surface, they germinate if the conditions are favorable. In the ecosystem, different substrates are attacked at different rates by consortia of organisms from different kingdoms. *Aspergillus* and other fungi play an important role in these consortia, recycling starches, hemicelluloses, celluloses, pectin and other complexes, polysaccharides and polymers. Maximum decomposition occurs when there is sufficient nitrogen, phosphorus and other essential inorganic nutrients (Bennett 2010).

In the early history of this genus, it was discovered that some *Aspergillus* could produce cleistothecia, which are common sexual reproductive structures in the Ascomycetes. Thus, it became apparent that a fungus previously placed in the Fungi Imperfecti (fungi that reproduce asexually, of unknown 'perfect' sexual stage) could undergo two different reproductive phases, sexual and asexual. Because the two phases are separated in both time and space, in other words, do not occur simultaneously, they are given two names. This early observation was the beginning of a dual naming system for the same organism, based on its modes of reproduction. The duality of names, however, was discontinued in 2013 with The International Code of Botanical Nomenclature, Article 59, Division II, Rules and Recommendations, Chapter 6, Names of Fungi with a Pleomorphic Life Cycle.

The commercial importance of *Aspergillus* is mainly due to its ability to produce the cellulase enzyme, which catalyzes the conversion of cellulose to glucose; glucose produced by cellulase can be fermented to ethanol, currently considered as a potential biofuel (Abdel-Azeem et al. 2016; Dimarogona and Topakas 2016). In addition to its ability to produce cellulases, which is of great concern to cultural heritage because of their destructive effects on cellulose in paper and textiles, *A. niger* also produces a number of metabolites, both primary and secondary, that are of enormous commercial value, widely used in the pharmaceutical and cosmetic industries (Kendrick 2000; Carlisle et al. 2001; Deacon 2006). Furthermore, *A. niger* has been used to study the proteolysis of cell walls in plants, to understand their biosynthesis and degradation. As a soil saprobe, it produces a wide array of hydrolytic and oxidative enzymes which are involved in the breakdown of plant lignocelluloses and are a subject of intense study in agriculture. In addition, *A. niger* has been an important model fungus for the study of eukaryotic protein secretion in general, as well as the effects of various environmental factors on suppressing or triggering the export of various biomass-degrading enzymes, molecular mechanisms critical to fermentation process development, and mechanisms involved in the control of fungal morphology.[5]

How these biodegrading enzymes affect paper's structure has been illustrated in the experimental stain production, in Chapter 2.4. The ability to produce cellulases

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[5] http://www.broadinstitute.org/annotation/genome/aspergillus_group/GenomeDescriptions.html (accessed 2 February 2023).

(Gamarra et al. 2010) places *Aspergillus* among species frequently found on paper artifacts; the other fungi found on paper and known to produce cellulases include *Trichoderma, Penicillium, Fusarium,* and *Humicola*. Interestingly, all of them are associated with the production of pigmentation in various colors: Trichoderma is associated with green-brown pigmentation in aerial mycelium, and Fusarium with magenta pigment secreted to the substrate; some of which have been reported in earlier studies (Kowalik 1980; Szczepanowska and Lovett 1992; Szczepanowska and Moomaw 1994; Pinzari 2018). In general, these fungi secrete three types of cellulases: (1) endo-b-1,4-glucanase (EG), (2) cellobiohydrolase (CBH), and (3) b-glucosidase (BGL) (Gincy et al. 2008), but *Aspergillus niger* is the most prolific producer.

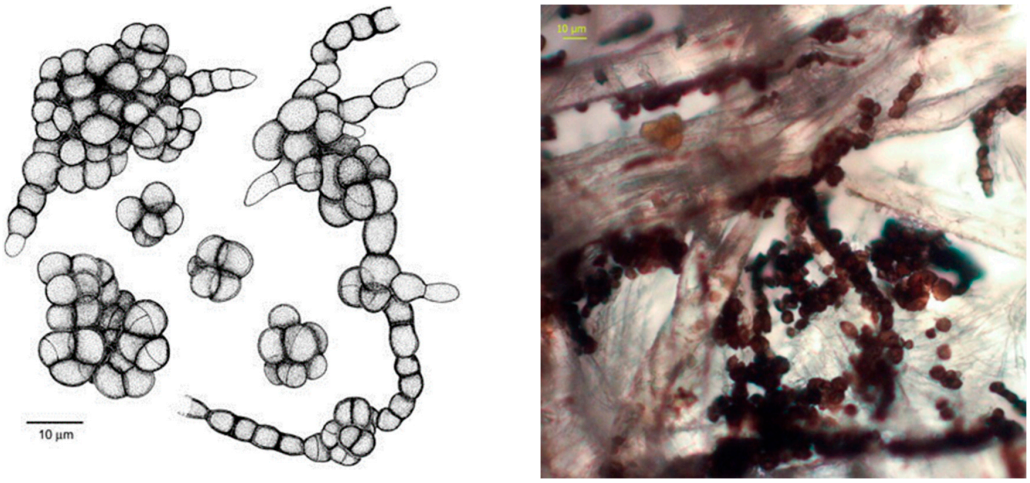
Both paper and textiles (cotton, jute, and linen), because of their chemical makeup (cellulose and starches), are especially vulnerable to *Aspergillus'* degradation. *Aspergillus* sp, in particular *A. niger, A. nidulans* and *A. versicolor*, were reported in various bibliographic references among species encountered on cultural heritage. In further discussions, the black pigmentation caused by the secretion of exoenzymes produced by *Aspergillus niger* will be particularly considered when looking at black stains in the context of cultural heritage artifacts.

Aspergillus sp. Identification

The defining characteristic of the genus *Aspergillus* is its spore-bearing structure (Figures 2.3 and 2.4). It is the most important microscopic feature used in *Aspergillus* taxonomy and identification. One of the hallmarks of *A. niger* is its dark brown-black spores, which are attributed to the presence of an extensive melanin coating (Sutton et al. 2009; Wargenau et al. 2011). However, strains of the *Aspergillus* genera may show various shades of green or yellow as well. The studied patterns of enzymatic activity and pigmentation of *A. niger* showed that nutritional composition was responsible for biochemical outcomes, one of which was manifested as color variation (Tamayo-Ramos et al. 2011). Knowing that color may be affected by external factors, the pigmentation of colonies needs to be cross-referenced with other identifying morphological factors in the identification process. This includes the colony growth rate and texture, colony characteristics, thermotolerance, degree of sporulation, production of sclerotia or cleistothecia and, on a microlevel, shape of conidial heads as well as the presence or absence of metulae between vesicle and phialides and phialides. In some cases, in the identification process, morphological features are supplemented with biochemical characteristics, such as secondary metabolite production or ultramicroscopic traits exemplified by spore ornamentation, including surface roughening (Kozakiewicz 1989; Samson et al. 2014). Genetic sequencing is one of the most reliable techniques in the identification of fungi species; however, the need for such analysis in the field of cultural heritage is usually reviewed on a case-by-case basis.

2.1.4. Meristematic Fungi

Black stains, other than those formed by the inclusion of single fruiting structures such as *Chaetomium* sp. or clusters of pigmented spores such as *Aspergillus niger*, can be attributed to black fungi that belong to the group of fungi called Dematiaceae. The different species are grouped under that name based on their common denominator, black pigmentation, resulting from their dark, thick, pigmented cell walls and hyphae. Dematiaceae is therefore not a taxonomic name but a name referring to a group of black fungi that represent different species. The other names associated with this group of fungi are meristematic fungi, microcolonial fungi (Sterflinger et al. 1999; Sterflinger 2006) or black yeast-like fungi (Yurlova et al. 2008; Sutton et al. 2009). Some of these names indicate forms of propagation such as the budding of cells in yeast and the ability to propagate as in filamentous fungi. The existence of two, or more, reproductive modes refers to the meristematic nature of these fungi. While in their filamentous mode they usually reproduce by the extension of hyphae, when in their yeast-like mode, their reproduction occurs by enlargement and subdividing isodiametric cells (Figure 2.12).



(**a**) (**b**)

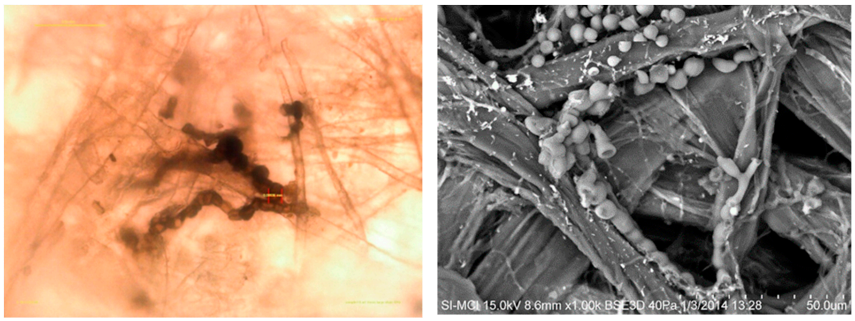
Figure 2.12. (**a**) *Phaeosclera dematioides*, UAMH 4265, clumps of meristematic cells. Source: Reprinted from (de Hoog 2000) (courtesy of G.S. de Hoog 2020). (**b**) Dark pigmented fungal cells in black stains on the 17th century Study Sheet, transmitted light, optical microscopy, scale bar: 10 µm. Source: Figure by author.

Dark pigmentation is associated with melanin, which makes these fungi particularly resilient in adverse environmental habitats. Meristematic fungi grow in extreme temperatures with a scarcity of nutrients and limited water availability, exposure to UV radiation, and oxygenic action (Gorbushina et al. 1993, 2003; Urzì et al. 1995; Sterflinger 1998; de Hoog et al. 1999; Zalar et al. 1999). Their presence has been reported on rocks and stones in diverse localities, from the desert of Arizona (Staley et al. 1982; Egidi et al. 2014; Isola et al. 2016) to Antarctic, from the Ivory Coast (Büdel et al. 2000) to Mediterranean countries (Wollenzien et al.

1997; Sterflinger and Krumbein 1997; de Leo et al. 2003). Those found on marble and granite monuments were responsible for stone deterioration by disrupting the coherence between crystals in a crystalline matrix (Sterflinger et al. 1999; Urzì et al. 2014). In contrast to well-researched black fungi on stone, Dematiaceous fungi on paper were mentioned only in a limited number of bibliographic references (Strzelczyk and Leznicka 1981; Pinzari et al. 2006).

Phylogenetically, meristematic fungi either belong to, or have close affinities with, at least three different orders of the fungal Kingdom: *Chaetothyriales* and *Dothideales*, with a large family of *Pleosporales* (Sterflinger et al. 1999; de Hoog et al. 1999; Selbmann et al. 2005; Ruibal et al. 2009; Egidi et al. 2014; Urzì et al. 2014; Teixeira et al. 2017). Their identification is difficult because of their meristematic nature, meaning their ability to convert from a filamentous form to yeast-like growth depending on the environmental conditions. To date, the molecular approach of nuclear sequencing enabled only partial taxonomic and phylogenetic positioning of meristematic fungi (de Hoog et al. 1999; Selbmann et al. 2005; Hyde et al. 2019).

In the course of this study, some characteristic features such as monilioid hyphae and branched or unbranched chains were identified in black stains on the cultural heritage material, using transmitted light microscopy combined with SEM analysis (Figure 2.13).



(**a**) (**b**)

Figure 2.13. (**a**) Transmitted light microscopy of black fungi in Case Study 3, Engraving 1920, illustrates monilial hyphae. Micrograph, scale 100 µm (for a detailed analysis of this structure, see Chapter 6). (**b**) Fungal monilial cells in the black stain on the 17th century Study Sheet (Case Study 1), seen in SEM micrograph, 1000×, scale 50 µm. Source: Figure by author.

Similar to the findings of stone-inhabiting black fungi, those growing on paper produced extracellular material outside the hyphae and conidia. These substances may protect microfungal structures from desiccation and, as in the case of Arctic conditions, from freezing (Selbmann et al. 2002).

2.1.5. Summary Points

1 A large number of filamentous fungi reported as occurring on cultural heritage material belong to Fungi Imperfecti (Deuteromycetes). However, they represent only a fraction of an enormously large Kingdom of Fungi and provide a sample

of a great variety of growth and reproductive patterns that may be encountered on cultural heritage material. Some of these patterns serve as diagnostic features.

2 The two species, *Chaetomium* sp. (Ascomycetes) and *Aspergillus niger* (Deuteromycetes), are commonly encountered on paper-based cultural material. *Aspergillus* species interact with cellulose-based cultural heritage by secreting cellulase enzymes that degrade cellulose. *Chaetomium* degrades cellulose, utilizing it as its essential nutrient.

3 Meristematic fungi (micro-fungi, black yeast) pose a challenge when their characterization is considered because even though they are present on cultural heritage objects, they have not been studied extensively. Most research has been carried out on the black meristematic fungi that are human pathogens; therefore, these reports served as references in discussing meristematic fungi occurring on cultural heritage objects.

2.2. Paper, an Overview

Paper is a web of fibers and fillers assembled with a great degree of heterogeneity, considering both the physical shapes and chemical composition. The air voids between fibers, referred to as pores, play an important role in how the paper responds to the environment. It is one of the oldest man-made materials, whose origin is conventionally dated to 105 AD, when paper was described for the first time by a Chinese script (Hunter 1978). Despite its long history, the scientific attempts to characterize paper's structure and link it with paper properties are relatively recent, beginning in the 1960s (Bristow 1986a).

Paper has been used for many applications, from a material on which art is created and documents are written, to clothing, umbrellas, and in balloons, produced in Japan and known as Fugo Balloons, that carried incendiary bombs during WWII. Each application requires different optical, physical, and chemical properties of paper to meet such diverse purposes. The properties of papers rely on the nature of the fibers that compose them, the processes that fibers undergo, and the characteristics of additives.

Paper is a two-phase system, solid and gas, with a three-dimensional structure of fibers and air voids between them. Voids play an important role in water adsorption and absorption, thus impacting the bio-susceptibility of paper. The surface of paper has different properties than the bulk; adsorption refers to surface-based processes, while absorption involves the entire bulk of the absorbing material. The surface energy of fibers plays a role in microbial attachment, as elaborated in subsequent chapters, in addition to detailing the role of surface topography, which directly impacts patterns of microbial attachment.

The smoothness or roughness of the paper surface is imparted by the application of sizing (or its absence) and different methods of flattening the paper fibers, while paper pliability, rigidity, density or weight are engineered during paper pulp processing. Because of the impact of sizing on paper characteristics, specifically when

interactions with water are concerned, papers are typically described in two broad categories as sized and unsized.

There are many different plants that supply fibers for the production of paper. Commercial papers use primarily wood-derived fibers, while artists' papers and paper used in some banknotes are made from cotton and linen (flax) fibers derived from textile rags. Thus, such paper is often referred to as 'rag paper.' The main difference between wood-pulp paper and cotton-based papers is their chemical content; wood fibers contain more lignin and hemicelluloses while cotton papers consist of nearly pure cellulose and no hemicelluloses. All plants that contain lignin also contain hemicelluloses, whereas cotton and other lignin-free fibers lack hemicelluloses (Emerton 1980; Bristow 1986a, 1986b). Paper made in the Far East regions, often referred to as 'Oriental paper', uses mitsumata or mulbury-fibers (common name kozo fibers) harvested from the inner bark of shrubs. Paper, therefore, can be referred to as Western or Oriental, depending on the geographic region in which it was produced and based on the characteristics of raw materials supplying fibers.

The paper-making processes serve as yet another broad criterion in describing the nature of papers, as hand- or machine-made. Examples of artists' (cotton) Western, hand-made, and machine-made papers, and one Oriental paper are examined and discussed further in case studies in Chapter 6 to determine what correlations may exist between the papers' structure, fiber type, or surface finish, and biodeterioration. The synergy of all these features affects microbial interactions with paper.

2.2.1. Characterization of Paper

Paper Fiber Structures

The basic unit of paper, a plant fiber, serves as the support onto which fungi are attached in biodeteriorated papers. The fiber structure has an impact on water adsorption, which directly affects biodeterioration; the fiber structure and surface morphology are discussed in more detail to determine what roles they play in biodeterioration processes.

The fiber consists of a multi-layered cell wall and lumen, making the fiber a semi-hollow, semi-tubular structure. The main plant fibers used in paper making differ in their structure depending on the source of the raw material; cotton and linen fibers will distinctly differ in their morphology and chemistry, setting them apart from wood-derived fibers.

Furthermore, the layers in the cell walls are distinguished by differences in the orientation of cellulose microfibrils. Their configuration has an impact on water adsorption, as elaborated in Chapter 2.3.

Cellulose Morphology

In a fiber, cellulose microfibrils are the basic reinforcing elements embedded in a stress–transferring matrix of amorphous polymers (Bristow 1986a). Cellulose exists in different phases, as crystalline and non-crystalline (Kolseth and de Ruvo 1986) (Figure 2.14). These disordered, so-called amorphous regions in cellulose are also more accessible to water than crystalline, consequently making them more prone to microbial colonization. The amorphous regions are also more susceptible to temperature changes, acids and enzymatic hydrolysis and are thus degraded by fungi at a greater rate than crystalline regions. Recent studies showed that cellulose hydrolysis mediated by fungal enzyme cellulases is typically 3–30 times faster for amorphous cellulose as compared to crystalline cellulose (Wertz et al. 2010). It is worth noting that cellulose is a resilient material, remaining stable under a low temperature and low acid concentration; however, the increase in adverse factors, such as a higher temperature or greater acid concentration, will eventually lead to breaking hydrogen bonds in cellulose structure.

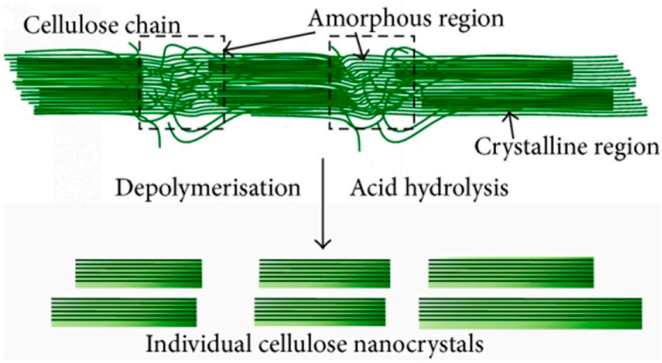


Figure 2.14. A diagrammatic model of amorphous and crystalline regions in cellulose fibril. Parallel lines indicate crystalline structure and 'flexing' lines indicate amorphous regions. Source: Adapted from (Lee et al. 2014) (open access, public domain, Creative Common Attribution License).

The complex interactions of water, cellulose fiber morphology, and fungi indicate that hydrolysis of cellulose induced by fungal enzymes is in direct correlation to cellulose imbibition and its degree of crystallinity. The phenomenon of imbibition by paper fibers is complex in itself and presents an area of extensive studies in the physics of paper (Krassig 1993; Alava and Niskanen 2006), some aspects of which are discussed in the subsequent sections (note: imbibition is an action, or process, of assimilating, taking in solution, or taking in liquid, especially the taking up of fluid by a colloidal system resulting in swelling).

Cellulose Chemistry

Cellulose is one of the most abundant natural polymers on earth; it is an important structural component in the cells of all green plants. As such, the research on cellulose is vast. In this brief overview, certain features are highlighted as the

most relevant to the interactions of cellulose-containing fibers with the biological living systems, such as fungi.

Cellulose is a polysaccharide in which the high content of hydroxyl groups (-OH) provides many possibilities for hydrogen bonding (Figure 2.15) (Klemm et al. 1998; Wertz et al. 2010). Hydrogen bonding, covalent bonding, and van der Waals forces (short-distance, chemical forces), which are considered in discussions of various cellulose models, play a major role in microbial adhesion to fibers' surfaces; some details regarding these are reviewed in Chapter 4.2.

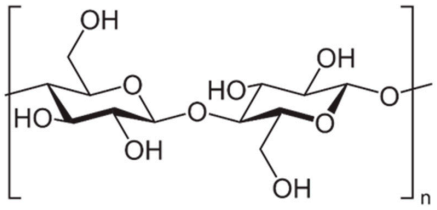


Figure 2.15. Cellulose molecule. Covalently bonded anhydroglucose units make up the cellulose chain. Each unit features three hydroxyl groups. Source: Public Domain, https://commons.wikimedia.org/w/index.php?search=Cellulose.Sessel.svg&title=Special:MediaSearch&go=Go&type=image (accessed on 15 January 2022).

Paper Fiber Content

Cotton and flax plants supply raw materials for producing high-end papers—'high end' because the high percentage of cellulose in paper makes it more durable and resilient to adverse environments than, for example, wood-pulp-based papers with a lower percentage of cellulose and higher content of lignin. Cotton fibers, which are the seed hairs of the plant *Gossypium*, are formed by the elongation of a single cell from the surface of the seed. Under a microscope, a cotton fiber appears as a very fine, regular fiber, resembling a twisted ribbon or a collapsed and twisted tube. These twists are called convolutions and serve as diagnostic features in the microscopic evaluation of paper's content (Figure 2.16). The same features are present in hand-made paper, indicating that the paper fibers underwent gentle mechanical processing, thus preserving the characteristic fiber morphology.

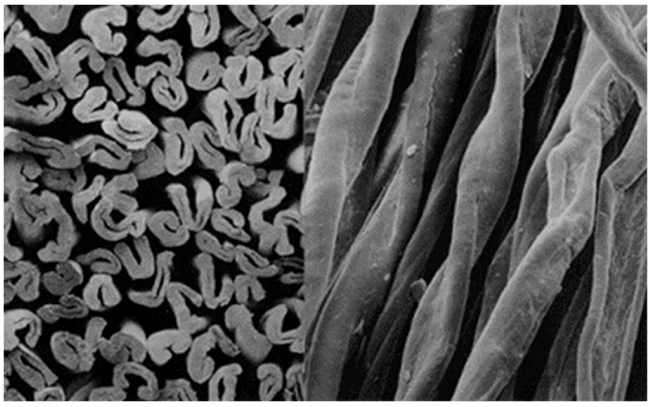


Figure 2.16. Longitudinal study and cross-sections of cotton fibers. The characteristic, ribbon-like twist is one of the identifying features of cotton fibers. The cross-section of cotton fibers shows the water entry areas. SEM micrograph. Source: Reprinted from http://www.freewebs.com/textile-technology/fibrescience.htm (accessed on 13 April 2012).

Cotton that is used for paper making is derived from new textile cuttings, old rags and linters. Linters are the shortest fibers in the harvested lot, generally 12–64 mm long and 20 μm wide (Emerton 1980). The dimensions of paper fibers are important to remember in order to distinguish them from fibrous filaments of fungi that may be present in paper. Chemically, cotton is almost pure cellulose with a small percentage of protein, pectic substances (congealed gum-like carbohydrates), ash and wax. The fibers are weakened and destroyed by acids but are resistant to alkalis (Dochia and Roskwitalski 2012).

Flax is a bast fiber used in textiles and in fine paper (so-called rag paper). The bast fibers are located in the stem of the flax plant (*Linum usitatissimum*). Bast is an old name referring to phloem fibers, which are bundles of fibers under the bark (epidermis and cortex layers) or stem's 'skin'. One of the characteristic features of flex fibers is nodules on the individual fibers (Figure 2.17). Chemically, the flax polymer is the same as the cotton polymer; both are cellulose polymers; however, the degree of polymerization in flax fibers is greater than in cotton fibers (Mahfuzul 2018).

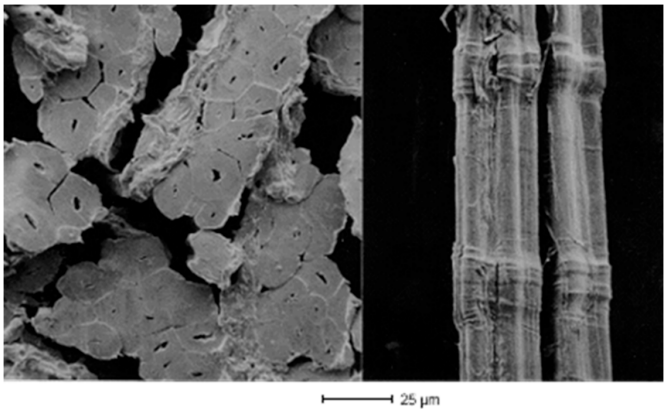


Figure 2.17. Flax (linen) fibers, often used in artist's paper, are seen here in an SEM micrograph. The morphological features of their surface (prominent nodules) and their cross-sections are used as diagnostic features in the comparative analysis of paper fiber content. Source: Reprinted from http://www.freewebs.com/textile-technology/fibrescience.htm (accessed on 13 April 2012).

'Oriental' Paper Fibers

There are three main types of plants listed as sources of fibers used for making Oriental paper: gampi tree, mitsumata shrub, and mulberry (kozo) bush (Sukey 1978; Collings and Milner 1978; Mizumura et al. 2015). All supply bast fibers, and similarly to flax, are extracted from under the 'skin' of the stem in bushes or from under the bark in trees. They differ in fiber length and width, which directly impacts the physical qualities of paper. In general, the Oriental papers encountered in heritage collections are made by hand, preserving the fiber length and structure, thus ensuring the strength of papers. Although the dimensions of fibers vary and broad ranges are listed, they are cited here to serve as a general guide that may assist in differentiating paper fibers from filaments of fungi mycelium. Sukey (1978), Collings and Milner (1978) listed the dimensions of mulberry fibers as, on average, 10 mm long and 30 microns wide, and mitsumata only 3 mm long and 9 microns wide. Both authors reported that other than showing thick walls when examined using transmitted light microscopy, the fibers do not have very distinctive morphological features and are thus not easily identified microscopically. Some features in mulberry fibers may include a transparent membrane in some places along the fiber wall. As for mitsumata, the fibers show a broader portion in the central part of fibers (Ilvessalo-Pfaffli 1995).

2.2.2. Paper Structure

Formation of Paper Sheets: Hand-Made or Machine-Made Papers

The hand-made process of making paper produces superior material characterized by an even distribution of fibers. The manual process ensures that a well-dispersed, highly diluted fiber suspension is drained evenly through a wire sieve (paper-forming mold). Fiber flocks are almost completely avoided; flocculation

is a process in which particles aggregate into small lumps. The measuring of particles of the flock size is important in the proper assessment of paper properties (Norman 1986; Alava and Niskanen 2006).

Although evenness of fiber distribution enhances the quality of paper, such as mechanical properties and suitability for printing in commercial applications, or as material for artistic creations, it does not seem to have an impact on the fungal growth or fungal pigment distribution, based on the experimental work that was carried by the author. This observation is exemplified in case studies where papers with different fiber contents, distributions, processing methods, and ages were affected by fungi, without any distinct correlation to the fiber distribution or processing of paper.

The oldest papers in the group of case studies reported here were a 17th-century Study Sheet and the Oriental paper (1847), both hand-made papers. The 17th-century paper was most likely used as an 'end-paper' in a book, showing an uneven distribution of fibers. The Oriental paper, used for a Japanese woodblock print, featured a fine, even network of fibers. The third paper used as a case study was a Western-type, machine-made paper, with a varied amount of sizing (1920 Engraving). All three papers were randomly stained by black fungal pigmentation. The patterns of staining could be attributed to the type of fungal species and water availability in paper rather than the fiber distribution in the paper sheets.

One common factor observed in the optical microscopy of those three papers was the partial attachment of fungal filaments (tubular and swollen hyphal cells) to the paper fibers and growth towards pores, airy and open voids within the paper matrix. A discussion of the attachment forms follows in the subsequent section.

Thickness and Density of Paper

In classical mechanics, the thickness of a structure is the distance between the two border planes defining that structure. Paper, however, composed of many discrete particles, does not fall into that definition. Instead, the density of the fiber distribution in the matrix (or, in thickness) is used as a criterion (Fellers et al. 1986). Density, a structural characteristic, is one of the primary parameters determining the mechanical properties of paper. A specific density can be achieved in the manufacturing process, using different methods of fiber beating, wet processing or finishing, such as calendaring. Calendaring in paper manufacturing refers to the finishing process aiming to smooth the surface of paper by pressing it between cylinders or rollers. The density, along with the surface finish, appears to have a direct impact on fungal growth, pigment deposition and, consequently, stain formation.

Paper Sizing

Historically, gelatin and starch were used as paper sizing. Gelatin is made of animal skin or bone and is therefore of a proteinaceous base. Starch is a polysaccharide derived from plants. It is estimated that on a commercial level starch sizing was abandoned in ca 14th century in favor of gelatin (Clapp 1972

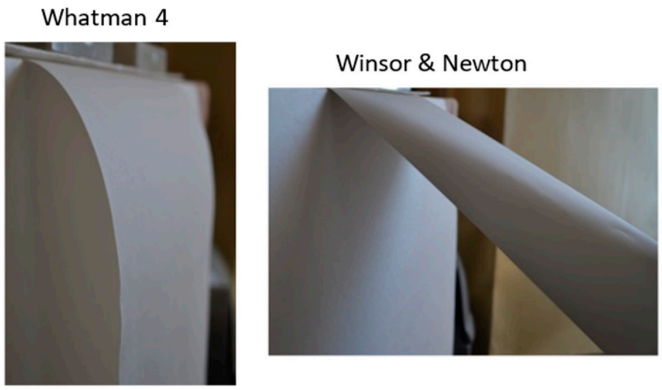
cited in James and Cohn 1997). However, today, both are being used in individual, small-production paper mills (Schwartzberg 1995). Some basic features of sizing are summarized in a tabular format below (Table 1).

Watercolor papers are heavily sized, so additives are applied in the process of paper making to reduce the paper sheet absorbency and make it hydrophobic (that is, water repellant). The addition of sizing permits an artist to modify an image painted in watercolor once applied on the surface, using water, without causing damage to paper fibers. Sizing of paper, or a lack of it, impacts the paper pliability, as can be seen in a simple test (Figure 2.18a). The measurements of paper pliability collected as shown in Figure 2.18b were applied in a bio-simulation experiment, discussed in Chapter 2.4. Internal sizing is added to the paper pulp after rinsing the pulp and before it is cast into the paper mold; external sizing is applied to the paper surface after the paper sheet is formed and has dried. Traditional sizing utilizes starch, gelatin, gum Arabic or rosin (Hunter 1978; Emerton 1980). These substances bond with cellulose via hydrogen bonding and form a film on the surface of fibers, thus modifying the entire paper surface. Because the presence of sizing has an impact on the paper–water relationship, it directly affects the biodeterioration of paper; a closer look at those relationships is elaborated in Chapter 2.3.

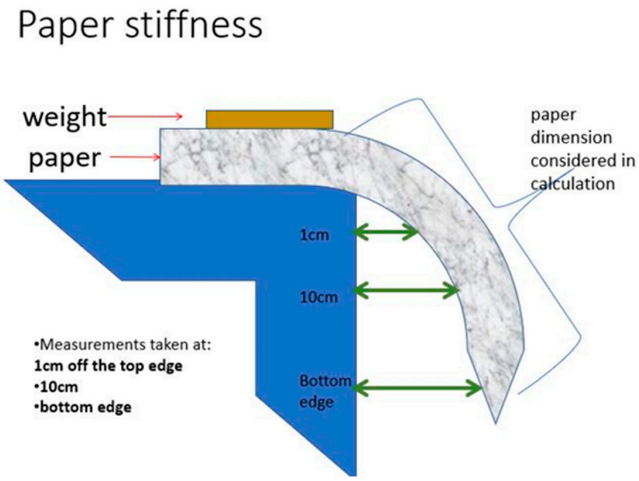
Table 1. Sizing of paper.

	Sizing of paper, as an action (verb), entails the application of a chemical in solution to a paper sheet or paper pulp to modify paper characteristics to meet desired end-product specifications.
Purpose of sizing:	The main objective of sizing paper is to change its water adsorption, aiming to make paper more water repellent to applied ink or printing media. Heavily sized papers are used for watercolors. Unsized papers are blotting papers that absorb liquids and filter papers, for example, Whatman filter papers.
Sizing agents:	Gelatin and starch are historically used sizing agents. Modern sizing agents include alkyl ketene dimer (AKD), alkyl succinic anhydride and acrylic co-polymers.
Types of sizing:	Surface sizing is applied after the paper sheet is formed. Internal sizing is added to wet paper pulp.
Test for presence of sizing	For starch: Iodine-potassium iodide solution in water. Detection limits: starch 0.01 μg. For gelatin:bicinchroinic acid solution and 4% copper (II) sulphate pentahydrate (note, this is only one example; other tests are available and can be carried out, depending on the needs).

Source: Table by author.



(**a**)



(**b**)

Figure 2.18. (**a**) Visualization of how the presence or absence of sizing impacts paper's response to relative humidity (RH) in the air. The Whatman paper, filter paper, used in laboratory analysis does not contain any sizing. It becomes soft and pliable when exposed to high RH. The Winsor & Newton paper used in watercolor painting is heavily sized to make the paper water-resistant, thus permitting watercolor artists to make changes without disturbing the paper surface. It remains stiff when exposed to high RH. (**b**) A simplified method of evaluating the paper stiffness of two model papers used in the Light Experiment, Whatman 4 (unsized) and Winsor & Newton (sized). Source: Figure by author.

2.2.3. Paper Surface

Forces of suction and compression that are applied during the paper-making process conform the surface fibers to the topography of support on which paper is formed. It could be a felt fabric or metal wire, depending on the process used during the sheet formation. The surface is irregular, and these irregularities and depressions may be seen as an extension of the pore system in the matrix of fibers. The surface roughness has a direct impact on fungi growth, as illustrated in Figures 2.20 and 2.21.

The surface topography, or roughness, is defined in terms of the reference plane brought into contact with paper under a defined pressure, as illustrated in Figure 2.19.

The surface topography can be measured with many different techniques depending on the purpose of a measurement. Earlier methods such as stylus profilers that traversed the surface have been replaced by non-contact, optical instruments that reflect light at different angles from the surface. A comprehensive review of surface analysis and measurement techniques that are currently used commercially are discussed by Preston in her monography, The Surface Analysis of Paper (Preston 2009).

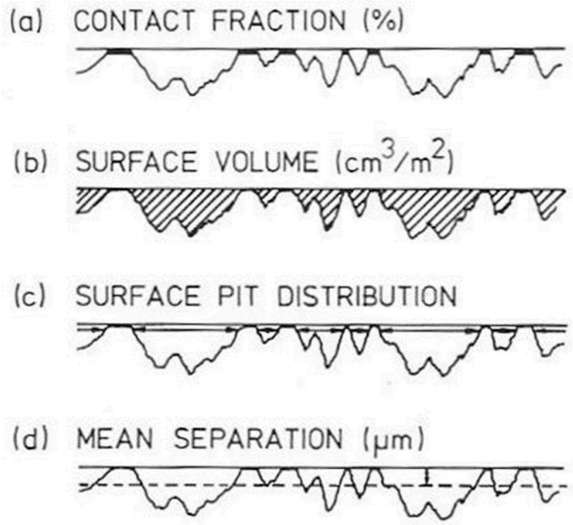


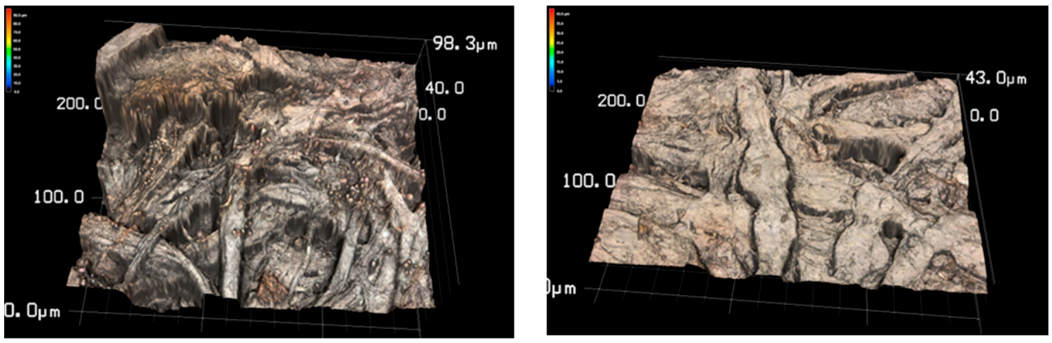
Figure 2.19. The diagram shows various ways in which the surface topography of paper can be defined and measured. Source: Reprinted from (Bristow 1986a, p. 172), used with permission.

Other methods, in addition to profilometers, characterize papers' roughness in terms of the volume of voids between the surface and the reference plane. In that approach, however, it is difficult to distinguish unequivocally between roughness and porosity since the surface pits are often a continuum of the pore network.

In industry, paper roughness is commonly determined by measuring the airflow between the paper surface and a reference plane under specific controlled conditions. The values are expressed in air resistance per time unit as min/ml, or as airflow units. There are several instruments for measuring air flow; in general, their principle relies on measuring the air forced between the surface of the paper and the measuring head to determine how quickly the air can pass through that junction (Preston 2009; Parker 1965, 1971; cited in Bristow 1986b).

(**a**) (**b**)

Figure 2.20. (**a**) Rough surface of paper (referred to as recto, face) with scattered fungal deposits from 17th century Study Sheet. (**b**) Verso of the same paper, smoother than recto, with a minimal number of fungal deposits. Macro-images 1:1. Source: Figure by author.



(**a**) (**b**)

Figure 2.21. (**a**) Three-dimensional topography captured with confocal laser scanning microscope shows rough surface with well-pronounced fibers, and numerous fungal cells. (**b**) Confocal laser scanning micrograph, 3D topography of the reverse side of the same paper, smoother than recto, seen in Figure 2.21, on which no fungal cells were detected. Source: Figure by author.

Another aspect of the paper's structure that has an impact on fungal growth and pigment deposition is the 'openness' of the fiber matrix, which enables fungi to access airy spaces necessary for their development. In the microscopic observations of the biodeteriorated papers, fungi showed positive chemotropism, which is a directional expansion towards chemical stimuli, in this case, oxygen in open pores, [6] once they established firm attachment to the substrate (Figure 2.22). This pattern of growth can be explained by the fact that fungi are aerobic and require oxygen for their development.

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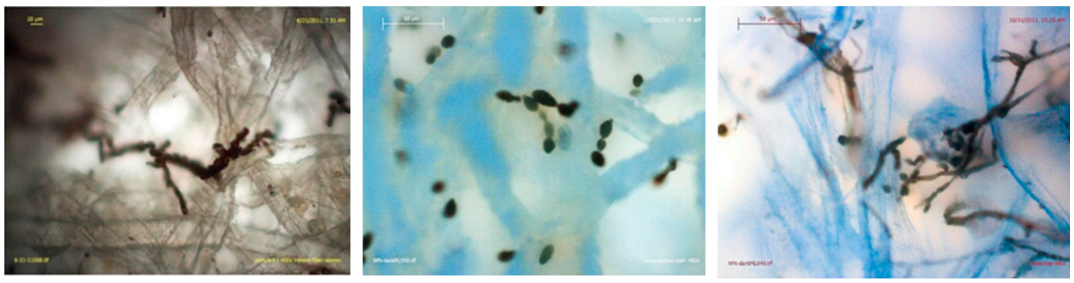
[6] Tropism (frequently used as a suffix); a turning or growth in response to an environmental stimulus. Positive tropism indicates response towards the source, negative tropism, away from the source of the stimulus (Ainsworth and Bisby 2008).



(**a**) (**b**) (**c**)

Figure 2.22. (**a**) A micrograph in transmitted light microscopy shows fungi chemotropism that was observed in fungal stains on the 17th century Study Sheet, scale 10 μm. (**b**,**c**) micrographs exemplify fungal chemotropism observed in fungi cultured in bio-simulation experiment, cultured on Whatman paper, grown in the dark. In both cases, the directionality of growth is towards air pockets in the paper matrix, referring to positive chemotropism. Scale: 50 μm. Source: Figure by author.

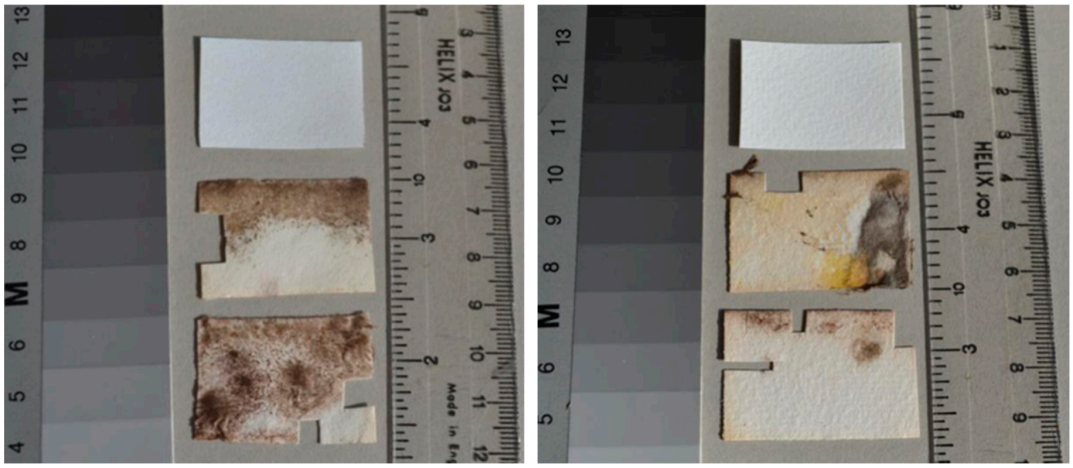
Paper is a network of fibers embracing a network of pores, qualifying paper as an aerogel (Bristow 1986b) or a two-system structure of solid and gas. The word 'pore' is used here to denote the macropores of interfiber spaces. The experiments carried out by other researchers showed that virtually all pores are accessible to liquid when exposed to one. This observation places the role of pores as an important feature in the process of fungi infestation as they have a direct impact on the sorption of liquids by paper. In most cases, during the paper-making process, the lumen of the fibers collapses during the manipulation of fibers (see lumen in cotton and flax Figures 2.16 and 2.17). However, if the fibers are not collapsed, the lumen is accessible through the pits of the cell wall, allowing water to penetrate into their inner structure. This may indicate that fungi search for air not only in pores but also in the lumen, where water could be trapped, thus promoting fungi growth.

Paper is a two-phase system (gas and solid) that is potentially absorbent; therefore, the sorptivity of paper is not only associated with the porosity of the material. In other words, not only pores between the fibers but also the fibers themselves play a part in the swelling of paper (Westman and Lindström 1981; Bristow 1986b; Alava and Niskanen 2006). These two types of sorption are at times referred to as interfiber and intrafiber penetrations.

Based on the experimental work of the researchers of paper, the dependence of swelling (volume increase) on water sorption into fibers and pores was confirmed and plotted (Bristow 1986b). A complex relationship between paper fibers and water in a paper system is examined in detail by Banik and Brückle (2011) in their book 'Paper and Water, A Guide for Conservators'.

The water-transporting properties of paper are one of the relatively little-explored areas of paper's physics (Alava and Niskanen 2006). Based on the author's observations, the path of paper absorbency appears to be in direct correlation with fungal growth. The observations were based on the experiment in which two papers characterized by different sorptions due to the presence of, or lack of, sizing

were inoculated with the same species (*Aspergillus niger*) and subjected to the same environmental conditions. The inoculant grew rapidly on paper with no sizing and showed very limited growth on paper that was heavily sized. Both selected papers were made from high-quality, cotton-based fibers. One of them, Whatman 4, is manufactured for laboratory use; as such, it is unsized to prevent any impurities that could potentially affect analytical results. The other, watercolor paper, Winsor & Newton, is heavily sized to prepare its surface for frequent changes in design that watercolor artists could make, using water and mechanical rubbing. The differences in fungi developments are illustrated in Figure 2.23.



(**a**) (**b**)

Figure 2.23. (**a**) Whatman 4, hydrophilic paper used in bio-simulation. The top sample is a reference; the one below shows fungal pigmentation produced by *Aspergillus niger* cultured in the dark. The one at the bottom shows pigmentation induced when culture was exposed to daylight. The intensity and abundance of dark pigmentation are prominent on this highly absorbent paper. (**b**) Winsor & Newton hydrophobic paper used in bio-simulation. The top sample is a reference; the one below shows fungal pigmentation produced by *Aspergillus niger* cultured in the dark. The sample at the bottom shows pigmentation induced when culture was exposed to daylight (note: in the second sample, the dark stain along the short edge and yellow discoloration were caused by microorganisms other than the inoculant). Source: Figure by author.

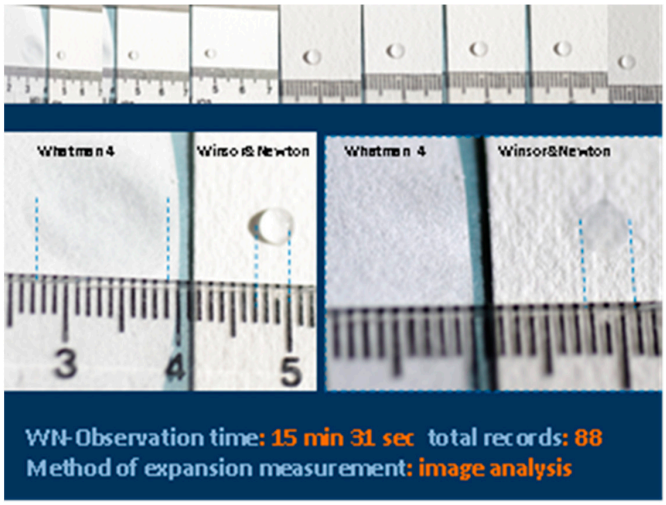


Figure 2.24. An illustration of water absorption timing on two papers, Whatman 4 and W&N used in Bio-simulation. Water was absorbed immediately on W4, while the time of absorption by W&N took 15 min and 31 sec (shown on right). The ability to absorb water has a direct impact on fungi growth. Source: Figure by author.

One of the plausible explanations for the difference in the absorption timeframe could be that water cannot easily enter the pore system of a sized paper and the only sorption that occurs is the one associated with diffusion into and swelling of the fiber system. It can be hypothesized that, in this case, the intrafiber diffusion led to swelling, which reduced the size of pores, and because of that, in the sized paper, water was unable to enter the pore space. Sorption, therefore, took place not by capillary action but by a diffusion process in a swollen paper. Sizing effectively prevented the entry of water into the pores and the uptake of water, in which case the update can be attributed solely to fiber sorption.

The above conclusions were derived using two papers as examples: one heavily sized (W&N) the other (W4) without any sizing. It is important to note that both papers represented two extremes of paper modification resulting from sizing. There are many types of papers that are sized lightly, or medium-sized, suggesting that fungal growth will vary when we consider a direct relationship between the paper sorption and sizing.

Paper Stiffness

Another way of evaluating paper characteristics is stiffness, which is defined as the bending moment per unit width of the specimen and per unit curvature of the specimen at the torque axis (Carson and Worthington 1952). Many sophisticated and complex tests such as ultrasonic measurements (Carson and Popil 2008) have been performed in the paper industry to determine the paper stiffness because it provides information about paper characteristics affecting its behavior in various applications. For example, stiff paper may indicate additives while pliable paper may be completely free of additives. The presence or absence of additives will in turn determine how ink will be accepted by paper or how paper will respond to higher

humidity in the environment, among many other industrially important concerns. The methods and instruments used in measuring stiffness are not uniform and, according to some reports, not completely standardized (L&W n.d.). A simplified fold test was performed to gain insight into the characteristics of the studied papers, by exposing them to elevated RH; the results are illustrated in Figure 2.24.

Assuming that the stiffness of paper indicates additives, more specifically sizing, one may conclude that such papers, with a greater amount of sizing, should support fungal growth. This line of thinking, that the presence of sizing, which would provide an additional nutritional supplement for fungi in case paper becomes wet, has prevailed in many biographical sources. However, this has not been confirmed by the experimental work in this study, especially evident in bio-simulation 2.4. On the contrary, the more absorbent paper with no sizing or additives supported fungal growth to a much greater degree than heavily sized paper, as evident in Figure 2.23; a detailed discussion of this observation follows in the Chapter 2.4.

2.2.4. Summary Points

1 Paper characteristics such as surface topography (roughness) and porosity appear to have a direct impact on fungal attachments to surfaces and how they grow and deposit pigmented cells.
2 A rough, fibrous surface of paper provides a more conducive environment for fungi attachment and growth, most likely resulting from self-preservation measures adapted by fungi towards the protection of spores against their removal by external forces, in a 'waiting' period before germination.
3 The roughness of paper is associated with a greater attraction of water, which is an important factor that promotes fungal growth.
4 Paper with open pores and a lesser amount of sizing provides an inducive environment for fungi growth. This may be explained by water sorption, on the one hand, and on the other hand, by the fungal physiology such as the need for oxygen during fungi development.

2.3. The Environment and Its Impact on Fungi Growth and Pigmentation

2.3.1. The Environment, an Overview

Relative humidity, light, temperature, and time, the main elements defining the environment, work in synergy in the course of the biodeterioration of cultural material (Figure 2.25). Each of these factors affects various stages of fungal development, leaving a different imprint on cultural objects. The environment and a porous, rough paper surface provide a natural habitat for opportunistic fungi.

Abdel-Maksoud (2011) appears to be one of very few authors who discusses the impact of the paper surface morphology in the context of biodeterioration. The extent to which fungi interact with the paper substrate largely depends on the environmental conditions. The alteration of one environmental factor triggers different fungi responses within the same species, expressed in varied growth

patterns, colonization, or pigmentation. This was evident in different responses of *Aspergillus niger* to the presence or absence of light when cultured in daylight and in dark conditions, as demonstrated in the bio-simulation experiment. The presence or absence of light had a particularly significant impact not only on the pigmentation but also on the secretion of enzymes to the paper substrate, and consequently a degree of degradation of the paper matrix, as discussed in more detail in Chapter 2.4.

In addition to the physical and chemical characteristics of the substrate, its acidity level and nutritional content, environmental factors contribute to the fungal growth, pigmentation, and density of colonies. The presence of sizing, as pointed out earlier, has been considered by some authors as supporting and increasing fungi growth (Ellis 1981; Florian 2002), which is disputed here.

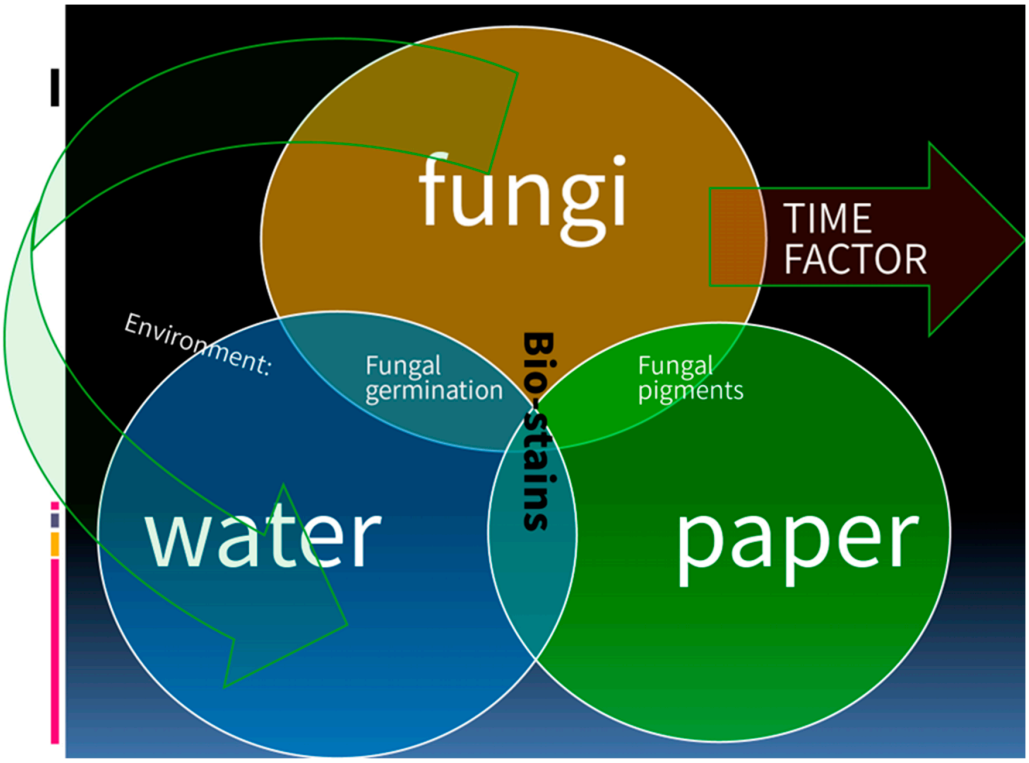


Figure 2.25. The interrelations of environmental factors, water, paper characteristics, and time of the fungal growth, as contributors to the formation of bio-stains Source: © Szczepanowska 2011.

The bio-simulation experiment that aimed to explore that relationship and the impact of sizing on biodeterioration was carried out in a monitored environment on two papers, sized (Winsor & Newton) and not sized (Whatman4). An additional factor considered during this experiment was light and its impact on both fungi growth and pigmentation. The black fungal stains were induced by *Aspergillus niger* cultured on those two papers placed on agar-agar, which served mainly to retain moisture on Petri dishes. This experiment revealed that neither growth nor pigmentations were promoted by sizing in paper samples; the only growth-promoting factor was the ability of the substrate (paper) to absorb water.

The observation that sizing does not promote fungi growth was confirmed by the analysis of fungal stains on historic papers, which occurred in natural conditions on the studied artworks on paper (Chapter 6). Furthermore, fungal stains on historic paper studied in detail in this monograph clearly showed that fungi were more prominent on the paper with a lesser amount of sizing and greater surface roughness. In the case of the experimentally induced stains, fungi grew more rapidly and produced more extensive pigmentation on paper that was not sized. The conditions and parameters measured in this experiment are reported in Chapter 2.4.

Many hypotheses have been proposed by researchers regarding the role that each of the environmental elements plays in the life cycle of fungi, yet many aspects still remain unclear. The difficulty is partially explained by different responses among species to the same environmental factors. Recent advancements in molecular technologies permitting in situ studies of microbes in their natural environment revealed that more knowledge is to be gained about microbial diversity and their responses to the environment. A brief review of the relative humidity, light, and temperature in the subsequent sections emphasizes the role each factor plays in fungal staining of the paper substrate.

Light was explored further in the bio-simulation experiment of stain production because it is known to have a significant impact on each phase of fungi life cycles and the production of secondary metabolites, which are often pigmented. The impact of light on pigmentation can be observed once the spores begin germination and hyphae begins to expand and form mycelium. The presence of liquid water is essential for the initial stage of a new life cycle. Water, water activity, relative humidity, and dew point are interdependent and will be reviewed first to provide a background and to understand how they interrelate to light and fungal pigmentation.

Fungi not only respond to the external environment and its changes but also modify the most immediate environment in areas of their growth to create conditions more favorable for their development. This may explain the finding of evidence of biogenic re-mineralization of calcium carbonate filler in paper (W&N) on which *Aspergillus niger* was cultured. This biogenic re-mineralization was observed on samples cultured in both daylight and in the dark, but to a different degree in different light conditions, as elaborated in Chapter 2.4.

The environment and characteristics of the substrate work in tandem, either promoting or prohibiting fungal growth and development; therefore, they must be discussed as two interdependent systems.

2.3.2. Relative Humidity, Water, and Dew Point

The concepts of the relative humidity and dew point are extensively researched in many branches of industry concerned with the effect of moisture on materials and human health. These environmental factors have been also discussed by several authors who explore optimal conditions for cultural heritage objects. Therefore,

only some aspects of these concepts are included here, based on how they relate to fungi-induced biodeterioration and bio-stain formation.

Relative humidity

Relative humidity is defined as the amount of water vapor in the air at any given time, usually less than that required to saturate the air. The relative humidity is the percentage of saturation humidity, generally calculated in relation to the saturated vapor density.[7]

Another common definition of relative humidity is the amount of moisture in the air compared to the maximum amount that the air can 'hold' at that temperature in the same 3D space. When the air cannot 'hold' all the moisture, it condenses as dew. The relative humidity is expressed as a percentage (RH%). The relationship between temperatures and relative humidity is illustrated in Figure 2.26.

Molecular water

Molecular water is referred to as a monolayer of water that condenses on a surface when material reaches dew point temperature. This occurs during rapid changes in temperature in the environment and when a particular material's moisture equilibrium is not reached.

Dew Point

Dew Point is a function of relative humidity and temperature. If the air is gradually cooled while maintaining the moisture content, the relative humidity will rise until it reaches 100%. This temperature, at which the moisture content will saturate the air, is called the dew point. If the air is cooled further, some of the moisture will condense as liquid water.[8] In other words, the dew point is defined by the temperature at which air must be cooled for water vapor to condense into liquid water. The condensed water is called dew when it forms on a solid surface. The dew-point temperature also depends on the moisture equilibrium in each material.

Dew formation may explain why microbial life is active in dry and hot desert conditions, such as in the tomb of Tutankhamen in the Egyptian desert. It can be speculated that rapid changes in temperature in diurnal cycles result in dew formation on the walls inside the tomb, sufficient to support the growth of fungi (Szczepanowska and Cavaliere 2003).

Another observation during the bio-simulation experiment was water condensation inside the Petri dishes that were exposed to daylight (as illustrated in Figure 2.27a), in contrast to a lack of condensation in plates cultured in the

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7 http://hyperphysics.phy-astr.gsu.edu/hbase/kinetic/relhum.html (accessed on 5 February 2013).
8 http://hyperphysics.phy-astr.gsu.edu/hbase/kinetic/relhum.html (accessed on 5 February 2019).

dark (Figure 2.27b). The water condensed in Petri dishes exposed to light due to fluctuations in temperature increased by the energy of daylight and cooling at night. The temperature inside the Petri dish when compared with the external environment adjusted at a slower rate to the diurnal changes, remaining warm when the night temperature cooled, resulting in condensation, and vice versa; once the external temperature increased during the day, the internal temperature in Petri dish was lower.

The dependence of temperature changes and water condensation as shown in the chart in Figure 2.26 is based on the fact that one can see a change from 30 °C during the day to 25 °C during the night, a change that was sufficient to result in water condensation. In the bio-simulation experiment, the values of light energy fluctuated between 0 Lux at night to over 9000 Lux during the day. The condensation was one of the contributing factors to the abundant fungal growth in aerial mycelium and expansion of colonies cultured in daylight.

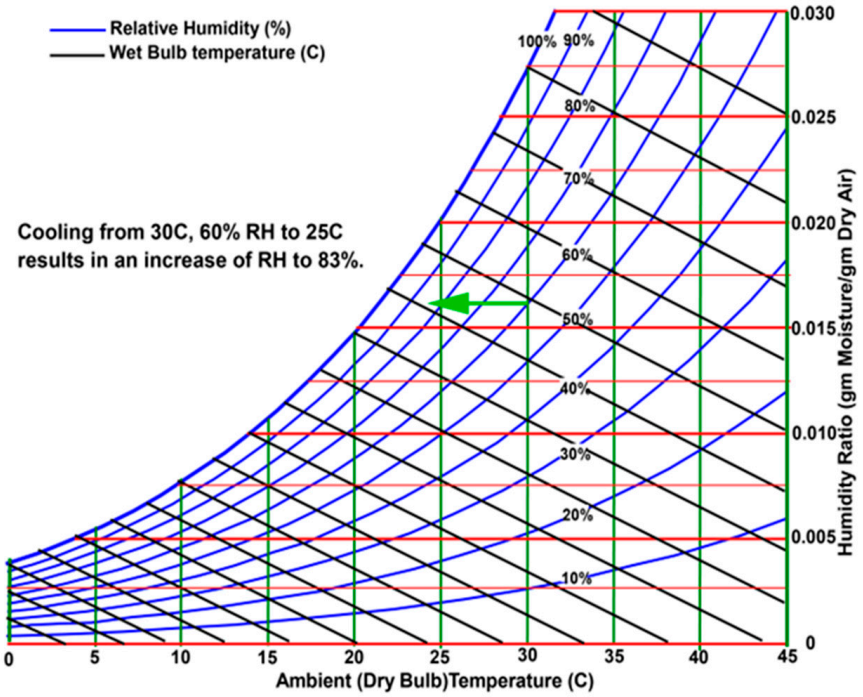
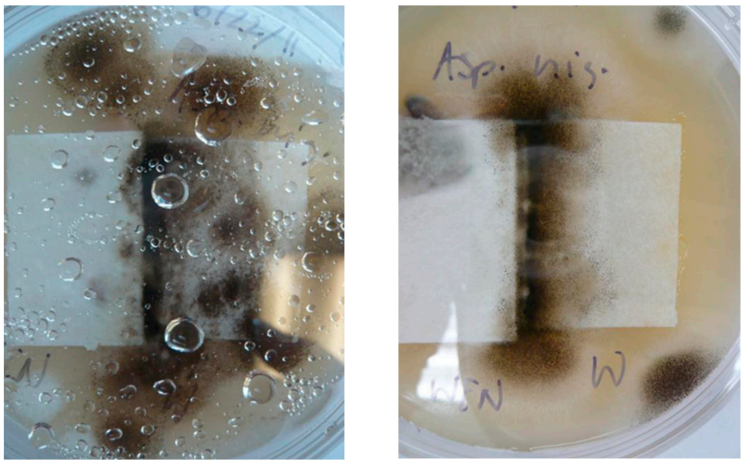


Figure 2.26. Calculation of the dew point in a given environment. Source: Courtesy of Dr. M. Mecklenburg 2019, used with permission).



(a) (b)

Figure 2.27. (**a**) Condensation of water inside a Petri dish during cultivation in daylight conditions; *Aspergillus niger*, 6-day cultivation. Conditions: visible light 0–9400 Lux, UV light 0–500 mW/M^2, RH in room 30–42% (100% inside the Petri dish), T in the room: 25–30 °C. (**b**) The *Aspergillus niger* cultured for 6 days in dark conditions. Water did not condense inside the Petri dish. This, combined with a lack of light, resulted in less-abundant growth and pigmentation. Conditions: light 0, UV light 0; RH in the room: 48–52%; T: 21–22 °C, nearly constant. Both images were taken with a Leica D-lux optical camera, scale 1:1. Source: Figure by author.

Based on the characteristics of the environmental conditions in both settings—daylight and darkness, and water condensation, or a lack of it—one may conclude that the pigmentation and growth pattern are governed by both light and water content, in addition to the absorption capacity of paper on which the fungi species were cultured.

The dew point is one of the concepts associated with fungal growth; water activity is another. Often, the elevated humidity is equated with the initiation of fungal infestation; however, that statement is true only as long as the elevated humidity leads to the formation of liquid water on surfaces; fungi will grow on surfaces that are wet, even if for a short period of time. As noted for the first time by Scott (Scott 1953), it is water activity, not water content, that is correlated with fungal growth. The concept of water activity (a_w) was developed to account for the intensity with which water associates with various non-aqueous constituents and solids. It is a measure of the energy status of water in a system, defined as the vapor pressure of a liquid divided by that of pure water at the same temperature; therefore, pure distilled water has a water activity of exactly one. Higher a_w values indicate more complex systems and tend to support more microorganisms. Bacteria usually require at least a $_w$ 0.91, and fungi at least a_w 0.7. Transversely, since then, it has been established that the growth of fungi and bacteria are inhibited at specific water activity values, below a_w 0.9 and a_w 0.7 for bacteria and fungi, respectively.

Water activity of 0.60 is considered the lowest limit for microbial growth. Below a_w 0.55 it was reported that all metabolic activity ceases and DNA is denatured.[9]

<0.9	most bacterial activity inhibited
<0.8	most yeast activity inhibited
<0.7	most fungal activity inhibited
<0.6	all microbial activity inhibited

Other studies indicated that for a given a_w and specific organism, large differences in the survival rate are a function of the nutritive solutes used to culture the microorganisms, thus suggesting that other components play a role in their survival, in addition to a_w (Mugnier and Jung 1985). These other factors include the temperature and acidity level (pH level) of the nutrient.

Paper, when viewed as a substrate for fungal growth, provides scarce nutrients and a minimal amount of water. Therefore, fungi growing on paper are known to be xerophilic, meaning 'dry loving'. Sporulation is a key event in the life of fungi, and how fungi solve the problem of sporulation in water-limited conditions affects fungal dispersion. In a simplistic explanation of this complex process, dormant conidia are formed containing all necessary mechanisms to begin a life cycle when conditions are conducive to this. The dormant conidia contain a complete mitochondrial respiratory system and electron-transport system that become active immediately upon spore activation (Brambl 2009; Borkovich and Ebbole 2010). Enzymatic subunit precursors synthesized and stored in the cytoplasm during conidia dormancy are imported into mitochondria upon the initiation of germination (Brambl 1981, 1985, 2004, 2009) and activated once water becomes available.

2.3.3. Temperature

Similarly to other environmental factors, temperature affects microbial life and cannot be considered alone without a synergistic effect of relative humidity and light. Decades ago, temperature was found to play a major role in spore germination, as reported by Schenck et al. (1975). Unsurprisingly, temperature has been utilized as the primary means of sterilization; high temperature leads to the deactivation of vital functions in spores. According to Burge (2006), types of microorganisms are classified according to their preference for a specific temperature as:

- Psychrophiles or cryophiles: with optima less than 10 °C reaching as low as −20 °C
- Mesophiles: with optima of room temperature range (18–22 °C)
- Thermophiles: with optima at or above 37 °C

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[9] www.ecometrex.com/moisture.html (accessed on 1 May 2020).

The optimal temperature range for the mesophilic fungi that grow on paper is considered to be average room temperature, 18–22 °C. The temperature above the tolerance level for specific species is damaging to their cells and may deactivate spore production and/or germination. For example, a one-hour exposure to 170 °C and three-hour exposure to 140 °C will kill many spores, while 4–6 h in 71 °C is suggested for the treatment of fungi in an infested house as an effective measure to deactivate the mesophilic fungi (Burge 2006). The effects of heat on fungi are related to the chemical reactions within their cells. For optimal growth, temperatures must be in a range appropriate for given species promoting the most efficient progression of the chemical reactions necessary for growth. As temperatures rise above the optimum level, the chemical reactions are less efficient, thus slowing the growth of microorganisms. Eventually, the temperature can reach a point wherein growth stops, and cell components begin to be damaged by heat. Proteinaceous contents of fungal cells denature when heated to the limit of their tolerance (Burge 2006).

The temperature has a particularly acute impact on spore germination and hyphae morphogenesis in filamentous fungi. In some cases, however, researchers observed that elevated temperatures outside the optimal ranges may activate germination. In other cases, exposure to colder temperatures may be required to achieve the activation of germination. *Cheatomium globosum*, one of the most common fungi found growing on paper, falls into the first group, where one hour of exposure to 37 °C was found to promote spore activation (Chapman and Fergus 1973). Another fungus producing dark-pigmented spores and commonly encountered on items of cultural heritage belongs to *Mucor* sp., *M. miehi*; it also showed the activation of germination at 45 °C with a 5 h exposure (Sussman 1976).

The effects of changing temperature were noticed with respect to the physiological developments of meristematic fungi (Chapter 2.1.5). In many cases of these dimorphic (yeast-filamentous) fungi, Anderson and Smith (1972) and Bartnicki-Garcia (1968) reported that the budding of yeast-like cells was promoted at elevated temperatures, whereas the filamentous phase performed better in lower temperatures. When cross-referencing that knowledge with the cell formation in paper, one may predict the temperature parameters in which the fungal infestation occurred. It is important to stress again that temperature is not alone in altering the physiological developments of fungi; it acts in concert with other environmental factors, one of which is light.

2.3.4. Light

Light is one of the most important environmental factors influencing the growth patterns of almost all organisms on Earth, including fungi. The molecular machineries translating light electromagnetic energy (photons) into the chemical language of cells transmit vital signals necessary for the adaptation of living organisms to their habitat. Light was found to have both stimulatory and inhibitory effects on fungi at all stages of their growth, development, reproduction, and

behavior (Tan 1976; Mehra et al. 2009). Fungi reactions to illumination are species-specific (Corrochano and Avalos 2010; Vitalini et al. 2010; Corrochano 2011) and wavelength-specific (Tan 1976). It has been reported over the years in number of studies concerned with the phenomena of responses to light that although light stimulated the production of some metabolites, such as in *Fusarium* (Avalos and Estrada 2010; Asthana and Tuveson 1992), it did not affect growth or sporulation in other species, such as *Myrothecium verrucaria* and *Pestalotia gracilis* (Yusef and Allam 1967). Furthermore, light was reported as prohibiting the growth of the pathogenic fungi *Botrytis cinerea* and *Trichoderma harzianum* (Paul et al. 2005), yet in another species propagated their growth and development. Among variations in fungi responses to light exposure are the volume and mode of bio-pigment production. The multi-faceted nature of light effects on the production of pigments was demonstrated in the bio-simulation experiment when *Aspergillus niger* was cultivated in daylight and in darkness; the details of this experiment are reported in the subsequent section.

The responses of fungi to light are initiated within minutes of exposure. The experimental work reported by Schwerdtfeger and Linden (2000), as referenced by Rodriguez-Romero et al. (2010), indicated that the intensity of response to light diminishes within 30–240 minutes from the initial exposure. During that short period of time, fungi alter their behavior to cope with the (often) harmful effects of light and prepare the organism for reproduction. The advancement in molecular biology pointed out that the presence of a number of different photoreceptors in each fungus suggests complex and diverse regulatory, light-dependent systems (Rodriguez-Romero et al. 2010; Tisch and Schmoll 2010; Schmoll 2011). However, despite the fact that large numbers of processes in fungi are light-dependent, the knowledge of the exact mechanisms of light perception and signal transduction in fungi is still limited.

Regarding the presence of photoreceptors, as mentioned earlier, many species of fungi utilize a number of different wavelength-specific photoreceptors during their development and growth. The core of all receptor types is a chromophore, a low-molecular-weight protein. Most phytochrome responses in fungi result in different gene expressions, explaining the mechanism of transferring the light signal into the nucleus where gene expression takes place. The microbial response to blue light (455–492 nm wavelength) is one of the best-studied among fungi's response to light; all other light-sensing mechanisms, which involve different wavelengths, are less well-studied or largely unknown. What has been discovered is that fungi appear to employ both plant- and animal-type photosensors (Tan 1976; Rodriguez-Romero et al. 2010). An intense study of fungi's response to light carried out in the 1960s to the late 70s was reported by Tan (1976), where the effects of each wavelength, UV, NUV, blue, and red/far-red have been associated with conidiation, sporangium initiation and ascospore formation.

The response to light differs according to the night–day cycle following the fungi's circadian clock and is temperature regulated, as pointed out earlier (Dunlap

and Loros 2006; Brunner et al. 2008). Furthermore, fungi sense not only light intensity but also directionality, resulting in a phototropic response. Filamentous fungi that are encountered on paper-based cultural heritage material may use light signals as an initiator for the emergence of hyphae from the substrate into the air, expanding onto the substrate's surface.

The presence of light has been reported in some cases by other researchers of biodegradation of cultural heritage as halting sporulation, which cannot be confirmed by the author's bio-simulation experiment and production of bio-stains on paper. However, what was observed in the course of this experiment, and what has been confirmed by other researchers, is that the same species react differently to light exposure when cultured with various nutrients, even with identical environmental conditions. It has also been reported that different genetic strains of the same species react differently to light. Considering the scarcity of publications reporting the effect of light on pigmentation in the context of cultural material, the bio-simulation experiment reported here is intended to expand the knowledge of this complex subject.

2.4. Bio-Simulation, Effect of Light on Fungi Growth, Pigmentation and Paper Alteration (Light Experiment)

2.4.1. Selection of Paper and Inoculant

The experiment was designed to demonstrate a combined effect of light and paper characteristics (presence of sizing and lack of it) on the pigment production and resulting modification of the paper surface and structure. *Aspergillus niger* was selected as an inoculant. The selection of this microorganism was dictated by the extensive knowledge about this species due to its commercial importance, as reviewed in Chapter 2.1.4. Another selective factor was its known ability to produce black pigmentation, which is a hallmark of this fungus, as indicated by its name.

The substrates on which *A. niger* grew were two papers, both made of good-quality, predominantly cotton fibers: Whatman4, laboratory filter paper; and Winsor & Newton, a watercolor paper. Both papers were also well-researched from a commercial standpoint, thus eliminating unwanted variables that could potentially derive from the manufacturing processes. To force the fungus into utilizing paper as its primary source of nutrient, only a simple agar-agar medium was used to retain the required level of moisture supporting the microorganism's growth.

2.4.2. Experiment Setup and Environmental Conditions

Two sets of sterilized papers were placed in two Petri dishes with agar-agar medium, inoculated with *Asperguillus niger* and monitored on a daily basis during a 13-day period. One type of paper, Whatman 4 (W4), is a laboratory-grade filter paper made from cotton fibers. The other paper, Winsor & Newton (WN), is a watercolor paper, heavily sized to permit artists to make changes in the course of creating

a watercolor painting, using water and at times mechanically rubbing the paper surface. The papers were sterilized prior to placing them on sterile agar-agar in Petri dishes, before inoculation. The setup of the experiment is shown in Figure 2.28 and the placement of paper samples is shown in Figure 2.29. The pure specimen of *Aspergillus niger,* obtained from a reputable supplier of pure cultures, was inoculated along the long edge of each paper, in the center of the Petri dish.

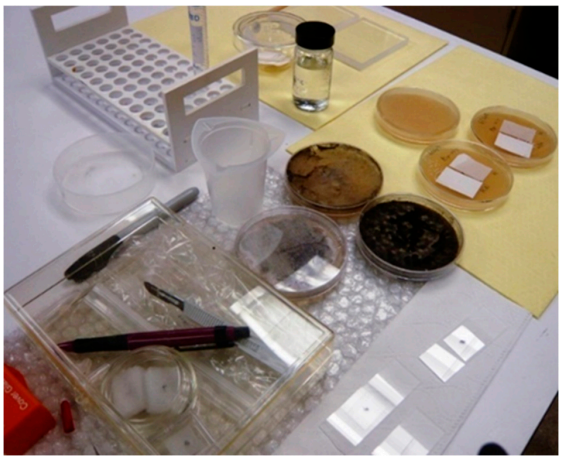


Figure 2.28. The setup of the bio-simulation experiment: Petri dishes, inoculating needle, pure inoculant *Aspergillus niger*, and sterilized model papers (Whatman 4 and Winsor & Newton). Source: Figure by author.

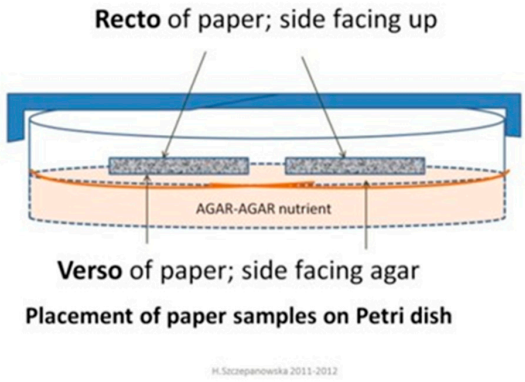


Figure 2.29. A diagrammatic illustration of paper sample placement in Petri dishes. Each set contained one sample of W4 and W&N papers. The samples were inoculated along long edges in the center of the plate. Source: Figure by author.

Each set of the inoculated sterile papers and agar-agar medium was cultured in two different light conditions; one set was exposed to daily cycling of light, on a window seal, and the other was cultured in a darkroom. The fungus development, colony growth, and pigment secretion were recorded digitally, on a daily basis, and the results are illustrated in Figures 2.30 and 2.31. The environmental conditions were monitored with two units, one in each location, collecting data for the relative humidity (RH), temperature (T) and light (L) over a 13-day period. All these parameters, in addition to colony expansion, were recorded on a daily basis.

In the first period of five days, the light induced rapid and greater pigment secretion into the substrate on the third day when compared with colonies grown in darkness during the same period. The intense expansion of colonies cultured in light was accompanied by the vigorous growth of the aerial mycelium and pigmentation. The colonies grown in darkness showed a modest amount of pigmentation until the fifth day, and the colony growth did not expand beyond the area of inoculation, along the edge.

After six days, colonies exposed to light intensified in pigment production, reaching a plateau around the seventh day, but the expansion of colonies was not observed. During the same period of time, the plates cultured in darkness showed steady expansion of both colonies and intensity of pigmentation. Colonies were much denser and pigmentation was more compact than in colonies cultured in light. This greater density was also observed upon the termination of the experiment, as shown in Figure 2.32.

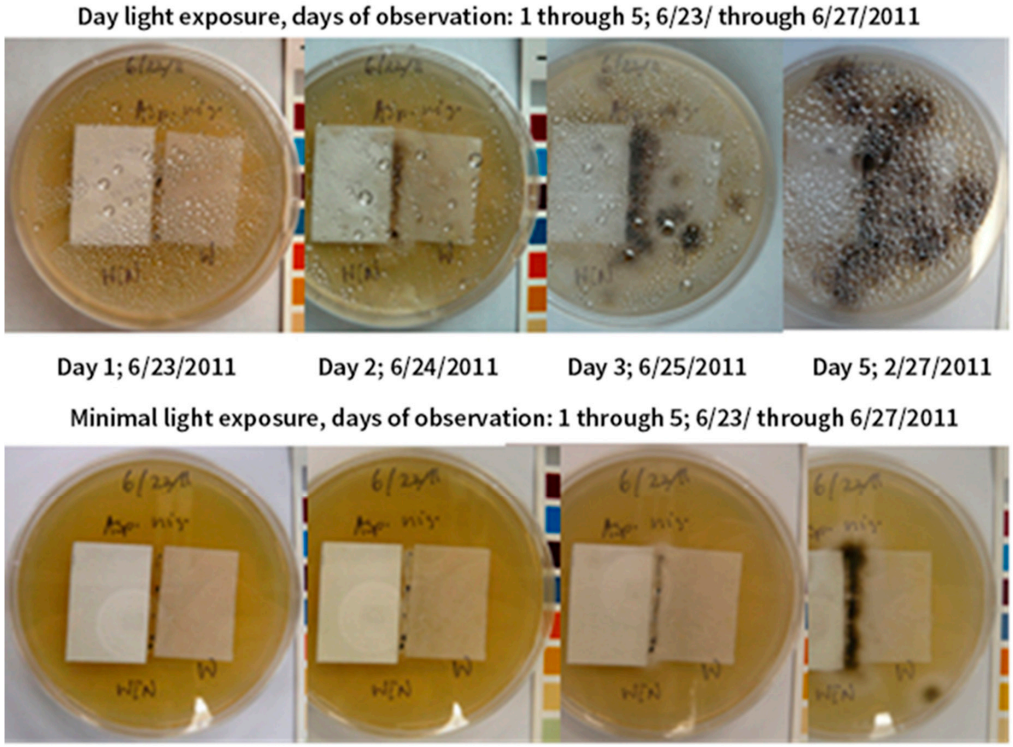


Figure 2.30. The first 1–5 days of cultivation in two light conditions. The upper row shows colonies cultured in daylight; the lower row shows colonies cultured in darkness. The W&N sample is on the left and the Whatman 4 samples is on the right in each Petri dish. The colonies grew rapidly in plates exposed to light and very slowly when cultured in darkness. The illustration is a composite of optical images taken with a Leica D-lux optical camera, scale 1:1. Source: Figure by author.

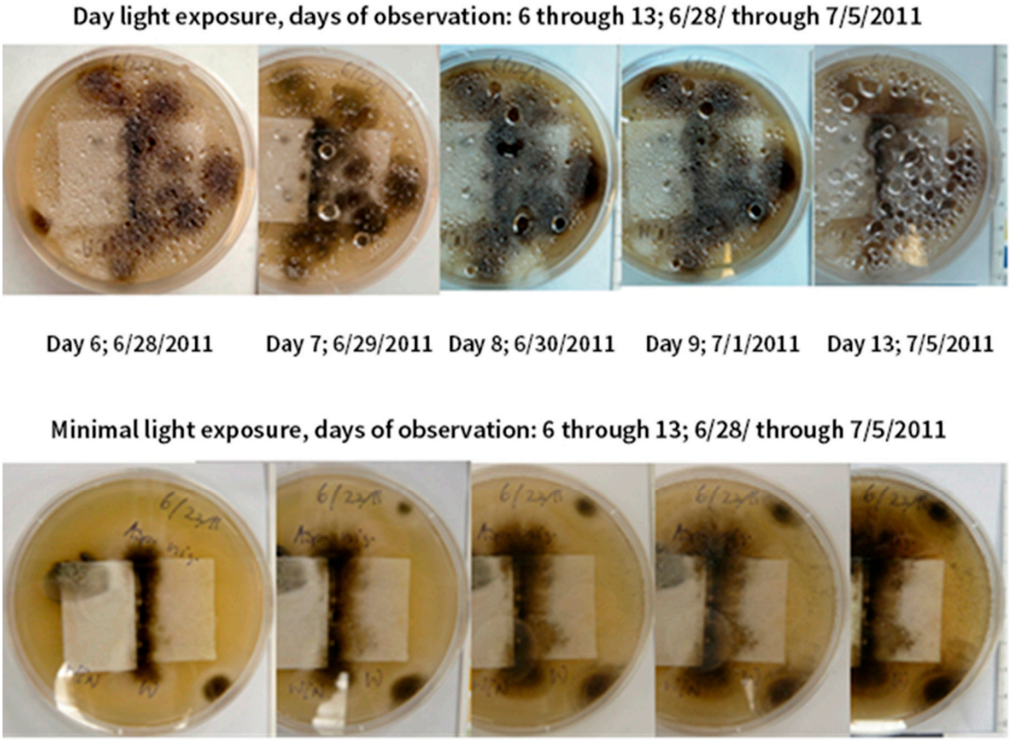


Figure 2.31. The progress of culture development recorded from day 6 to 13 in light (upper row) and dark conditions (lower row). The intensity of pigmentation in daylight colonies reached a plateau between the 7th and 8th day, and the colonies did not expand. In plates cultured in darkness, the pigmentation continued to intensify until the termination of the experiment on the 13th day, and colonies were also expanding. The illustration is a composite of optical images taken with a Leica D-lux optical camera, 1:1. Source: Figure by author.

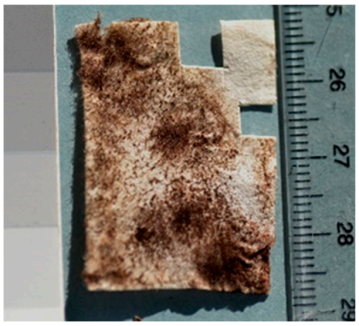
Figure 2.32. (**a**) The W4 paper with *A. niger* cultured in daylight for 13 days upon the termination of the experiment. (**b**) The same paper, W4, on which fungus was cultured in darkness over a period of 13 days. A distinct difference was observed in the density of colonies, their pigmentation, and pigment secreted to the paper substrate. Optical camera images were captured with a Leica D-Lux optical camera, scale 1:1. Source: Figure by author.

2.4.3. Summary of Observations and Measurements

The bio-simulation experiment generated observations that were evaluated with a focus on three aspects: (1) dependence of bio-pigment production on exposure to light, in daylight and in darkness; (2) correlation between the paper characteristics and fungi growth cultured in those two conditions; and (3) alterations to the paper morphology and chemistry caused by fungi. Although the analysis of each aspect follows in greater detail, to derive quantitative and statistical results, each aspect needs to be examined in a separate experiment, eliminating all other variables. The observations reported in this monograph are meant to merely signal a multitude of changes that are triggered by fungi infestation of paper and interdependence of all factors in the bio-pigment production, all of which contribute to fungi stains on paper.

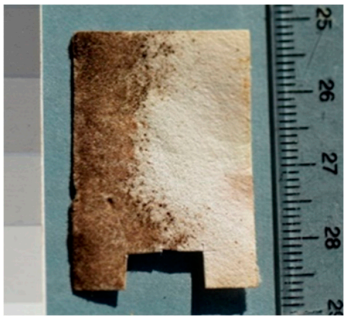
The Impact of Light on Pigmentation and Morphology of Fungal Colonies

The growth of colonies and ratio of pigment production were distinctly more rapid in the first 5 days on plates exposed to daylight than on plates cultured in dark. In daylight, the colonies grew rapidly, reached a peak in the first 7–8 days, and produced dark, loose and airy aerial growth. Figure 2.33 shows the pigmented W4 papers after the termination of the experiment on the 13th day of cultivation. Figure 2.34 illustrate Winsor & Newton (W&N) paper used as a substrate in culturing *Aspergillus niger* in light and dark conditions over a 13-day period.

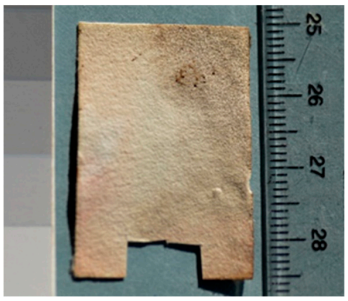


(**a**) W4 paper, recto, side on which fungi grew in daylight.

(**b**) W4 paper, reverse side of the same paper; a lesser amount of pigmentation was secreted than on recto.

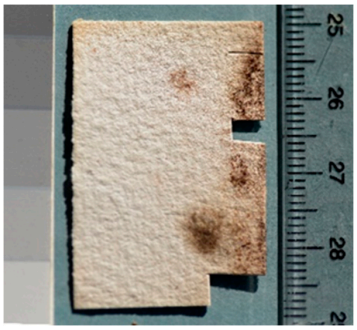


(**c**) W4 paper, recto, fungus was cultured in dark.

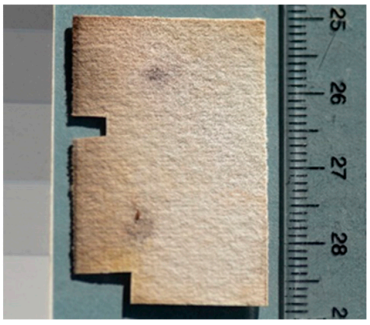


(**d**) W4 paper, reverse side of sample on which fungus grew in dark.

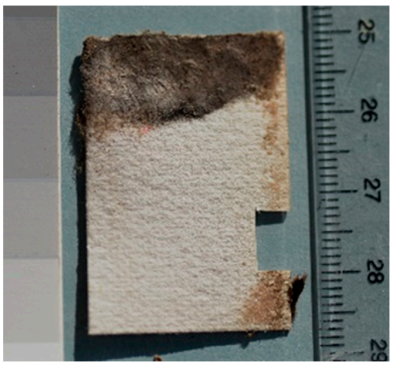
Figure 2.33. Two samples of W4 paper with *A. niger* cultured in daylight (**a**,**b**) and in darkness, with no light (**c**,**d**) upon the termination of the 13-day bio-simulation experiment. (**a**,**c**) show recto, the front side on which fungi grew; (**b**,**d**) show the reverse sides of each sample. In both cases, cultures in light and dark, the discoloration of paper was more prominent on the front side (recto) than on the reverse. The reverse was directly placed on agar-agar in Petri dishes. Source: Figure by author.



(**a**) W&N paper, recto (side on which fungus grew) in daylight.



(**b**) W&N paper, reverse side of the same paper; a lesser amount of pigmentation was secreted than on recto.



(**c**) W&N paper, recto, fungi was cultured in darkness. The dark colony along the top was a contamination and not the inoculant. Inoculant was applied along the vertical, longer edge and showed meager growth.

(**d**) W&N paper, reverse side of sample on which fungi grew in darkness.

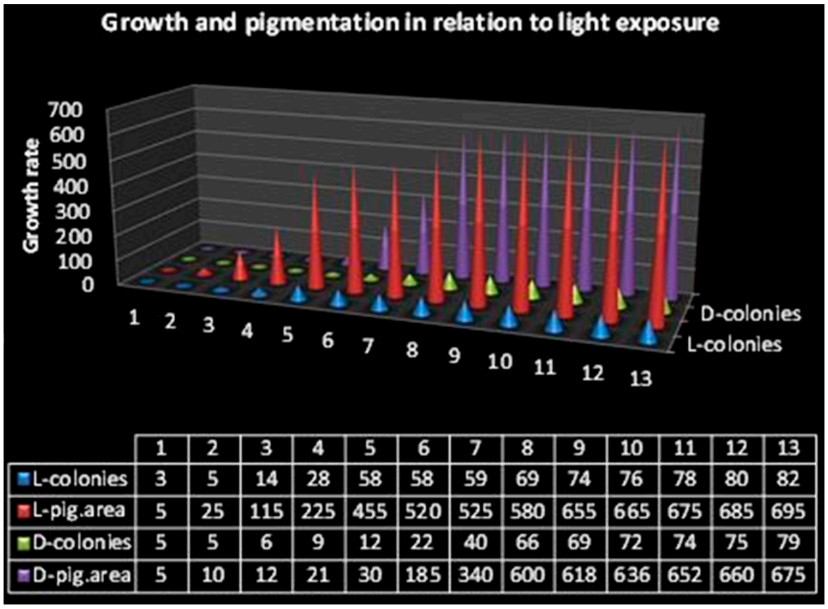
Figure 2.34. Paper samples of Winsor & Newton (W&N) used in culturing *Aspergillus niger* in light and dark conditions shown upon the termination of the 13-day cultivation period. (**a**,**c**) show the front side of papers on which fungi grew, and next to each one, the reverse sides of the same papers. (**b**) Colonies on recto were small and migration of bio-pigment to the reverse side closely corresponded to the pattern of growth on recto. Heavy growth on one of the samples cultured in darkness resulted from contamination rather than inoculation. The same can be said about the yellow stain on the reverse seen in (**d**). The area of investigation was limited to the long edge of the sample where the inoculant was applied; that area showed minimal growth. Source: Figure by author.

The two sets of paper samples were imaged—recto and verso of each. Each set showed evidence of pigmentation, but with a varied intensity and pigmentation pattern on each side, recto and verso, and changes directly corresponding to the two light conditions.

In dark conditions, colonies grew slowly and at a lower expansion rate; however, they produced more compact aerial mycelium at the end of the experiment than colonies cultured in light. The pigmentation produced by fungi growing in darkness appeared denser at the end of the experiment, when compared with the staining produced by fungi cultured in light during the same time period. Interestingly, the growth and pigment secretion in colonies cultured in darkness continued through the 13-day duration of the experiment until termination. In daylight cultures, the

colonies and pigmentation reached their peak around the 7–8th day and showed no expansion or increase in pigment intensity in the following days.

In colonies grown in darkness, the intensity of pigment secreted to paper reached the same level as those cultured in daylight at the end of the experiment; statistical calculations illustrate this trend computed as a graph (Figure 2.35).



	1	2	3	4	5	6	7	8	9	10	11	12	13
L-colonies	3	5	14	28	58	58	59	69	74	76	78	80	82
L-pig.area	5	25	115	225	455	520	525	580	655	665	675	685	695
D-colonies	5	5	6	9	12	22	40	66	69	72	74	75	79
D-pig.area	5	10	12	21	30	185	340	600	618	636	652	660	675

Figure 2.35. The growth of colonies and pigmentation in relation to light exposure. L stands for light and D for dark conditions. The measurements were collected over the 13-day duration of the bio-simulation experiments. Source: Figure by author.

The pattern of growth on both sets, cultured in light and in darkness, was more prominent on the side of papers faced up, allowing microorganisms access to air, when compared with the side that was placed against the culture medium, agar-agar. This may confirm the need for fungi to access air as an essential component in their development.

Impact of Paper Features on Fungal Pigmentation

Whatman4 paper, which is an unsized, fibrous, laboratory-grade filter paper, supported rapid development, abundant growth of colonies, and pigmentation in both daylight and dark conditions. Whatman4 paper absorbs water rapidly. The watercolor paper, Winsor & Newton, on the other hand, which is heavily sized, supported minimal and slow growth in the same light conditions and over the same time period of cultivation. The impact of the speed in which the fungi grew directly affected the paper structure, as seen after the termination of the experiment (Figure 2.36a,b). The rapid growth was accompanied by the intense production of enzymatic secondary metabolites which degraded the paper structure (Figure 2.36b).

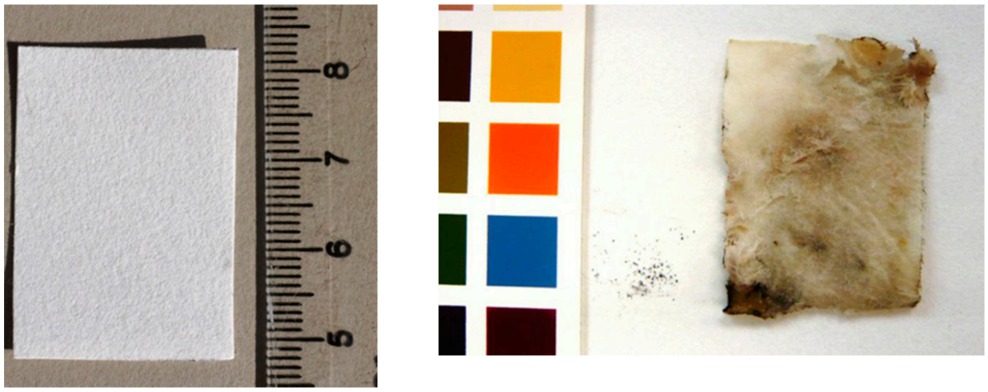
The ability to absorb water by W4 paper was the main factor supporting fungi growth. Conversely, the presence of sizing in W&N prohibited water sorption, thus

significantly slowing down the fungi development on the watercolor paper. The mechanism of water sorption has been discussed in Chapter 2.2.3.

Although all components were sterilized, minimal contamination was observed on W&N paper in the form of small, round, red and yellow colonies of Actinomycetes. These organisms, showing morphological features similar to bacteria and filamentous fungi, are omnipresent. They are characterized by the bright colors of their colonies. Actinomcetes were present on W&N paper and not on W4 paper, as confirmed by optical microscopy of the brightly colored areas on W&N paper.

Alteration of Paper by Fungi

The paper substrate was affected structurally and chemically by the metabolites secreted as byproducts of fungi metabolic processes. This became evident once the paper samples were removed from the culture plates upon the termination of the experiment, after fungi had grown on paper for 13 days. The changes in the paper structure were clearly visible when comparing the control paper, before its exposure to fungal activities (Figure 2.36a) and after fungus had grown on it (Figure 2.36b). This observation has far-reaching implications in practical scenarios, for example, during rescue operations of paper-based collections after flooding or the suppression of fire, which often involves heavy use of water and, consequently, results in fungi infestation.



(a) (b)

Figure 2.36. (**a**) A control sample used as a reference shows W4 paper before inoculation. (**b**) The same paper upon the termination of the bio-simulation, on which *A.niger* was cultured in light for 13 days; it shows the reverse side of the sample. The rapid growth of colonies was accompanied by intense production of pigmented metabolites, which severely disintegrated the paper. Paper was partially fused with agar-agar in the Petri dish. Images were taken with a Leica D-lux optical camera, scale 1:1. Source: Figure by author.

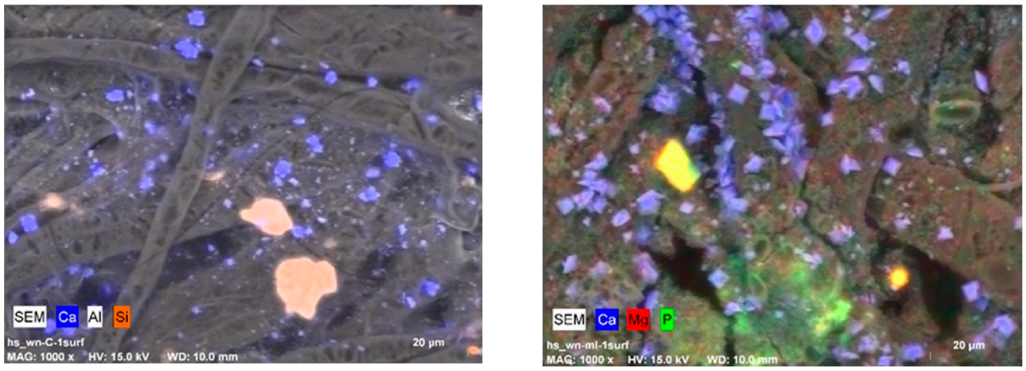
The alteration of paper was observed on both W4 samples, in light and dark culturing conditions; in both cases, fungi grew in greater abundance on W4 than on W&N and caused more distinct alteration to the paper, greater in light than in dark conditions. The paper fibers were so weakened in light-culture that some were

separated from the sample bulk and adhered to the agar-agar medium. This was not observed on W4 paper on which fungi were cultured in darkness.

Several modes of examination were applied to document and capture the extent of alterations caused by fungi. Transmitted light microscopy and SEM-VP examination were carried out on all samples, which confirmed significant changes to the surface and damage to paper fibers in the matrix. The interface of fungal deposits and paper was captured and measured with a non-contact, white-light profilometer. X-ray tomography was carried out on a synchrotron, at the European Synchrotron Radiation Center, Grenoble, France, which is discussed in detail in Chapter 5.3.3.

In addition to the structural alteration, a chemical change was observed on W&N paper, which contained a filler, calcium carbonate. The chemical change was observed on both sample papers used in light and dark conditions. The energy dispersive spectroscopic (EDS) analysis of the control samples, before their use as fungal substrates, showed the presence of calcium as an element of the typical filler, dispersed randomly in the matrix (Figure 2.37a). The SEM-EDS examination of the same paper W&N after being used in fungi cultivation revealed that the calcium particles underwent biomineralization resulting in calcium oxalate. This was confirmed by the characteristic shape of calcium oxalate crystals, tetragonal, 'envelope-shaped', and the elemental composition confirmed a high percentage of calcium and oxygen (Figure 2.37b). The crystal formations were more pronounced in the paper on which fungus was cultured in daylight. The crystals were smaller on the papers used in dark conditions.

The formation of calcium oxalate by fungi metabolic activities can be explained by the ability of *Aspergillus niger* to produce oxalic acid, resulting from its metabolic processes. In higher plants, the biomineralization of calcium oxalate is a known phenomenon and is reported by many researchers, among them Khan 1995; Gadd 1999, 2007; and Pinzari et al. 2010. The process is interpreted as part of the plant's mechanism during which excess chemically active Ca is removed via the precipitation process into highly insoluble salt of calcium oxalate (CaOx). That way, Ca is no longer osmotically or physiologically active. Adapting that notion to the morphology of fungi, this mechanism seems to be a plausible explanation for the adaptability of fungi: by chemically binding calcium, they 'deactivate' and reduce alkaline environment, thus creating a more favorable, slightly acidic environment. The SEM-EDS micrographs and chemical maps visualize that biomineralization of calcium into calcium oxalate (Figure 2.37b).



(**a**) (**b**)

Figure 2.37. (**a**) The W&N paper before inoculation with *Aspergillus niger*. Calcium-based filler is shown as amorphous deposits. SEM-EDS, scale 20 µm. (**b**) The W&N paper after 13 days of cultivation; fungi biomineralized calcium into calcium oxalate, seen as well-defined crystals. SEM-EDS, scale 20 µm. Source: Figure by author.

One difference, however, is worth noting, which is the crystal forms observed on papers used in cultures in daylight and darkness. It appeared that the crystal clusters of calcium oxalate were more randomly dispersed and less defined on paper used in daylight culture than those on paper used in dark cultivation; calcium oxalate crystals in dark-grown fungi were well defined as tetragonal, with an envelope-like shape and larger crystals.

A meaningful interpretation of the results requires familiarity with the sample's characteristics, especially the chemical composition of additives. Furthermore, replication of the experiment to ensure that the target topography is properly measured and alterations are documented is essential.

The biomineralization of the calcium carbonate filler in Winsor & Newton paper has been reported here to illustrate one of the effects of fungi growth on paper, and the role light plays in these alterations. Remineralization of calcium carbonate to calcium oxalate, although researched in other fields, has not been investigated extensively in the context of the biodeterioration of cultural material; one report focused on paper documents, authored by Pinzari (Pinzari et al. 2010), and another one focused on the formation of calcium oxalate on mineral surfaces of cultural heritage monuments (Ciccarone and Pinna 1992).

2.5. Summary Points

The main observation points have direct implications for predicting the outcome of fungi infestation of paper-based collections after exposure to water in floods or in rescue operations after fire events.

1 The environmental factors, characteristics of the substrate, type of microorganism and time factor work in synergy towards biodeterioration; therefore, their accumulative contribution must be considered when biodeterioration is evaluated.

2 Rapid growth and intense pigmentation can be attributed to fungi's response to daylight energy and the ability of paper to absorb water. The paper that quickly absorbs water (exemplified by W4) also supported rapid and intense growth of fungi in daylight and in darkness. The paper that did not absorb water due to heavy sizing (exemplified by W&N) supported minimal growth of fungi in both light and dark conditions.

3 Fungal growth is not always associated with the presence of sizing, as was reported based on results of fungi growing on paper with heavy sizing (W&N) that repelled water and consequently did not support fungal growth.

4 Fungi grew vigorously on the surfaces of paper directly exposed to light, almost no mycelial growth was found on the opposite side placed directly on a solid agar-agar medium. This observation indicated that light acted as a promoting factor for the growth and pigmentation; bibliographic sources indicate that light is known to act as a promoter or prohibitor of fungi growth. The bio-simulation reported here illustrated a promoting role of light on fungi development and production of pigmentation.

5 The intense and rapid colony growth and pigment production that occurred in daylight conditions corresponded with the greatest level of structural degradation of paper, most likely resulting from an intense production of metabolites.

6 The chemical characteristics of calcium-based filler in paper were altered by fungi that grew on paper in daylight and dark conditions. The formation of crystalline calcium-oxalate was evident in papers stained by fungi cultured in both light conditions, although more prominent crystals were formed in papers with cultures grown in darkness. This may lead to the conclusion that a longer time of culturing allows better formation of calcium oxalate.

7 The time factor plays an important, multi-faceted role in the biodeterioration process and can be evaluated based on different criteria; one is the intensity of pigmentation, and another is the degree of paper deterioration. In the experiment discussed in this monograph, the intensity of pigmentation was greater in the initial stage of growth and daylight exposure, but physico-chemical deterioration appeared to have increased with time.

8 The complex and multidisciplinary nature of investigating paper biodeterioration requires the application of various analytical instrumentations. They facilitate gaining a better understanding of the environmental impact on fungi, on the one hand, and on the other hand, modifications of paper substrates induced by fungi. Each set of factors should be examined with consideration given to the interactions of variables to fully assess their individual impact and contribution to the biodeterioration processes.

3. Fungal Stains on Paper

3.1. Origins and Definitions of Stains

Although staining is a perceptual phenomenon, an attempt is made here to define and describe it in relation to paper and paper-based substrates, with a focus on stains induced by fungi. The interpretation of a stain's significance depends on the context in which it was formed as well as criteria of its evaluation, aesthetic considerations, and values of the one who describes it.

A stain is a mark created on a surface; below the surface, it may penetrate the substrate, and in some cases, it may migrate to the reverse side. Many synonyms of the word 'stain' bear connotations pointing to the origin of the mark and mode in which it occurred, and may suggest the shape of its footprint. A smudge, for example, can be caused by liquid or dry matter smeared over the surface and suggests an elongated rather than round mark due to the action involved in its making. A splash suggests that a liquid caused the stain and that its footprint could be round, if the liquid fell straight in a perpendicular direction, or elongated, if the liquid came in contact with paper from an angular position.

Stain formation, shape, appearance, and behavior depend on the interplay of many factors, which can be categorized into four main areas:

1. The substrate on which a stain is formed (in this case, paper);

    1.2. The materials that make a stain (liquid or dry matter, in this case, solids such as pigmented mycelium or spores, or liquids such as secreted products of metabolism);
    1.3. forces at the interface of the staining material and substrate (in this case, paper and living systems, fungi);

2. The kinetics of material causing a stain.

The stain description depends on the material that produces the stain, and whether it is a liquid or solid, or dry in nature.

A liquid stain depends on the liquid's viscosity and material's porosity/wettability. Liquid stains produced by fungi result from the secretion of products of metabolism into the substrate and often penetrate into the bulk of a substrate, and even through the entire bulk marking the reverse of a substrate.

A dry stain depends on the pigmented particles' size, compactness of the substrate upon which they fall, surface characteristics of particles and substrate, and their affinity to the substrate. Fungi produce 'dry stains' by the deposition of pigmented spores on surfaces and/or pigmented fruiting structures formed in the paper matrix.

The stain shape and its footprint, profile and depth of penetration reflect an interplay of all of the above-listed elements. The possibilities seem countless, nearly infinite, if one considers the rapid development of new materials (substrates), novel

liquids and methods of their application, and the complexity of living, biological systems that produce pigmentation. The most frequently encountered shapes of fungal stains reflect fungi patterns of growth, both superficial and in the matrix of paper.

The stain–substrate interface is governed by the electron charge of the surfaces coming in contact with each other. They could be considered in the context of physico-chemical forces, gravity, capillary action, physical/chemical affinity of materials, and environmental conditions such as relative humidity and temperature or light energy. The electron-charge characteristics of spore surfaces are discussed in a separate chapter in the context of microbial adhesion to surfaces.

Considering the multitude of factors playing a role in the formation of stains, for clarity and simplicity, the stain will be referred to in this text as an individual phenomenon shaped by a particular set of circumstances and environmental parameters. The openness of this approach seems suitable for fungi-induced stains as it encompasses many variables resulting from continuous changes in living systems, such as fungi.

3.2. Fungi-Induced Stains

Bio-stains represent the discoloration of a substrate uniquely associated with the metabolic activities of fungi, bacteria, yeast, or algae. Pigmentation produced by microorganisms results from the complex bio-chemical processes occurring in their cells and has recently found its application in numerous areas of industry. Fungal pigments are secondary metabolites, proteinaceous enzymes, involved in many vital functions of fungi critical to their survival (Turgeon and Bushley 2010; Rodrigues 2016; Abdel-Azeem et al. 2016).

Pigmented metabolites are produced in abundance and in a great variety by many species. Dark brown secondary metabolites are known as naphtoquinons (Medentsev and Akimenko 1996); that and other colors, including red, yellow, and green, are species-dependent and modified by growth conditions. It is exemplified by the red complex of monoscorubrin and rubropunctatin found in *Monascus* (Hajjaj et al. 2000), or aurofusarin and/or bikaverin, also red pigments, found in *Fusarium* (Sorensen et al. 2012). Pigmentation may vary within the same species, and *A.niger* can serve as an example in which its genotypic differences result in a broad gamut of pigmentation (Tamayo-Ramos et al. 2011; Flores-Gallegos et al. 2016).

Fungi have the ability to produce a plethora of secondary metabolites, typically dependent on their stage of development, and as pointed out earlier, environmental factors, ranging from nutrient concentrations to light levels and a range of temperatures. Pigment production is a characteristic feature of certain species, although it may occur in response to stress, or as a protection against external factors such as UV radiation or extreme temperatures. The commercial importance of fungal pigment production dictates the direction in which the changes in their

growth conditions need to be taken. Defining such conditions is a result of extensive research predominantly in the field of medicine and agriculture.

The recent revival of utilizing natural, stain-forming materials includes pigments produced by fungi; many branches of industry adapt fungi-produced colorants. Those that have been used in Asian cuisine as food colorants for centuries were recently approved by the European food industry (Ali and Bashier Saleh 2006 cited in Mapari et al. 2006; Abdel-Azeem et al. 2016; Pennerman et al. 2016). Chemically stable bonds that characterize fungal pigments gained attention in commercial leather tanning and textile dying (Velmurugan et al. 2010). Moreover, fungal pigmentation is of interest in medical fields as a source of antibacterial and antifungal agents, while in agriculture, fungal pigments may be suitable as phytotoxins and insecticides.

Contrary to the importance of pigmented metabolites in industry, fungi-induced discoloration on cultural heritage material is undesirable. It alters not only the surface of artworks, sculptures, monuments, art on paper and documents on parchment but is also often associated with enzymatic digestion resulting in acid-related weakening of their structure (Gorbushina et al. 1993; Szczepanowska and Moomaw 1994; Szczepanowska and Lovett 1992; Szczepanowska and Cavaliere 2000, 2003; Szczepanowska et al. 2013; Szczepanowska et al. 2015). Pigmentation induced by microorganisms is nearly impossible to remove if secreted to the substrate. The unsuccessful effect of chemical means, such as the use of solvents, is due, as mentioned earlier, to the stability of fungal pigments' chemical bonds. The physical means, such as superficial cleaning or reduction, can be more successful if discoloration occurred on the surface and resulted from the dispersion of dry particles of pigmented spores.

Cultural heritage collections affected by microbiological infestation are altered, often irreversibly, which diminishes their aesthetic and artistic quality and lowers their market value. Studies of the aesthetic impact of the bio-deterioration of cultural heritage items proliferated during the last two decades, but mostly with respect to stone and wood deterioration (Gorbushina et al. 1993; Kumar and Kumar 1999; Santos et al. 2010; Ciferri et al. 2000). The study of leather and parchment bio-deterioration is represented only by a small number of authors; one of the most comprehensive works is a monograph by Strzelczyk and Karbowska-Berent (2000).

Relatively few researchers have dealt with fungal pigmentation secreted into paper and the physical disintegration of paper caused by microorganisms (Szczepanowska 1986; Szczepanowska and Moomaw 1994; Zyska 1997; Szczepanowska and Cavaliere 2000, 2012; Michaelsen et al. 2010; Principi et al. 2011; Pinzari 2018; Puskarova et al. 2018). To date, the most comprehensive review of the existing bibliography on fungi-induced stains was compiled by Melo et al. 2018. It shows a broad range of variations among reported stain pigmentations produced by the same organisms, indicating that changes in conditions directly affect the color of pigments. For example, *Asperglus niger*, which is characterized by its black color when cultured in optimal conditions (22–24C, RH 60–65%), has been reported as

producing yellow and green stains (Melo et al. 2018). This disparity of observed differences in pigment production confirms the impact of external and nutritional factors on pigment production.

Even though the surface is of essential importance in artworks and is usually the first affected by bio-deterioration, no research has reported a correlation between paper's surface characteristics and bio-deterioration. This study therefore constitutes the first attempt to formalize a systematic approach to the study of the impact of the surface morphology of paper on its bioreceptivity in the context of cultural heritage. This systematic approach, which is developed based on cellulose material, can also be applied to other types of cultural heritage collections.

Among the great diversity of fungi-induced stains, two types, rust-colored foxing (reviewed briefly for a comparative purpose only) and black stains on paper (discussed in detail), are the focus of this monograph. The most prevailing are rust-colored foxing stains, which have been the subject of research for decades. Black stains, which are encountered on paper nearly as frequently as foxing, have been researched to a minimal extent; this study is filling in that gap with a detailed analysis of the black stains produced by Dematiaceous (black) fungi.

3.3. Foxing Stains

Foxing stains are small, ca. 1–2 mm in diameter, of rusty color, and resemble the color of fox fur, which accounts for the origin of their name. Foxing has been studied since 1930, yet its origin still remains controversial, as evident in a comprehensive bibliographic review by Choi, which covers foxing research from 1930 to 2003 (Choi 2007). The most prevailing hypothesis associates foxing with the presence of fungi and metal impurities, or the presence of metallic inclusions in paper. Some authors attribute fungi as being the main source, while others lean towards rusting of iron inclusions, independently from biological influence. Consequently, no single definition has been formulated as to what foxing stains are. The most significant research findings on the biological origin of foxing and fungi-causative association were reported by Arai (Arai et al. 1988; Arai 2000).

The included illustration shows foxing that is, beyond any doubt, caused by fungi, based on fungal mycelium and aerial growth that were identified in the areas of foxing stains (Figures 3.1–3.3).

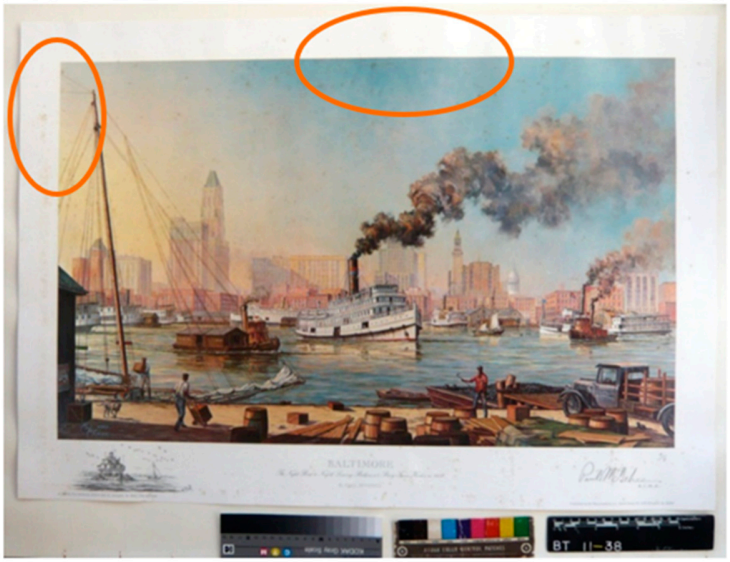
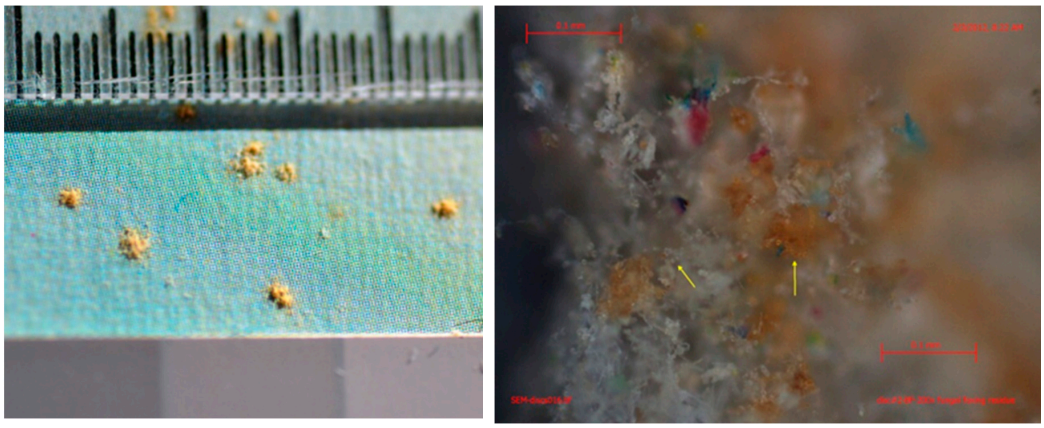
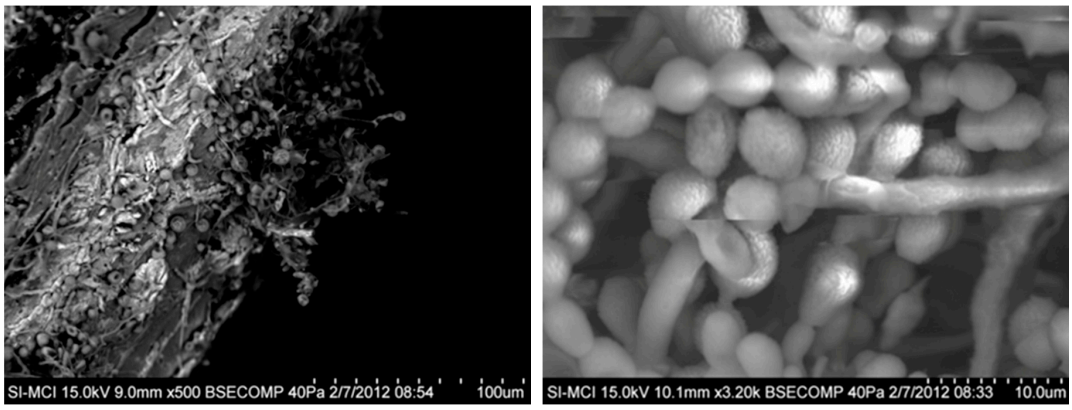


Figure 3.1. A commercial chromolithograph shows foxing stains scattered around the edges; details are shown in Figures 3.2–3.5. Artwork by P. McGehee, *Baltimore*, 18 3/4″ × 30″. The areas with high concentrations of stains are indicated by oval demarcations. Source: Figure by author.



(**a**) (**b**)

Figure 3.2. (**a**) Detailed view of foxing stains and aerial mycelial growth on the chromolithograph (Figure 3.1) in areas of foxing stains; scale 5 mm, macro-image with a Leica D-lux optical camera, scale 1:1. (**b**) Reflected light micrograph of fungal residue, found in foxing stains on the margin of the chromolithograph (Figure 3.1); scale 0.1 mm. Source: Figure by author.



(**a**) (**b**)

Figure 3.3. (**a**) SEM micrograph of a cross-section of paper with foxing stain shows paper fibers overgrown with mycelial and fruiting structures visualized as small, spherical formations; scale 100 μm. (**b**) Detail of spores from the foxing stain, collected from the chromolithograph shown in Figure 3.1, SEM micrograph, scale 10 μm, spore size 5–7 μm. Source: Figure by author.

One of the explanations for the rusty color pigmentation of foxing most likely points to the characteristics of the complex extracellular materials produced by fungi and their interactions with materials, impurities and/or additives in the paper matrix. The hypothesis of the interaction of fungi metabolites with impurities in paper was proposed by the author in 1994 (Szczepanowska and Moomaw 1994). The more recent studies of fungal secondary metabolites affirm the production of siderophores, which show high iron chelating activity (Borkovich and Ebbole 2010), thus leaning towards the earlier proposed interaction of fungi and inclusions in paper. Iron, which is an essential component for almost all life forms and is abundant in nature, is not easily available to bioactivities due to the low solubility of iron oxides. Fungal (also bacterial and yeast) siderophores acquire iron from its mineral phases such as oxides and hydroxides (Miethke and Marahiel 2007; Kreamer 2004; Raymond et al. 2003). Due to the extraordinary thermodynamic stability of soluble siderophore–iron complexes, following the research finding of Kreamer (2004), it is possible that foxing stains are formed as iron-concentration spots, thus explaining the formation of rusty-looking stains.

Another path of foxing stain formation was proposed by Arai (2000), who attributed the color of foxing to the interaction of amino acids produced by fungi and their chemical reaction with β-D-glucose in the cellulose chain. Arai attributes the coloration of foxing stains to the presence of brown organic compound melanoidines in fungal structures. The formation of brown-pigmented melanoidines was also confirmed in a different field, in food processing, and is discussed in that context by He-Ya Wang et al. (2011). However, the attribution of foxing stains to fungi reaction with sugar in the cellulose chain has not yet been confirmed outside the theory proposed by Arai in 2000.

Some of the reported studies of foxing also indicated a high concentration of copper in the areas of stains (Tang and Troyer 1981 cited in Choi (2007)). Another author links the molecular and phylogenetic investigation of fungal pigment formation to the presence of multicopper oxidase (MCO) genes (Tamayo-Ramos et al. 2011). Yet another explanation could be the activity of chelating functions of siderophores, which show strong binding activities to copper (Turgeon and Bushley 2010).

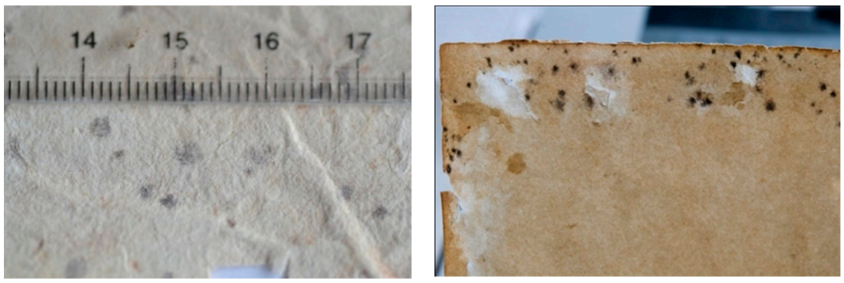
Foxing spots investigated in the course of this study (as a side observation to the main focus, black stains) showed that fungi grew indiscriminately, without any apparent dependence upon the type of paper or the presence of artistic media. As illustrated in the example here, foxing was observed on the paper margin as well as on the printed media (Figures 3.1 and 3.2). Foxing stains were reported by the author on other media in an earlier publication discussing bio-pigments on pastel paintings (Szczepanowska and Cavaliere 2003). In both cases, as earlier and currently reported, it seemed that the paper characteristics were not correlated with foxing stains as far as the surface and matrix were concerned. The paper used as a substrate for pastel was hand-made; in this study, the paper was calendared and heavily sized in commercially printed chromolithograph. The investigation of stains in SEM–VP of the cross-sections of the latter one in areas of foxing (Figure 3.3a,b) showed fungal filaments intertwined with the paper fibers. Often, the stains penetrated through the entire paper matrix and appeared on the reverse side as colored spots.

The conservation literature reported various methods used effectively in the removal of foxing stains. These included oxidizing bleaches employed during conservation treatments or mechanical removal of metallic inclusions in foxing that was 'non-biologically' formed (Choi 2007). The illustrated examples of fungal mycelium intermingled with paper fibers clearly exclude the possibility of the complete removal of fungal structures from the paper matrix. However, chemical reduction with conservation-approved bleaching agents may reduce the coloration of fungi-induced foxing.

3.4. Black Pigmented Fungal Stains

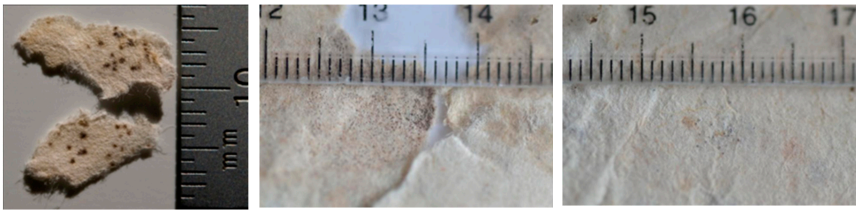
Black pigmentation produced by fungi is associated with the presence of melanin in fungal cells. The natural melanin pigments of vertebrates, insects, plants, and microbial organisms serve vital roles in camouflage, in sexual behaviors or in the protection against solar radiation. Melanin, having such significant implications, has been studied extensively across a broad spectrum of scientific disciplines. The mechanism by which melanin granules are assembled in pathogenic fungi and incorporated into their cell structures is still little known (Eisenman et al. 2005). In recent studies, utilizing solid-state nuclear magnetic resonance spectroscopy, it was suggested that melanin in fungi is tightly bound by covalent forces in fungal cell walls and that polysaccharide components may facilitate that attachment of pigment to cell walls (Zhou and Liu 2010).

The notion that pigment is tightly bound in fungal cells has been supported by this study. Black pigmentation was confined to the cell walls of mycelia, spores, and the walls of fruiting structures. The examples of black stains found on cultural material and investigated in this study represent various shapes, intensities, and forms, which are seen in Figures 3.4 and 3.5 and analyzed in the subsequent chapters. Fungal forms found in the studied black stains on paper were evaluated with multivariate techniques including the surface morphology and topography of the paper surface with bio-deposits. Resulting from these investigative approaches, a strategy for the systematic examination of the biodeterioration of cultural heritage material is proposed in Chapter 6. Some of these techniques were reported by the author in separate publications (Szczepanowska et al. 2013, 2015).



(a) (b)

Figure 3.4. (**a**) The 17th century Study Sheet shows stains with a diameter of 1 × 3 mm, semi-round, formed by clusters of black spores in the paper, as revealed during analytical examination. Stains were present primarily on one side of the paper sheet. (**b**) Detail of a 1920 engraving shows black stains, diameter 1 × 3 mm, semi-circular with rugged edges. Analytical examination with LM, CLSM, and SEM revealed chain formations of fungal cells intertwined with paper fibers. Stains were formed on both sides of the paper sheet. Source: Figure by author.



(a) (b) (c)

Figure 3.5. (**a**) The distinct black inclusions were identified on the 20th century silkscreen. Well-defined, spherical spots of pigment secretions each measured 0.2 mm × 0.3 mm. (**b**,**c**) show black stains formed by clusters of individual, dark fruiting bodies of smaller diameters than the ones seen in Figure 3.5, ranging from 0.01 to 0.03 mm. The stains in each case were caused by different factors: intensity of black secretion (**a**), concentration of pigmented bodies (**b**) and defined pigmented, individual fruiting structures (**c**). Source: Figure by author.

The black stains studied in this monograph were formed by fungi-pigmented, swollen mycelium, individual fruiting structures embedded in paper, and secretions of the products of metabolic activities. Rather than one type, a combination of stains

in the form of solid deposits and discoloration is typically present on the actual objects of cultural heritage, as discussed in detail in Chapter 6, Case Studies. Black staining is only one example of many possible colors that fungi produce. A great variety of fungal pigments and their potential correlation with the chemical composition of the substrate on which they grew has been reviewed in Melo et al. 2018.

3.5. Summary Points

1 All black-pigmented fungal structures, single cells and chain-like deposits were characterized by thick walls and defined cellular forms, as evidenced by analytical investigation of the selected fungal inclusions.
2 Based on multivariate analysis, black pigmentation was attributed to the meristematic fungi and perithecia of *Chaetomium* sp. (see Chapter 2 for details on its morphology).
3 Secreted pigmented material adhered to paper fibers. The pigmentation that was secreted varied in color intensity; some were significantly lighter than that in the fungal cells and could be attributed to extracellular material. The function of extracellular material secreted by fungi is discussed further in the context of microbial adhesion to the host material in Chapter 4.2.

4. Paper and Fungi Interfaces

4.1. Fungi and Paper Interfaces; an Overview

The interaction of fungi and bacteria with surfaces is of great importance in the study of biofilms in medicine, agriculture, and industry. Attachment to surfaces and the formation of biofilms are associated with the pathogenesis of both animals and plants. Consequently, a substantial body of literature in the fields of medicine and agriculture has been concerned with understanding the complexity of microbial adhesion to surfaces in order to prevent their pathogenic effects in humans and plants. However, as indicated in the introduction of this study, there is no reported research on interfaces of microbes and cultural heritage material.

The few available publications on the biodeterioration of cultural heritage are focused on describing the visual effects of microbial growth without attempting to explore phenomena occurring on interfaces or mechanisms of interactions with the substrate. Furthermore, most of the published articles discussing the biodeterioration of cultural heritage resulting from infestation by black-pigmented fungi deal mainly with the biodeterioration of stone, and only a few with the biodeterioration of paper and other materials. The mechanisms of the biodeterioration of paper remain largely unresolved.

This study is attempting to fill that gap by exploring the multitude of phenomena occurring at interfaces of bio-interactions with the substrate on which fungi grow, in this case paper. Here, the terms 'substrate' and 'paper' are used interchangeably. Paper is made from plant-derived material; therefore, some of the processes observed in the pathology of plants are used here in parallel to elucidate the biodeterioration of the cellulose-based cultural material. It is worth noting that some of the fungi growing on cultural material are also human pathogens; the biosafety of handling fungi-infested objects is discussed in a separate chapter.

Fungi and the substrate on which they grow are systems of solids interacting with water and air; therefore, some laws of physics, mechanics, and chemistry governing the interfaces of two- or three-body systems can apply (such as solid:solid, solid:gas:liquid, solid:gas and gas:liquid) (Figure 2.25). However, challenges lay in the fact that microorganisms are living systems and undergo continuous changes during their development. Consequently, the dynamic interactions between heterogeneous paper and microorganisms involve a multitude of forces. Furthermore, fungi produce pigments in different parts of their structures and/or secrete them as colored metabolic products into a substrate, thus forming stains. These stains may be superficial or may form liquid-like markings, as elaborated in Chapter 6.

4.1.1. Fungi (Solid), Air (Gas) and Paper Substrate (Solid) Interfaces

Fungi interface with the environment are biochemically complex and multifunctional. Fungi interface with air during spore dispersal and again when fungal hyphae begin to grow and protrude into the air above the substrate's surface.

The first contact of fungal spores with a substrate atfungi:substrate as solid:solid phases (Figure 4.1) is of great importance because it relies on forming attachments to surfaces necessary for further growth and development; it involves the interaction of physical forces and chemical changes. The attachment is facilitated by the adhesion of fungal spores to a substrate. Another type of fungus and substrate interface is formed by hyphae growing into its bulk, and chemically breaking down the substrate, which is then utilized as a nutrient source. The cycle of growth involves a variety of complex bio-mechanisms and kinetic forces; therefore, all of the interfacial phenomena are interconnected.

Fungi disperse spores or conidia via water or wind. Survival strategies of fungi facilitate dispersion with specialized spore/conidia-bearing structures and mechanisms (i.e., appendages) that ensure their successful dissemination. Survival strategies developed among fungi that pertain to this study are concerned with interfaces of fungal elements with the substrate (paper), such as spore adhesion to the substrate and germination, cell surface and pigmentation, surface area of colony expansion, and growth patterns. All of these interactions are discussed in the context of interfacial dynamics of biomass and heterogeneous, cellular substrates of paper (Figure 1.1). Furthermore, each of these interactions results in a different type of substrate alteration due to depositions of pigmented spores and/or enzymatic reactions of fungi with the substrate.

Pigment production and subsequent substrate alterations follow fungal growth and development that is marked by distinct stages:

1 Spores/conidia landing on a suitable surface (liquid water needed) (Figures 4.1 and 4.5).
2 Adhesion to surface and germination (Figures 4.2, 4.3, and 4.6).
3 Growth of hyphae expanding into mycelium (Figures 4.4 and 4.8).
4 Formation of fruiting structures with spores and conidia.

4.1.2. Fungal Spores: Substrate Interface

Fungi growth begins once spores find a suitable surface onto which they adhere. Two essential elements are required, hydrophobicity of the surface and molecular water (Hazen et al. 1988; Rosenberg and Doyle 1990; Wessels 1997; Azeredo and Oliveira 2003; Filonov 2003; Smits et al. 2003; Schumacher et al. 2008). It is surprising, however, that none of the authors dealing with the hydrophobicity of surfaces point out that molecular water is available precisely because the surface is hydrophobic. A hydrophilic surface would absorb water, leaving no liquid film on its surface. Hydrophobicity has been discussed by researchers in the context of complex cell communication (Martin et al. 2017) and an electro-charge of the spores' surface

in aqueous environments. The views on the role of hydrophobicity in microbial adhesion have been debated for at least two decades, and recent developments are beginning to recognize the contribution of other forces besides hydrophobicity in the process of microbial adhesion.

Once the spore lands on the surface, and if the environment is favorable, enzymatic reactions occur very quickly, within the first 3 minutes of the contact, resulting in a firm adhesion of the spore to the surface (Figures 4.1 and 4.2). The extracellular material, which functions as an adhesive, undergoes polymerization and becomes insoluble (Figure 4.3) (Epstein and Nicholson 2006).

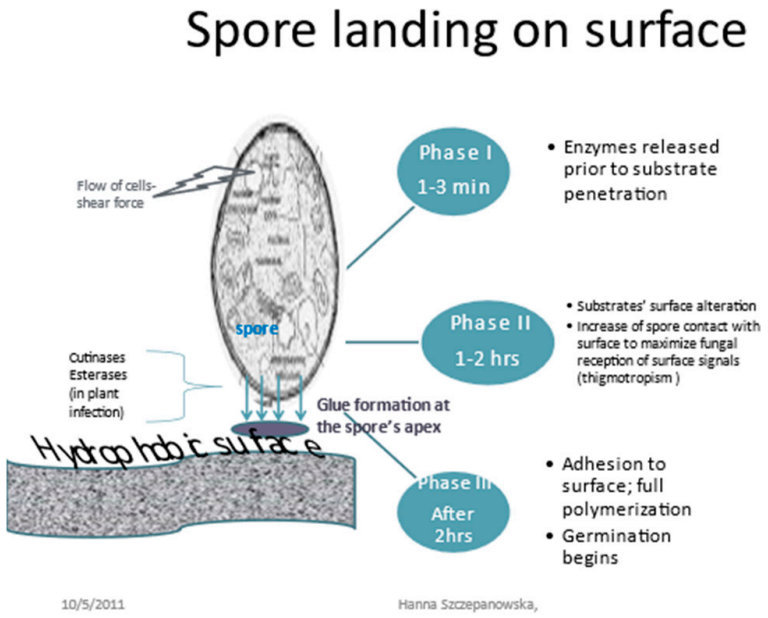


Figure 4.1. Conceptualized sequence of events during the initial phase of a spore contact with substrate's surface. Source: Visualization of description in Epstein and Nicholson 2006; © Szczepanowska 2011

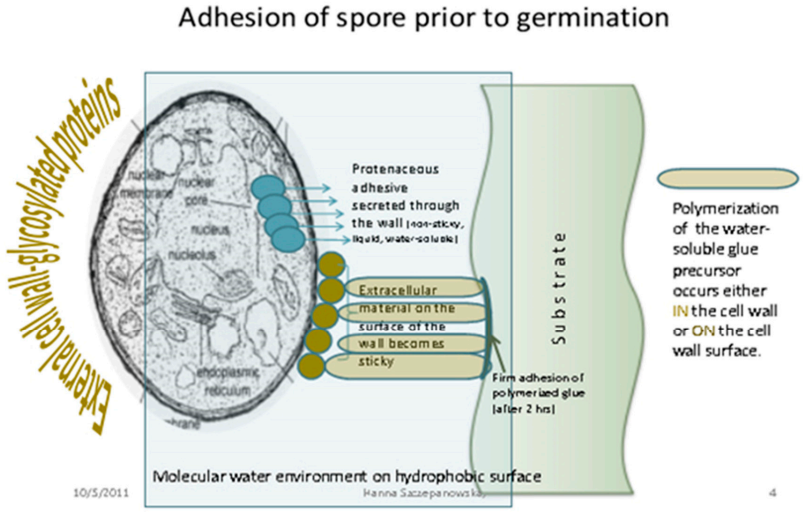


Figure 4.2. Conceptual representation of extracellular material excretion and adhesion of a spore to a surface. Source: © Szczepanowska 2011. Visualization based on description in Epstein and Nicholson 2006.

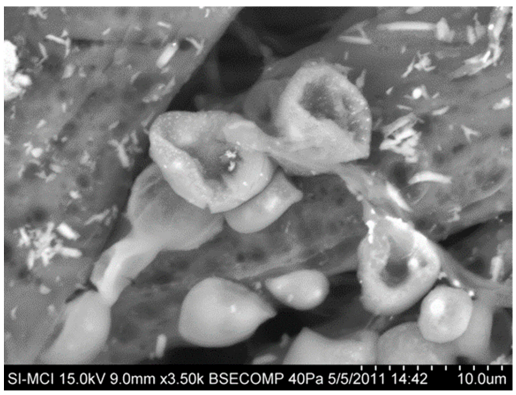


Figure 4.3. Black-pigmented fungal cells are attached to the surface of paper fibers. Extracellular secretions and energetic forces hold the cells in place. Stain type A, the 17th century Study Sheet, detailed analysis in Chapter 6.1, SEM-VP, scale bar: 10 μm. Source: Figure by author.

The secretion produced by a spore that changes its characteristics from a water-like soluble substance to an insoluble polymer once it passes through the cell walls remains an unresolved phenomenon. Because of is potential applications in the medical field, it has been studied extensively as a substitute for traditional sutures.

4.2. Microbial Adhesion to Surfaces

The adhesion of microbes to substrates has been studied since 1924 (Rosenberg et al. 1991). It has been discussed in terms of both chemical and physical interactions depending on who is investigating the issue (Donlan 2002). Spores and hyphae attachments are aided by the production of an adhesive-like extracellular material (Gamarra et al. 2010). The phenomenon of the attachment process is also attributed to the hydrophobic characteristics of spore and hyphae surfaces.

Surface cell hydrophobicity and its role in microbial adhesion are not well defined among researchers, just as the adhesion phenomenon itself cannot be well defined. The definition often depends on who is describing it.

Physical chemists agree that hydrophobic interactions between surfaces depend largely on the unique properties of water itself (Duncan-Hewitt et al. 1989). Others argue that the hydrophobic nature of bio-cells is imparted by the unique structure of small proteins, hydrophobins, unique to filamentous fungi and lichens, congregating on the surface of spores and hyphae, forming a thin layer of film (Geertsema-Doornbush et al. 1993; Wösten et al. 1994; Wösten and Scholtmeijer 2015; Wessels 1997, 2000; van der Mei et al. 1998; Doyle 2000; Vadillo-Rodrıguez et al. 2003; Epstein and Nicholson 2006; Kwan et al. 2006; Wang et al. 2010; Urbar-Ulloa et al. 2019). This film, by changing the surface into hydrophobic, reverses cell walls' wettability (Figure 4.4). In general, authors associate the hydrophobic effect with the characteristics of small proteins, hydrophobins' surface energy, and ionic interactions between fungi and surfaces to which they are attached. A conceptualization of hydrophobic interactions of fungal walls with the environment and substrate is

illustrated in Figure 4.4, based on one of the early publications where hydrophobins are discussed in depth (Wessels 1997).

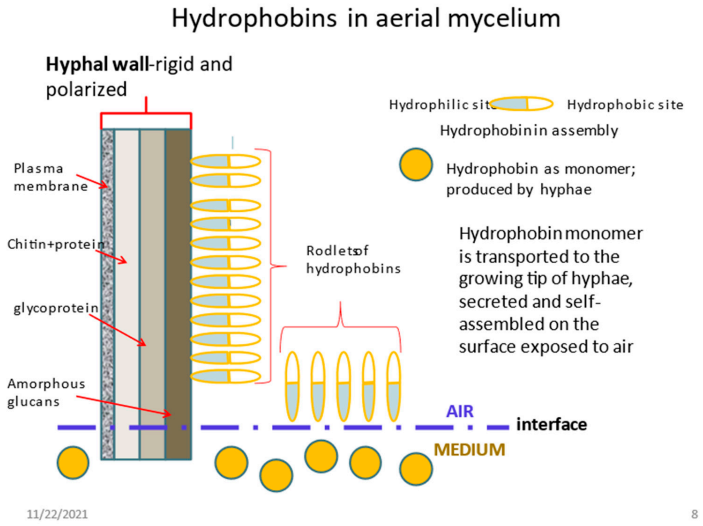


Figure 4.4. Conceptualized model of the hydrophobin rodlet monolayer on the surface of aerial hyphae. The synthesized monomer of hydrophobin is transported to the growing tip, most likely via exocytotic vesicles, and secreted on the surface. The monomers are then self-assembled when reaching the interface between the hydrophilic wall and hydrophobic air. Here, they form an insoluble amphipathetic monolayer with the hydrophobic rodlet-decorated side facing the air and changing wettability of surfaces. Source: © Szczepanowska 2011; visualization of description in Wessels 1997.

The hydrophobins that mediate fungal adhesion to surfaces were found in the aerial hyphae wall that makes up fruiting structures and on the surface of spores (Wösten et al. 1994; Wessels 1997; Kwan et al. 2006; Epstein and Nicholson 2006; Wösten and Scholtmeijer 2015). The term 'hydrophobins' was first used by Rosenberg and Kjelleber in 1986 (Wessels 1997). As mentioned earlier, they are unique to filamentous fungi (Hazen et al. 1988; Wessels 1997; Epstein and Nicholson 2006). The hydrophobins are small proteins made up of ca. 100–250 amino acids. The mechanism of how hydrophobins adhere is of interest to various biotechnical immobilization applications; consequently, they have been studied extensively, primarily in the field of infectious diseases in humans and in agriculture, where the adhesion of pathogens is the first phase of the host's invasion, leading to disease (Doyle and Rosenberg 1995; Doyle 2000; Inoue et al. 2016). The understanding of their nature has been elucidated over the past two decades due to genetic sequencing. For example, Wang et al. (2010) found that several types of proteins readily adsorb onto hydrophobins, but only under defined conditions of pH and ionic strength. The value of pH and ionic forces, such as the Coulombic charge, are among the factors identified in surface adhesion interactions (Figure 4.5).

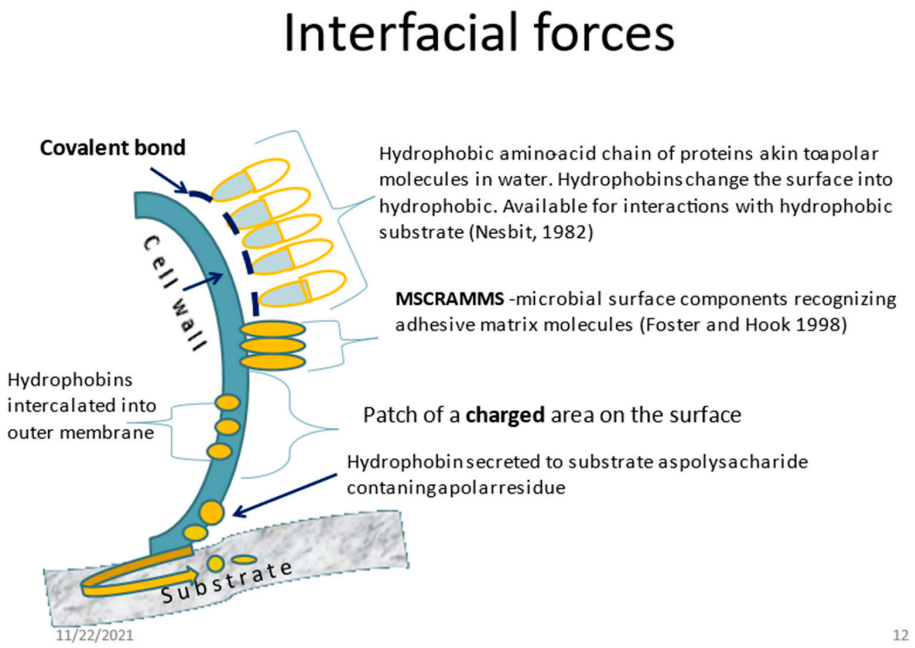


Figure 4.5. Visualization of interfacial forces between fungal structure and paper. Source: © Szczepanowska 2011;.based on description in article by Doyle 2000.

One fact remains unchanged: the adhesion of spores or conidia to the surface of a substrate is an essential step in the life cycle of a fungus. It is a process that occurs rapidly once the spore lands on the surface in a favorable environment. Enzymes and proteinaceous materials are involved in producing extracellular material that attaches spores to surfaces. Upon contact with favorable surfaces, conidia release adhesive material localized in a droplet at the spore apex, which some authors named Spore Tip Glue (STG) (Schumacher et al. 2008; Figure 4.6). Histochemical investigations of STG indicated the presence of proteins and carbohydrates. With the aid of transmission electron microscopy, two phases in the STG at the tip of dry, mature conidia were revealed on the outer side of the intact fungal cell wall. These surface-active substances affect the adhesion of conidia to hydrophobic surfaces, stressing the importance of hydrophobic interactions (Schumacher et al. 2008). Similarities of such adhesion were documented in black stains on the 17th-centruy hand-made paper, Study Sheet No. 1 (Figure 4.7).

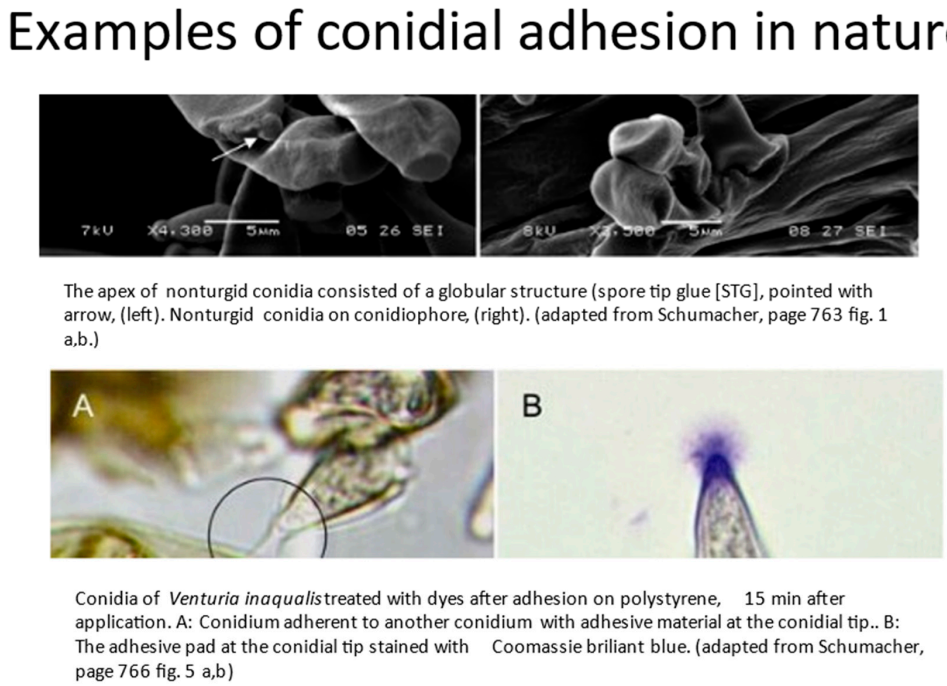


Figure 4.6. An illustration of an apical secretion of adhesive-like extracellular material glue, at the spore tip Source: Adapted from (Schumacher et al. 2008), used with permission.

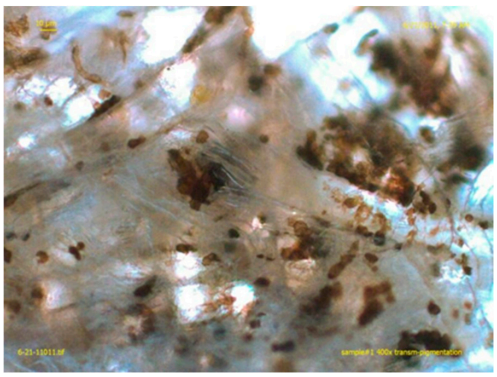


Figure 4.7. Pigmented deposits in the areas of black stains on Study Sheet No. 1 are most likely extracellular secretions. The preliminary conclusion is based on the amorphous shape of pigmented residue accompanying the defined in-shape conidia. Transmitted light micrograph, scale 10 µm. Source: Figure by author.

The studies of bacterial and fungal adhesion by numerous researchers indicated that a number of cell surface's physico-chemical factors contribute to the process of adhesion. In addition to extensively debated surface hydrophobicity, the presence of extracellular polymers and cell surface charge has been considered (Busscher et al. 1991; Azeredo and Oliveira 2003). The latter determines the electrostatic interactions between the cell and the substrate (van Loosdrecht et al. 1989).

In the most common situations, i.e., aqueous media with pH near neutral, the microbial cells and solid substrates are negatively charged. This means that the surface charge has a repulsive effect and acts contrary to adhesiveness. However,

small patches of positively charged areas on the spores, according to the most recent discoveries, may be sufficient to induce attachment. The extracellular polymers secreted by fungal cells (spores and hyphae) contribute to the adhesion process. Extracellular materials are not only important to the adhesiveness, but they also determine the structure of biofilms. Hydrophobicity is important in the first stage of contact between spores and substrate, while the extracellular biopolymers are equally important in the early and final stages of the bio-film formation. Attempts were made by some researchers to measure the attachment of microbial spores and cells to surfaces using the contact angle method, which is recognized as the most reliable way to determine cell hydrophobicity (Doyle 2000). However, the results were not entirely successful due to the small size of cells (3–9 μm) and the need to remove them from their natural environment, which altered their responses.

4.3. Role of Surface Topography in Fungal Growth

The most intimate contact of fungi with the surface has an impact on the directionality of hyphae expansion. Knowing that fungi grow by extension of mycelium (discussed in detail in Chapter 2.1) the hyphal wall acts as an interface between the fungus and its environment. It responds to the environmental stimuli by the directionality of its growth; the responses are species-specific and impacted by the direction and types of stimuli.

Chemotropism is a reaction triggered by a chemical present, in this case illustrated by fungi growth towards oxygen-rich spaces. It was observed in the fungi's directionality towards open paper pores. The surface topography was reported by other researchers as oriented directionality in plant pathogens, which follow the surface groves towards stomata, a natural opening in plant leaf surfaces that is used by fungi to enter the host (Leong et al. 2002; Kwan et al. 2006). A visualization of this phenomenon is illustrated in Figure 4.8.

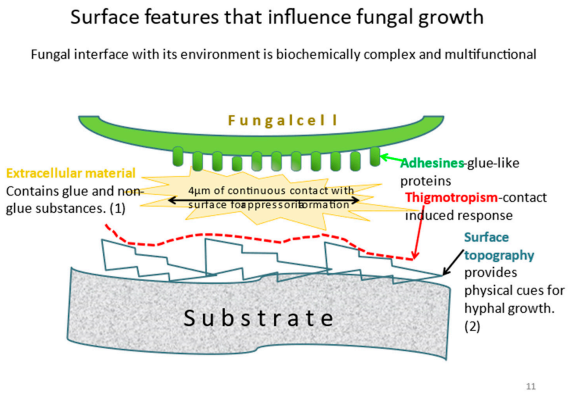
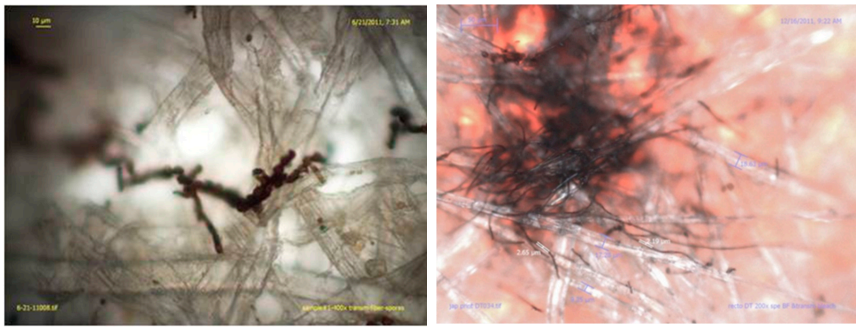


Figure 4.8. Visualization of fungal interactions with the substrate's surface (not to scale). The typical size of fungal spores ranges from 5 to 9 μm. Spores flatten upon contact with the surface to enlarge the area of attachment. Source: © Szczepanowska 2011; drawings based on description in article by (Nicholson et al. 1988; Pascholati et al. 1993; Hoch et al. 1987).

The response to the surface topography is termed 'thigmotropism' and was observed in the fungal growth on the studied artworks (Figures 4.9 and 4.10). The hyphae are not growing towards 'stomata', as observed in plants, since the paper fibers are not living structures; however, the rough surface of paper fibers seems to be utilized by fungi as anchorage permitting them to expand their growth in certain directionality. Figures 4.9 and 4.10 illustrate fungal tropism captured on micrographs during transmitted and reflected light microscopic examination.



(**a**) (**b**)

Figure 4.9. (**a**) Dark pigmented cells arranged in chains trailed on the paper fibers, utilizing their rough surface as anchorage. Some cells appear to be attached to the surface of fibers; others trail either towards another surface or to more airy pores in paper. Evidence of thigmo- and chemotropism on the 17th century Study Sheet (stain type A). Transmitted light micrograph, scale 10 μm. (**b**) The area stained by dark-pigmented fungal filaments concentrated at attachments of the fruiting structures of Chaetomium. The filaments migrated through the bulk of the substrate to the other side of the paper, possibly to access more open airy spaces. The 1847 Japanese woodblock print. Transmitted light micrograph, scale 50 μm. Source: Figure by author.

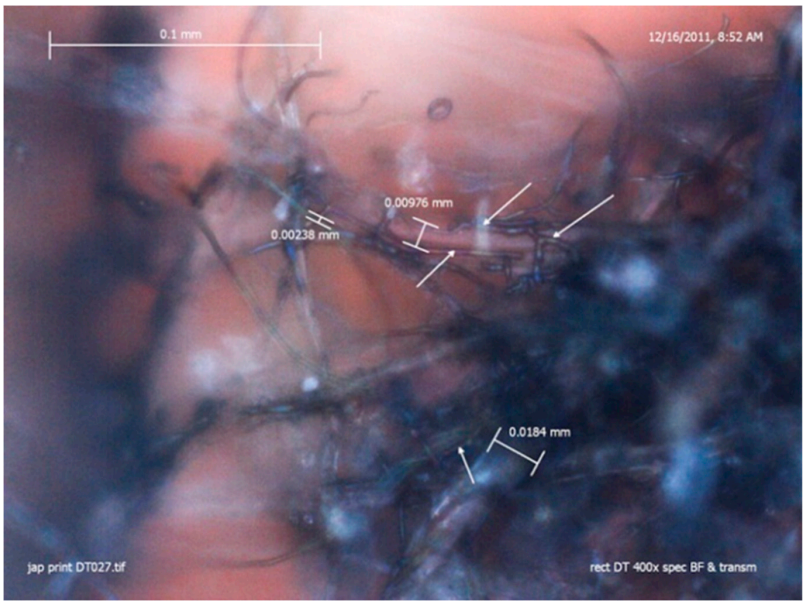


Figure 4.10. A detail of the stain shown in Figure 4.9: dark filaments' interaction with paper fibers. Similarly to other examples, the filaments trail the paper fibers and appear to be attached to the surface of these fibers. It is an example of thigmotropism, fungi utilizing the roughness of the fibers' surface as a physical anchorage. Transmitted light micrograph, dark field. Scale 100 μm. The thickness of paper fibers ranged from 9 to 18 μm while fungal filaments' average thickness was 2 μm. Source: Figure by author.

The evidence of fungal tropism illustrated in Figures 4.9 and 4.10 was found on samples extracted from the Study Sheet, Study Case No. 1, the 17th-century hand-made paper, the 19th-century Japanese woodblock print, and Case Study No. 2. In the first example (Figure 4.9a), the mycelial cells, swollen and in chain formations, grew towards air pockets in pores of the paper matrix, demonstrating a form of chemotropism. The other examples demonstrate thigmotropism, utilizing surface roughness, and chemotropism observed in colonies growing more freely, facing the airy-side of paper, shown on the Japanese woodblock print 1847 (Figures 4.9b and 4.10). Thigmotropism was also evident in colonies cultured in the bio-simulation light experiment, discussed in Chapter 2.2.

4.4. Summary Points

1 The adhesion of microbes to surfaces involves a multitude of factors, both physical and chemical in nature. External stimuli trigger complex changes inside a spore from the moment it chemically 'recognizes' the surface as a suitable environment. Once the favorable environment is recognized, the attachment occurs quickly and is estimated to be completed within 1–3 hours. This knowledge is essential for mobilizing a fast response for paper-based collections after their exposure to water.
2 The environmental factors work in tandem with the topography of the substrate's surface, both playing a decisive role in the initial stage of fungal development.

3 Attachment to surfaces is one of the most important events in microbial life because it facilitates the initiation of the new life cycle. Once the discovery of surfaces that prohibit microbial attachments to surfaces is made, the world will be free from illnesses caused by microorganisms.

5. Strategy for Investigating Biodeterioration of Cultural Heritage Material

5.1. Examination Approach

It is often difficult to determine the cause-and-effect relationship in the biodeterioration of cultural heritage material because of the multitude of factors contributing to that phenomenon. As pointed out in previous chapters, the environment, substrate, and fungi are the main three entities involved in the complex process of biodeterioration. Each of these entities requires a different set of analytical tests; only some can be applied to the analysis of fungi and substrate simultaneously.

An additional complication limiting the analytical examination of material is inherently associated with restrictions governing the sampling of original cultural heritage material. The procurement of samples from an artifact of historic value, even if only of microscopic in size, interferes with the original object and consequently requires a thoughtful plan and justification for carrying out analytical examination. Written permission for sampling an artifact is essential and must be obtained from the custodian who holds the legal responsibility for the object; this may be a collection's curator, a registrar, or the museum director, depending on the museum's structure and policy (Szczepanowska 2012).

Considering the complexity of biodeterioration and sampling restrictions, the ideal investigation should: (1) include the availability of a suite of analytical instruments that generate information about both the microorganisms and the substrate on which they grow, and (2) be performed without the need for sampling the substrate (in situ, non-invasive).

The application of light microscopy poses a challenge for the examination of artworks that cannot be sampled. They require custom-constructed support to carry out an examination. A practical solution to this is illustrated in Figure 5.1.

Figure 5.1. The Japanese woodblock print is examined on a support that maintains its flatness. The application of light microscopy poses a challenge for the examination of artworks that cannot be sampled. They require custom-constructed support to carry out an examination. Source: Figure by author.

Based on that premise, relying on multi-sensor analysis, and limited sampling, the examination techniques meeting the above criteria were reviewed, and the results of their applications were reported in Chapter 6. The investigative approach begins with macro-scale analysis and progresses into more-detailed, micro-scale analysis. The results of macro-analysis provide a base for proceeding further in depth, to instrumental and chemical analysis of microstructures guiding the choice of analytical methods.

It is essential to understand the context of biodeterioration, such as characteristics of the substrate, in this case paper, and ideally, the environment to which the substrate was subjected. The history of conditions to which any infested paper was subjected is rarely attainable and can be deduced, at times, based on visible signs marked on the paper itself. These signs may be of a chemical nature, such as water stains and discoloration, and/or physical, such as planary distortions, tears and disturbances of paper fibers such as abrasions. The characteristics of paper, which includes its surfaces, bulk composition (fiber type) and presence of additives (sizing, fillers), all directly influence the development of biofilms; therefore, their characterization is essential for the proper interpretation of analytical results of biodeterioration. In particular, surface features of paper impact the formation and distribution of biodeposits; therefore, new methods of surface analysis were tested as tools for providing insights into paper interactions with fungi.

5.2. Examination Methods of Paper

Paper is a composite material manufactured to meet the end user's demands and is engineered to serve many applications (detail discussion, Chapter 2.2: Paper, an overview). Consequently, the paper industry offers a broad array of testing methods to evaluate how the final products meet specific criteria. Many techniques, however, even though they are called 'non-destructive', require sampling of the original material, which is considered invasive in the field of cultural heritage. The term 'non-destructive' in commercial and analytical use means that the sample material is not destroyed during analysis and can be re-used again.

In general, chemical and physical properties of paper are tested, on the surface and in bulk. The interpretation of all tests relies on the information that each one supplies; test results cannot be viewed in isolation.

The fibers, fillers, and coatings, the main building blocks of paper, are affected by the environment, especially moisture content, requiring a particularly regimented set of testing conditions and careful interpretation of the results. For a meaningful interpretation of the test data, paper should be conditioned for at least 24 hours in a controlled and monitored environment to ensure that a varied moisture content does not alter the results.

The surface characteristics of paper play an important role in fungal attachments and affect fungal growth, as mentioned in the preceding chapter. The topography of the surface will impact measurements of microstructures, such as fungal deposits;

therefore, the review of techniques used in the examination of paper is limited to those that are the most adequate for the surface examination of cultural heritage objects. Furthermore, the limitations in sampling the cultural heritage material prevent the application of industrial tests carried out on the bulk of the material.

Paper itself is rough and porous, as is the coating applied on paper. Differentiation of porosity and roughness of either depends on the technique used to detect and quantify them. A comprehensive review of methods that apply to the analysis of paper surface can be found in Preston's 'Surface Analysis of Paper' (Preston 2009, Imerys Minerals, Ltd.) A brief review of different methods was prepared based on that and other publications. The selection of the reviewed methods was tailored to surface characterization, both physical and chemical. Many analytical tests of paper are used in the paper industry, depending on the desired final features of paper and expectations of the end user.

In general, the techniques and instruments applied to the testing and analysis of the paper surface were:

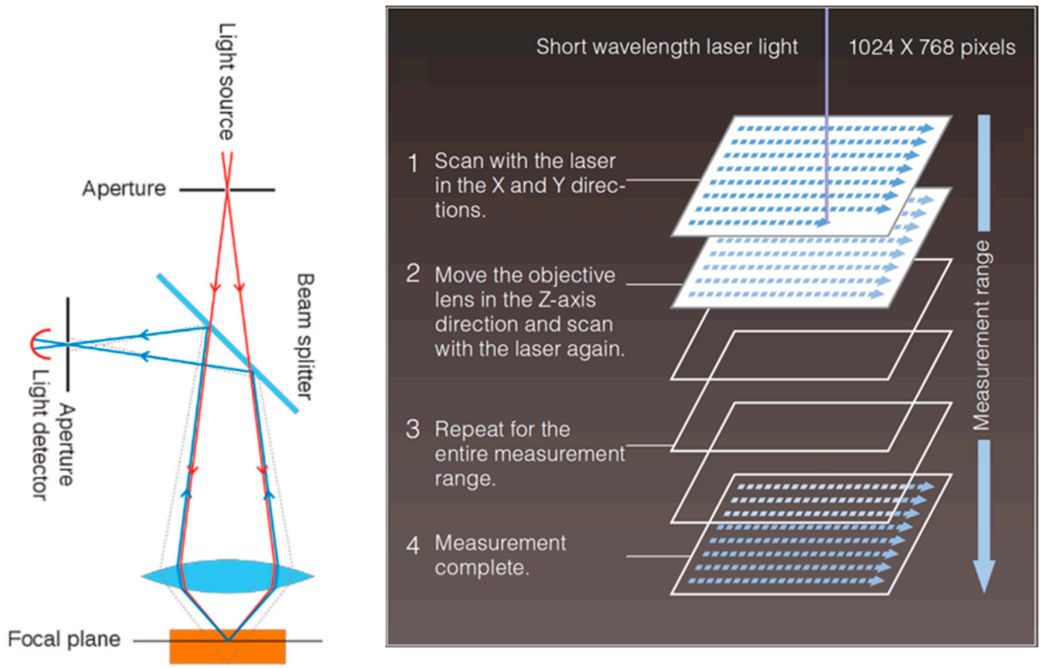
- For surface metrology and topography: optical and digital microscopy (light microscopy (LM), scanning electron microscopy (SEM), light profilometers, confocal light scanning microscopy (CLSM)
- For the chemistry of the surface: spectroscopic techniques (Fourier-transform infrared spectroscopy with attenuated total reflection (FTIR-ATR), SEM with energy dispersive X-ray spectroscopy (SEM-EDS), photo acoustic spectroscopy (PAS), X-ray photoelectron spectroscopy (XPS), secondary-ion mass spectrometry (SIMS), and Raman).

Some of these techniques were applied in this study, and the results were compared and evaluated to determine their practical benefits in the examination of cultural material. In recent years, the surface topography of paper has been measured with laser and stylus profilometers. A comprehensive review of this relatively new approach to the examination of paper surface can be found in Mercier et al. (2022). The promising results of utilizing laser profilometers in the examination of biodeteriorated paper were reported by the author in 2013 (Szczepanowska et al. 2013).

The non-contact measuring mode of optical instruments is a particularly desired feature for cultural heritage material. It eliminates the sampling of objects, permitting their examination in situ.

The use of confocal laser scanning microscopy is one example of surface analysis that combines both optical and laser-based modes for measuring the surface features. The principle of confocal microscopy relies on using point illumination and a pinhole in an optically conjugated plane in front of the detector to eliminate out-of-focus signals (Figures 5.2 and 5.3). The light emitted from a point source falls onto the surface; when reflected back, it reaches the detector. Because only the light that is very close to the focal plane can be detected, the image's optical resolution, particularly in the sample's depth direction, is much better than that of the wide-field

microscopes. However, because the light from the sample's reflection is blocked at the pinhole, the increased resolution is at the cost of decreased signal intensity, requiring long exposures. The greatest advantage is that the instrument's setup allows the examination of the paper document or artwork without the need for sampling the original material.



(**a**) (**b**)

Figure 5.2. (**a**) A diagram showing the principle of confocal light microscope operation. Chromatic confocal probes are single-point optical sensors built around a confocal coaxial setting that use chromatic dispersion and decoding to obtain the surface distance. The depth direction (y), measured in/through a transparent material, detects several interfaces between materials and, therefore, allows the thickness to be calculated. The metrological characteristics of chromatic confocal probes are close to those of stylus probes and are often used as a non-contact substitute on stylus profilometers (after (Blateyron 2011) in (Leach 2011). (**b**) The principle of CLSM VK-9700 operation (courtesy of Keyence Microscopy). With this instrument, light scans across a target in the x and y directions using a laser of choice. A light-receiving sensor gathers data from the reflected light on a pixel-per-pixel basis, in a roster fashion. After completing the scan in the X–Y planes, the lens is driven in the Z-direction and collects scans. From this information, the microscope can construct a high-resolution, fully focused 3D image with height information associated with each pixel. Source: Figure by author.

Figure 5.3. (**a**) An artwork with foxing stains is examined using a scanning laser microscope. This microscope permits carrying out the examination without removing samples from the original material. The metal ring shown here was used as a weight to ensure an even scan of an undulated paper; its use is optional. (**b**) An example of an image produced by confocal scanning laser microscopy. Paper fibers are covered by fungal conidia. Instrument used: Keyence, 3D Laser Scanning Microscope, VK-9700. Scale 10 µm. Source: Figure by author.

In confocal, white-light profilometry, the surface topography is measured in the x, y and z direction based on the confocal chromatic imaging principle. The operation of the instrument and the examples of data generated by these measurements are illustrated in Figures 5.4–5.6

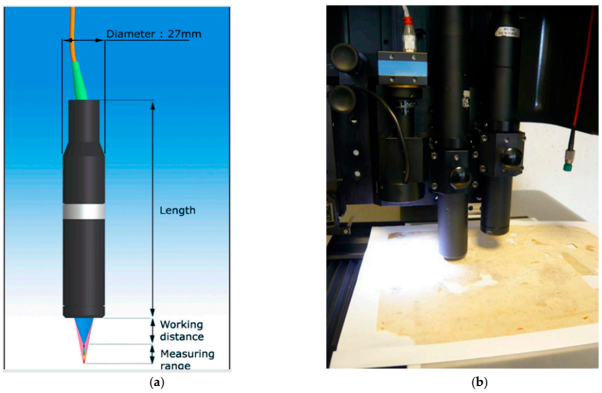


Figure 5.4. (**a**) Diagram of a stylus used in the white light confocal profilometer (WLP). Source: Courtesy of Alexandre Dembicki, Altimet Co. Spot size 2 µm. Area measured in most samples: 3 mm × 3 mm. Spacing between each scan: 1 µm. The scanning or rostering path, see Figure 5.5. (**b**) the 17th century Study Sheet, measurement of black fungal stainss with CLA Altisurf500. The advantage of this instrument is its ability to measure the intact object, without the need to extract samples. As only one point in the sample is illuminated at a time, 3D imaging requires scanning over a regular raster (i.e., a rectangular pattern of parallel scanning lines). The achievable thickness of the focal plane is defined by the wavelength of light and also by the optical properties of the specimen. The thin optical sectioning makes these types of microscopes particularly good at 3D imaging and surface profiling of samples. Source: Figure by author.

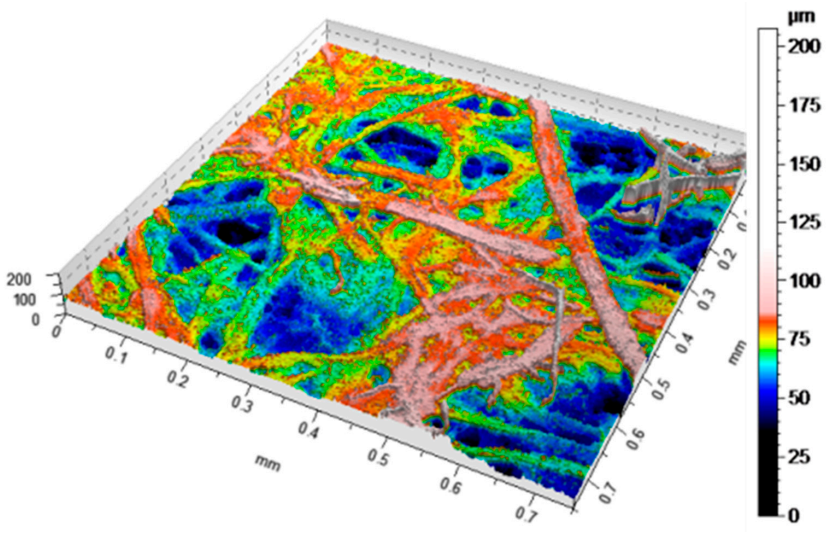


Figure 5.5. A 3D mapping of paper with black fungal stains generated by WLP. Note: spherical particles of conidia along the paper fibers shown in orange, at 'elevated' level. The map was created based on the reflected intensity of light emitted from the sensor. The light was sent through the probe and 'landed' on the samples' surfaces, which was then reflected by the sample. From this reflection, two types of information were gained; one was a topography map based on the calculated distance (illustrated here), and the other was an intensity map based on the percentage of reflected light. The latter is an indication of the percentage of light absorption or diffusion. Source: Figure by author.

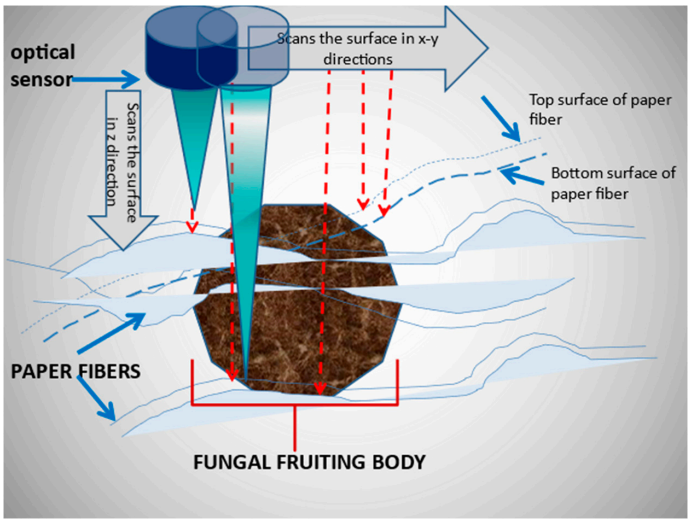


Figure 5.6. A diagrammatic illustration of the interaction of WLP with a large fungal fruiting body in paper. The large fungal inclusion is represented by a spherical, brown shape, and the light emission from the optical sensor/head, represented here by conical shapes, moving across the surface. Source: Reprinted from (Szczepanowska et al. 2013).

The surface morphology was studied further using *Scanning electron microscopy* (SEM). SEM and attached EDS detector are considered a standard method for the study of paper morphology, characteristic features of fibers used in paper

making, and the chemical composition of paper fillers, coatings, and other additives (Figure 5.7a,b). SEM typically measures surface topography on a much smaller, spatial scale than a stylus and optical instruments but can be used over relatively large surface-ranges. The main drawback of SEM is that it is essentially a two-dimensional technique, although 3D information can be obtained from some surfaces by tilting the sample and using a stereo-imaging or angle-resolved scanning techniques (Goldstein et al. 2003). SEM has been used in the examination of microbiological specimens for over five decades. The advantages of using SEM in such analysis have been widely reported (Collins et al. 1993; Bozzola and Russell 1999).

SEM can be enhanced with cryogenic capability (Cryo-SEM), allowing the examination of frozen samples. SEM can also be carried out in a low vacuum, in so-called environmental SEM, known also as variable-pressure SEM (VP-SEM), or field emission SEM (FE-SEM), which significantly reduce the buildup of electron charge that often occurs during SEM examination of paper. The micrographs generated during SEM examination used in this study demonstrate the capabilities and suitability of this technique in the examination of paper with bio-deposits, particularly for the detection of how the particulates are distributed and how they correlate with paper topography. The attachment of an energy-dispersive spectroscopy (EDS) detector to SEM permits chemical analysis and mapping of elements in the examined sample. In the course of SEM-EDS examination, the biogenetic reformation of the calcium filler in paper overgrown by fungi was detected (Figure 2.37).

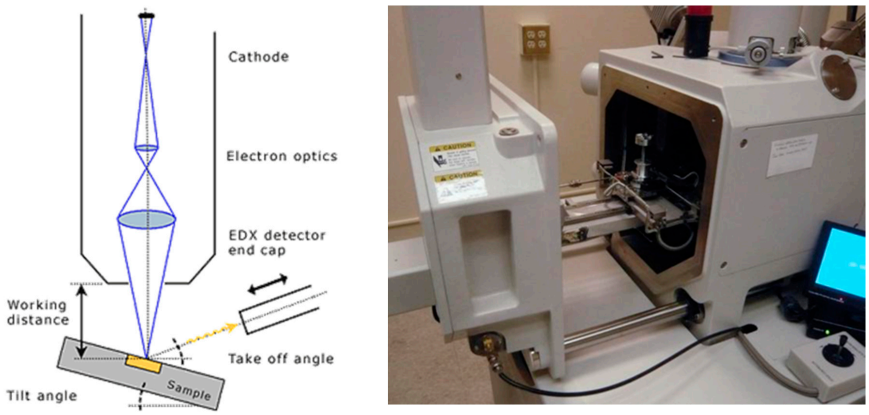


Figure 5.7. (**a**) Diagram showing the path of electrons falling on a sample inside SEM. Source: Reprinted from www.bruker.com (accessed on 1 May 2020). (**b**) The open chamber of the SEM where a sample is placed on a column, in the center of the chamber. Source: Figure by author.

The analysis of the chemical composition of the paper surface relies on infrared (IR) spectroscopy. This method allows the identification of individual components, binders, such as in sizing, and contaminants. Fourier transform infrared (FTIR) spectrometry is one of the most common spectroscopic techniques used within the

family of IR and is utilized to identify unknown organic and inorganic species by comparing the resulting spectra with spectra of known materials, available from reference libraries. The degree of surface sensitivity of the instrument is controlled by the types of accessory used to hold the sample. Measurements are usually collected with an attachment of the attenuated total reflectance (ATR) module. It provides information from the few microns of the surface and just below the surface. However, the quality of the spectra obtained depends upon direct contact with the sample; thus pressure applied to the sample to ensure contact measurements may alter the measured material; without pressure, the data may be of poor quality. FTIR-ATR requires a small sample of the original material, which is not always permissible in the examination of historically valuable material.

Photoacoustic spectroscopy (PAS) is another variant of IR spectroscopy, utilizing interferometry and aspects of photothermal technique for depth profiling of materials. It involves the application of a modulated IR beam to the surface, resulting in a modulated temperature change in the sample. The gas inside the test chamber reacts to the variations in temperature change, causing pressure waves, which are recorded. This technique, similarly to FTIR-ATR, requires sampling of the original material. Because the sample placed in the chamber is not destroyed during testing, the method is referred to as 'non-destructive' from an analytical perspective, but it is considered destructive simply because a microscopic piece needs to be extracted from the original artifact.

Raman spectroscopy has been applied in the analysis of paper, as a complementary technique to the IR-based group of instruments. Raman is sensitive to non-polar molecules, whereas IR spectroscopy is only sensitive to polar species. Raman uses a single-wavelength laser light to excite the sample, ensuring more intense light, better focusing and superior spatial resolution. The wavelength is selected based on the type of analyzed material. Recent developments in portable Raman technology permit the examination of cultural heritage objects in situ, making this technique even more attractive. There as several variations of Raman utilized in paper surface analysis; one of them is confocal Raman microscopy, used in the chemical assessment of inks. It scans the samples in the x, y and z directions, providing information on the distribution of pigments in coatings and under the layer of inks.

X-ray photoelectron spectroscopy (XPS) is a surface-sensitive technique providing quantitative elemental data and the chemical environment of these elements. This method involves irradiation of the surfaces of samples with nano-energetic X-rays and the analysis of emitted electrons based on their kinetic energy. A standard X-ray source causes cellulose to degrade, which is eliminated using the monochromatic X-ray source. XPS can determine the surface composition of coated papers, differentiating their mineral composition and binders regardless of the mechanical processing applied to the surface such as calendaring.

Secondary Ion Mass Spectrometry (SIMS) utilizes an energetic pulsed ion beam of cesium, gallium, or oxygen to remove molecules from the very outermost surface of the material in a sample (Figure 5.8. The very top of the examined surface is disintegrated upon contact with the particles used in the beam. The released fragments, atoms, clusters of atoms or molecular fragments provide information about the analyzed materials based on the ratio of molecular mass to charge. The SIMS combined with time-of-flight analysis is considered one of the most sensitive surface analysis techniques. The particles removed from atomic monolayers on the surface (secondary ions) are accelerated into an internal 'tube', and their mass is determined by measuring the exact time at which they reach the detector, thus explaining the term 'time-of-flight' (Figures 5.8 and 5.9). Three operational modes are available using ToF-SIMS: surface spectroscopy, surface imaging and depth profiling (Mogk n.d., accessed on 15 January 2021).

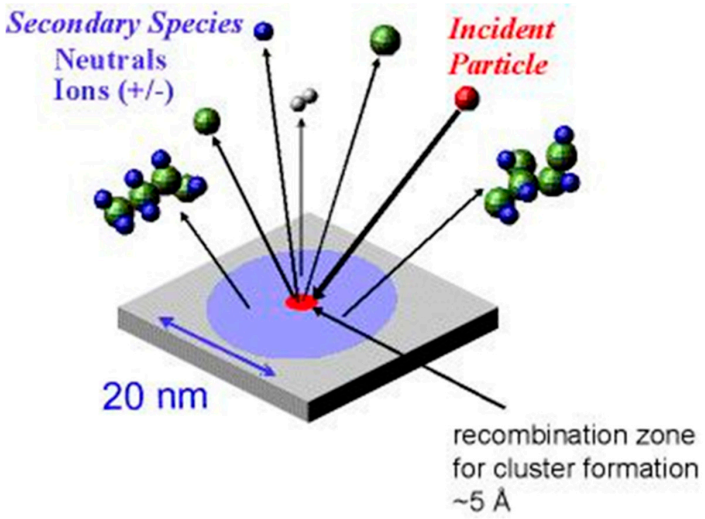
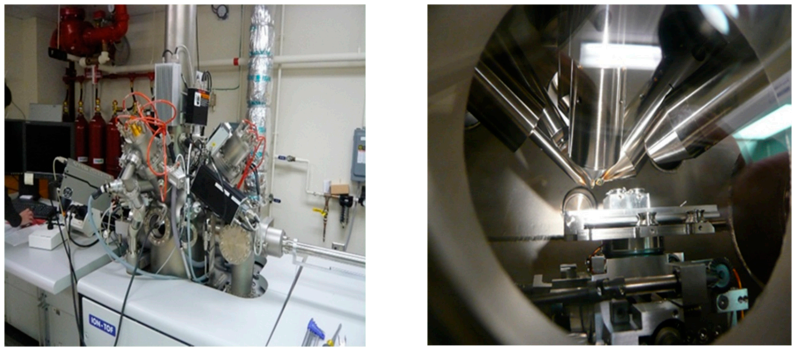


Figure 5.8. A schematic drawing showing ToF-SIMS principle of analysis. Secondary ion mass spectrometry (SIMS) detects very low concentrations of dopants and impurities. It can provide elemental depth profiles over a depth range from a few angstroms (Å) to tens of microns (μm). The sample of interest is sputtered/etched with a beam of primary ions (usually O or Cs). Secondary ions formed during the sputtering process are extracted and analyzed using a mass spectrometer (usually a quadrupole or magnetic sector). The secondary ions can range in concentration from matrix levels down to sub-ppm trace levels. Source: Reprinted from (Mogk n.d.) http://serc.carleton.edu/research_education/geochemsheets/techniques/ToFSIMS.html (accessed on 15 January 2021).



(a) (b)

Figure 5.9. (**a**) The overall view of the ToF-SIMS analytical instrument at NMNH Smithsonian Institution, Washington DC. It was used in the analysis of samples from Study Sheet 1. (**b**) The interior of ToF-SIMS shows a complex configuration of the detectors and examination stage. Source: Figure by author.

In the paper examination, ToF-SIMS is used to evaluate the spatial resolution and chemical composition of additives to the surface, such as surfactants or inks, among others. This technique was found to effectively show the distribution of filler in paper, but not the ink on the surface (Preston et al. 2000). ToF-SIMS was applied in this study to chemically identify the presence of organic, fungal growth in black-stained areas. Relying on the principles of ToF-SIMS spectrometry, which detects very low concentrations of impurities, the analysis detected the fungal residue, as shown in Figure 5.10 (Szczepanowska and Goreva 2014).

Although it has not been widely adapted by other researchers in the field of cultural heritage, ToF-SIMS might become a valuable technique in defining the relationship between fungal growth and the paper substrate. The chemical images generated by collecting a mass spectrum at every pixel as the primary ion beam by rostering across the sample surface are shown in Figure 5.10.

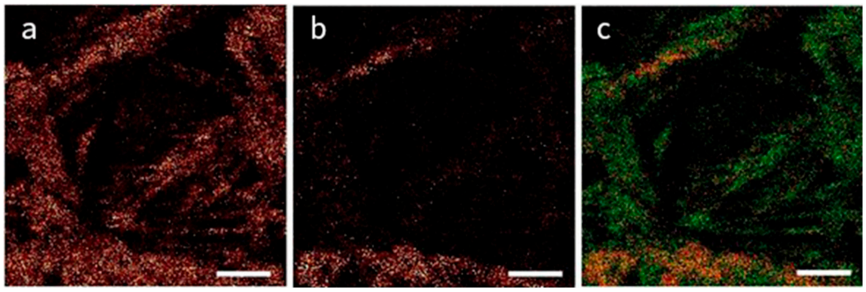


Figure 5.10. ToF-SIMS ion images of a paper fragment with black fungal stains show: (**a**) composite ion image of co-localized N-containing organic compounds; (**b**) ion image of C3H7O+ (an organic fragment of cellulose); (**c**) color overlay of results in A (green) and B (red). White scale bars = 40 μm. Detection of N-rich areas indicates the presence of organic fungi growth. Source: Reprinted from (Szczepanowska and Goreva 2014).

5.3. Methods Used in the Investigation of Fungi on Paper

Determining the presence or absence of fungi is typically the main objective of an examination of culture heritage that shows signs of biodegradation such as discoloration, staining, weakened structure combined with discoloration and colored, powdery deposits on the surface. The determination as to what types of fungi species are present is a secondary objective; the knowledge of what species of fungi are growing on cultural heritage does not change the strategy of their remediation and removal. Therefore, the review in this study is focused on detecting and recognizing the morphological features that indicate the presence of fungi and have a practical application for handling cultural heritage objects in museum conditions. Phylogenetic and chemical methods are briefly discussed as available techniques for identifying fungi species if there is a need to analyze fungal infestation in depth.

A general analytical approach may be classified into three main methodological sets for examining the morphology of aerial structures, mycelium, and fruiting bodies:

- microscopy (T LM—transmitted light, SEM)
- surface metrology for the detection and measurement of fungi deposits (CLSM, optical profilometers)
- cultivation of species (to isolate an organism of interest)

Approaches for more specialized analysis include:

1. Proteomics, relying on MALDI
2. Mass spectroscopy, MALDI, GCMS, and ToF-SIMS
3. Genetic sequencing and DNA methods
4. X-ray microtomography utilizing a synchrotron electron beam

5.3.1. Examination of Morphological Features Using Microscopy

Direct examination of fungi may be accomplished by either mounting hyphal strands or fruiting structures in a drop of solution on a microscope slide or by the use of 'tape lifts' if the surface of an artifact permits direct contact. If the latter, it needs to be executed with the utmost care to ensure that the paper of an artwork is not lifted along with the fungal residue.

Aerial hyphae may be harvested from a substrate with fine forceps, a surgical scalpel, inoculating needle, or sterile cotton swabs to be examined directly as collected or inoculated onto agar plates. Minute samples of infested paper or secondary supports may be treated in a similar manner.

In microscopic examination, using a transmitted light microscope (TLM), the samples are placed in a drop of sterile water or a 0.1% acid fuchsin solution. Gross morphological features are readily visible under a magnification of 450× to 500×. The size and features of fruiting bodies, such as the ascocarps of *Chaetomium* or the conidia of *Alternaria*, are easily identified. In addition, the features of hyphae, such as

the presence or absence of septations, branching and attachment of the conidiophores, may be observed.

Tape lifts use a 1–2-inch strip of clear cellophane tape touched against aerial mycelium (and not touching the surface of an object, if possible) growing on deteriorated paper or another support. The tape, with mycelium held fast to the adhesive side, is then pressed on a glass microscope slide and examined directly with a transmitted light microscope. With tape lifts, fungi producing distinct morphological features, such as *Chaetomium, Aspergillus, Penicillium, or Alternaria*, may be recognized immediately. The tape method, however, may not be appropriate for sampling fragile and deteriorated papers or surfaces of other cultural heritage objects because fragments of the original material may be removed in the course of sampling.

Surface Metrology Methods

Digital surface microscopy that relies on a white-light source captures an overview of fungi deposits, which guides more detailed examination using confocal microscopy, preferably in nano-scale units, laser scanning microscopy, and surface profilometers. Because fungi deposits on paper are measured using the same surface metrology instruments that are used in the examination of paper, confocal laser scanning microscopy and optical profilometers, both methods and instruments are described in detail in Chapter 5.2, detailing the examination methods of paper. However, considering that fungi aerial mycelium and spores are usually in the range of 2–9 microns, nano-scale measurements are the most accurate in capturing the morphological features of fungal spores.

Culturing of Sampled Fungi

Samples of living fungi may be cultured on agar-based media and grown prior to an examination for two reasons: one is to assess their vitality, and the other is to determine the specie for further morphological characterization, if needed. The dried specimens may be scraped from paper or another substrate with a sterile needle or scalpel and inoculated directly onto agar-based media. Several media are used for culturing fungi, the most common of which are corn meal agar, potato dextrose agar, Sabouraud's dextrose agar and Czapek Dox agar. Cultures are typically incubated at room temperature (22–24 °C) for several days, depending on the species; 22–24 °C is an optimal temperature in which fungi growing on paper thrive.

It is often necessary to subculture original samples that were most likely composed of more than one species in order to select an organism of interest. When hyphal strands begin to appear, they are removed from the original culture and transferred onto new media to prevent cross contamination of species.

5.3.2. SEM, the Scanning Electron Microscopy

The use of light microscopy for the exact identification of some fungi, especially *Fungi Imperfecti*, often does not produce precise enough results. For detailed determination of some features, such as spore/conidial shape, precise dimension, and ornamentations that may occur along the conidiophores, scanning electron microscopy is preferred.

The scanning electron microscope is a useful tool that shows the surface morphology and topography of objects, such as the wings or scales of an insect, the surface of petals, leaves and stems, and of particular interest in this work, the vegetative and reproductive structures of fungi. The electron beam is directed onto the sample and produces a topographic view of the surface. The column of a typical SEM is inside the metal housing of the instrument. At the top is an electron gun that produces a beam of electrons that travel down the column to the specimen. The column is equipped with electromagnetic condenser lenses that control and accelerate the beam of electrons; the beam can be concentrated on a small area of the specimen to be examined at high power or can be spread out over a large area to be observed at lower power. The objective lens system, acceleration, and pressure in the chamber are controlled by the focusing knobs on the control panel.

To obtain a view of the entire top surface of the specimen, the incident beam from the electron gun is scanned across the specimen in a raster pattern in synchrony with the scanning of the cathode ray tube (CRT) beam across the screen. The magnification of the image and the portion of it visible on the screen are controlled by electronic circuits operating on the signal produced by the detector. In order to view different parts of the specimen, the holder with the specimen can be moved, rotated, and tilted into different positions. The digital images are then captured by a number of software programs, computerized, stored and/or printed, and analyzed, if additional detectors are attached to SEM.

Traditionally, the biological samples of fungi required preparation before their examination with scanning electron microscopy, mainly to retain their morphological features when exposed to high pressure in the SEM chamber, which would result in deformation and a loss of identifying features. This process required the preparation of small agar embedded samples (2 mm^2) supporting mycelium with fruiting structures to undergo a 'fixation', dehydration in alcohol or acetone, at a critical drying point of fungi specimens. The SEM microscopes today, especially those that operate in low vacuum, eliminate the lengthy preparation process. They are typically referred to as 'environmental' or variable-pressure SEM microscopes.

The scanning electron microscope (SEM) employs the use of electrons rather than photons of light as the energy source for the production of photomicrographs or digital images. Because the electrons are energetic, magnification within a much higher range and with greater resolution is attainable. If fungi are observed in the environmental or variable-pressure (SEM-VP) microscope, coating the samples is not required because the vacuum is low and thus leads to a reduced beam interaction

with the sample, in turn reducing the electron charge. The accelerated voltage used in environmental SEM is low, reaching 3–5 kV, which is much lower than 15–50 kV as in traditional SEM; thus, the samples remain stable and do not undergo deformation of their morphological features. The lower kV energy permits to get closer to the surface, providing more information about surface features. The advantage of the environment SEM was pointed out by Collins already in 1993 (Collins et al. 1993). The digital images of various hyphal and fruiting structures captured electronically are referred to as SEM micrographs. In this study, such images were collected under varied magnifications ranging from 35× to over 5000×.

5.3.3. X-ray Microtomography on Synchrotron Beamline

X-ray microtomography was carried out using an electron beam at synchrotron designated beam line ID19, at the European Synchrotron Facility ESRF in Grenoble, France. The ID19 is a high-resolution diffraction topography beamline. It is a multi-purpose long (145 m) imaging beamline for radiography (absorption and phase contrast imaging) microtomography designated for diffraction imaging topography, analyzer-based imaging (Figures 5.11 and 5.12). The beamline can work in the energy range of 6 to 100 keV, but most of the measurements of paper were performed in the 10–35 keV range. The monochromatic beam originates from a double Si 111 crystal monochromator ($\Delta E/E\ 10^{-4}$).[10]

The X-ray absorption tomography has been used during the past two decades to characterize the architecture of cellular materials of a different nature (Maire et al. 2003). However, its application to the characterization of biodeteriorated cultural material is a novel approach. Ideally, the best way to analyze the architecture in the modeling of cellular materials is through the use of the three-dimensional information provided by this new, tomographic technique to produce a finite-element model of the actual architecture. The general principle of the tomography technique relies on an X-ray source and employs a rotation stage and a radioscopic detector. A complete analysis is made by acquiring a large number of X-ray absorption radiographs (in this case, it was ca 1500 slices) of the same sample under different viewing angles. A final computed reconstruction step is required to produce a three-dimensional visualization of the local absorption coefficients in the material, which gives, indirectly, a picture of the structure. This technique was utilized for the analysis of stains on the 17th-century Study Sheet (Figure 5.13, and 6.17, 6.18).

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[10] www.esrf.eu (accessed on 15 January 2012).

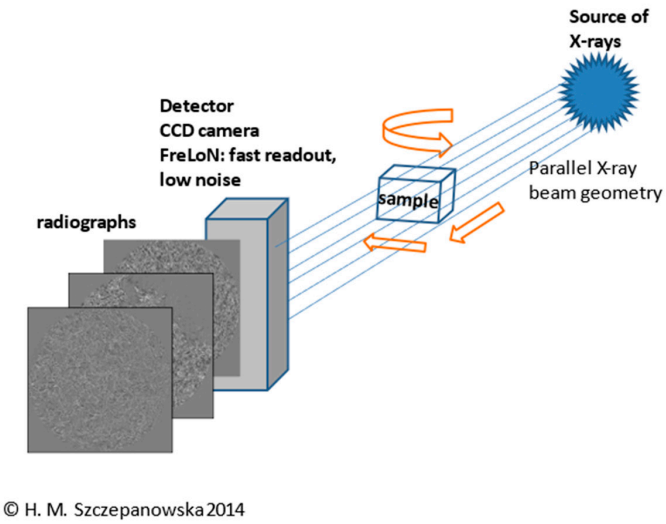


Figure 5.11. Beam directed to the sample. A schematic (not to scale) diagram of the X-ray microtomography experimental setup. The synchrotron produces a parallel X-ray beam so the sample is not magnified on CCD detector, FreLoN camera. The parallel, monochromatic beam setup ensures accurate quantitative reconstruction of the microstructure in the sample. Source: Reprinted from (Szczepanowska et al. 2015, JAAS, p. 653).

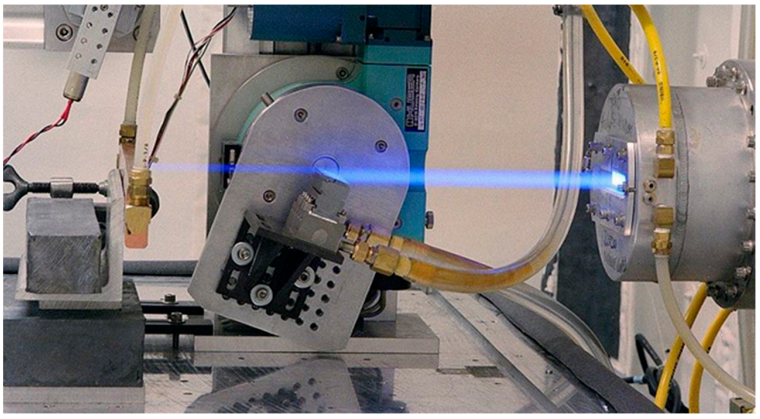
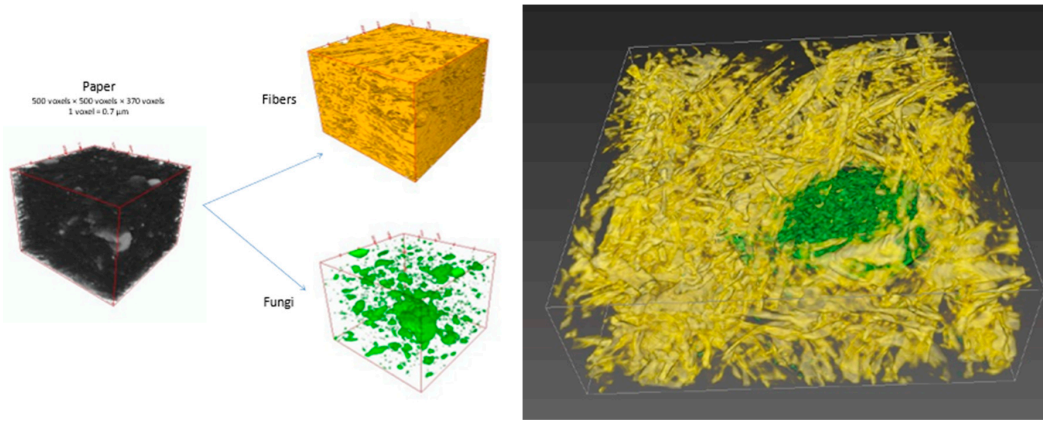


Figure 5.12. The beam is directed to the sample from the right and captured, on the left side of this image, by a radioscopic detector CCD FReLeon camera. Source: Reprinted from ERSF website, open source accessed on 15 January 2012.



(**a**) (**b**)

Figure 5.13. (**a**) An illustration of the 'separation' process of paper matrix and fungi before an overlay is constructed as seen in Figure 5.19. Source: visualization constructed based on XµCT data courtesy of Dr. J. Bloch. (**b**) A 3D visualization of fungal particles in the paper matrix constructed based on X-ray microtomography data from one of the fungal stains on Study Sheet No. 1. Source: Reprinted from (Szczepanowska et al. 2015).

The parameters of the measurements relied on the 6 MeV electrons that produced hard X-rays. The absorption images were collected using a fast-readout 2048 CCD camera. The images included in this text were generated from 900–300 slices (Szczepanowska et al. 2015). The ultimate outcome was to generate a 3D visualization of fungal deposits in the paper matrix. One of the stains on Study Sheet No. 1 is shown in Figure 5.13.

5.3.4. DNA Technology, Genetic Sequencing

Although the utilization of morphological and physiological characteristics remains the foundation for the classification of fungi, the use of DNA technology is rapidly emerging as a reliable means of species identification. Now more than ever, the ability to identify fungal species is imperative in the areas of animal and plant pathology; therefore, numerous fields of medicine and agriculture study invasive fungi-induced infections; the field of the conservation of our cultural heritage is following these steps. The use of morphological data coupled with molecular genetics offers a valuable set of criteria upon which fungal identification is possible (Begerow et al. 2010; Powers-Fletcher et al. 2016; Abdel-Azeem et al. 2016).

DNA bar coding uses short, standardized segments of the fungus genome for the purpose of identifying species. Also known as DNA-based identification, this method is particularly suitable for fungi with large numbers of species or those groups for which the identification of species is difficult to determine due to the subtle differences within their populations (Burnett 2003). This method is particularly suited for the genus *Aspergillus*, which includes over 335 species (Gomez et al. 2016). In 2005, when the first high-throughput sequencing was introduced, *Aspergillus* genomes were studied using this technique, thus officially establishing *Aspergillus*

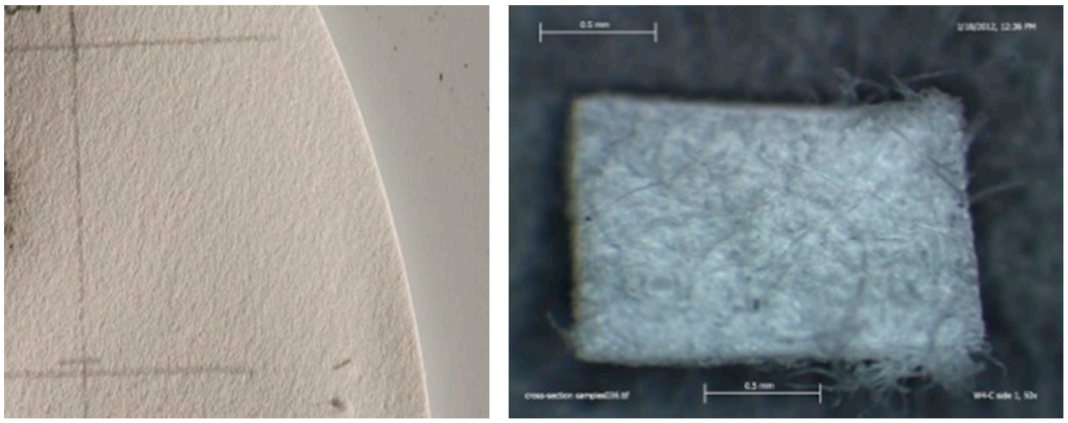
as the leading filamentous fungal genus for comparative genomic studies (Shamim et al. 2016).

5.4. Practical Application of Paper Surface Examination

To illustrate the practical application of methods and techniques whose principles were discussed earlier as typically used in the examination of paper and bio-infested paper artifacts, a closer look is offered into the characterization of papers discussed in this monograph and used in the light experiment (Chapter 2.4): Whatman4 (W4) laboratory-grade filter paper and Winsor & Newton (W&N) watercolor paper (Figures 5.14 and 5.15). It is a summary and compilation of complementary information offered by each technique about the surface topography and matrix of papers. Although both types of paper appeared to display rough and fibrous surfaces, closer microscopic examination revealed differentiation in fiber compactness and smoothness between the sized paper of W&N when compared with the unsized, fibrous filter paper of W4.

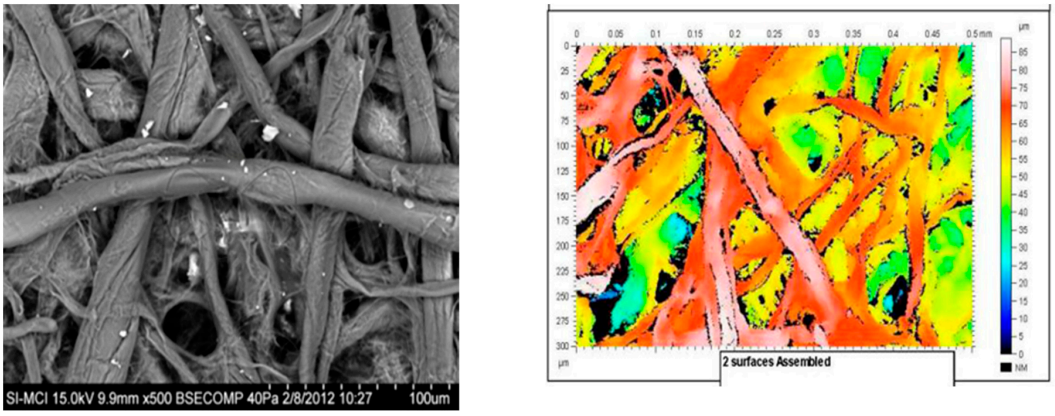
The surface morphology and topography are particularly important when fungi infestation is considered, as discussed in detail in Chapter 2.2.3. To reiterate, a rough surface provides a natural environment for air-disseminated fungal spores as they land, protecting them from removal by air movement or other mechanical means, such as brushing.

The macro- and stereo-microscopy of the W4 paper (Figure 5.14) showed the fibrous surface of well-defined fibers that can be distinguished as individual strands. The fibers' surface morphology examined with SEM-VP revealing well-preserved features, thus indicating a careful manufacturing process of the paper pulp (Figure 5.15). As anticipated, no presence of sizing was detected in EDS analysis. Additional information about the fiber distribution and surface topography was obtained by measuring the paper features with the white-light profilometer (Figure 5.15b). The metrology map illustrated interrelations between fibers, showing those that are on top, coded in warm colors, orange and yellow, and those in a lower plain, coded in green or blue color. The software Mountain Map, which is used for processing surface metrology data, shows peaks and valleys in the measured materials, in a similar fashion as geographic maps are showing mountains in warm colors and valleys in green to blue. Figures 5.14 and 5.15 show surface characteristics of Whatman4 paper documented with various examination techniques; surface features directly impact fungal growth.



(**a**) (**b**)

Figure 5.14. (**a**) Macro-image of Whatman 4 paper shows fibrous, rough surface that provides natural environment for air-disseminated fungal spores, protecting them from removal by air movement. Leica D-lux digital camera, 1:1. (**b**) A stereo-micrograph of Whatman 4 paper sample, scale bar: 0.5 mm. The fibrous surface topography is well defined. Source: Figure by author.

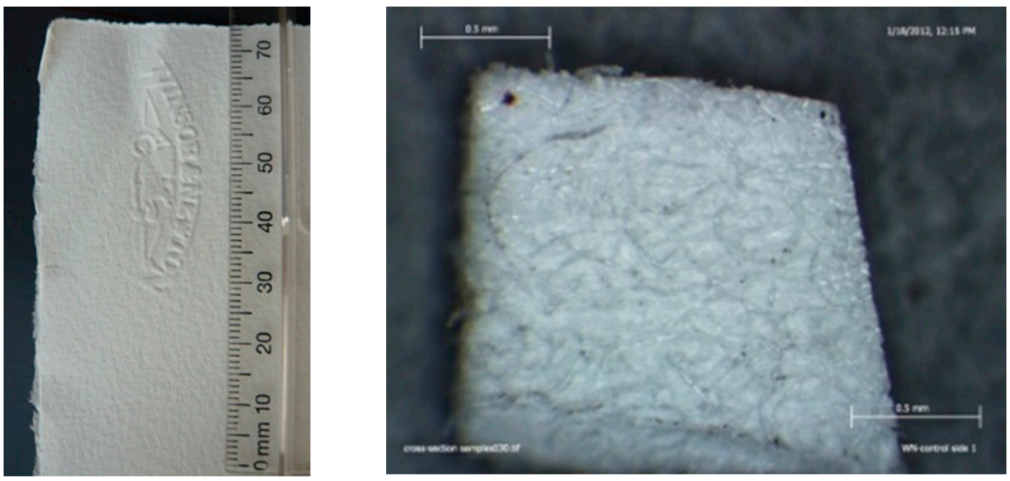


(**a**) (**b**)

Figure 5.15. (**a**) The surface morphology captured on SEM-VP micrograph shows well-preserved fibers. There is no evidence of any additives, which was confirmed with EDS analysis. SEM-VP, scale bar 100 µm. (**b**) The 3D surface topography of an area 500 µm x, 300 µm y, 85 µm h, show well-defined individual fibers, loosely interconnected and well-articulated. Surface morphology was generated based on data collected by white-light profilometer (WLP). Source: Figure by author.

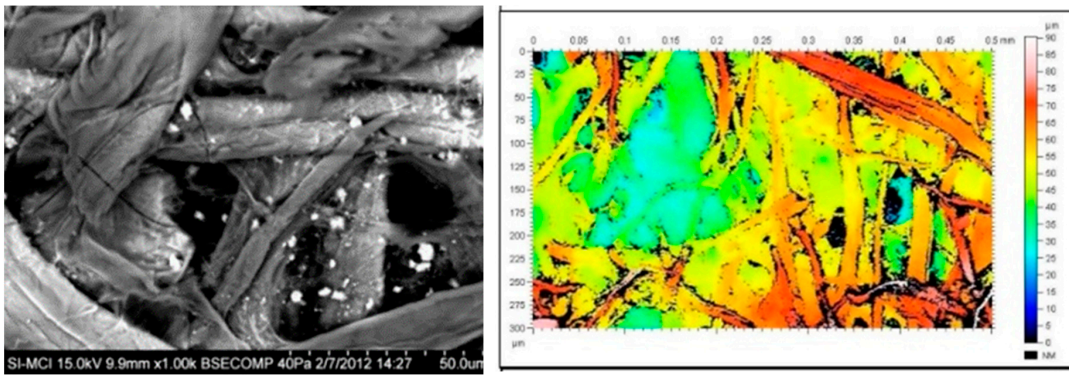
The same examination protocol was applied to the documentation of W&N paper, from macro- to micro-imaging and to profilometry measurements. Additionally, similarity to the results obtained from the examination of Whatman 4 paper, stereo-microscopy and SEM-VP micrograph analysis of W&N paper showed well-defined fibers and their surface morphology (Figures 5.16 and 5.17) The SEM-VP micrographs revealed additives in W&N paper, which were analyzed chemically. The inter-relationships of fibers showed on the maps generated by the white-light profilometer areas confirmed greater compactness, based on the fibers' topography,

by a 15 micron difference in the highest points. In the case of W4, the highest point was 85 micron, while in W&N, it did not exceed 70 microns.



(**a**) (**b**)

Figure 5.16. (**a**) The macro-image of W&N paper. The surface, under low magnification, was rough and fibrous. Leica D-Lux digital camera, 1:1. (**b**) The stereo-micrograph of W&N paper showed a fibrous surface, yet more compact than W4; scale 0.5 mm. The compactness prevented water sorption and did not promote fungal growth. Source: Figure by author.



(**a**) (**b**)

Figure 5.17. (**a**) The surface morphology of W&N captured on SEM-VP micrograph shows well-defined and preserved fibers. The small particles in the paper matrix indicate calcium-based filler, based on EDS data. SEM-VP, scale 50 µm. (**b**) The 3D surface topography of area 500 µm x, 300 µm y, 85 µm h. The highest detected level of fibers reached 70 µm. Surface morphology was generated based on data collected by WLP. The fibers were more compact and the surface was smoother in W&N than in W4. Source: Figure by author.

The evaluation of surface topography and morphology is typically the first step in assessing paper's features, which may contribute to predicting patterns of fungi infestation.

6. Genesis and Morphology of Fungal Stains; Investigation of Three Case Studies

The three case studies were selected to illustrate the variations in bio-infestation of different paper substrates and advantages of a systematic protocol of investigation that could lead to the development of mitigation strategies. All three objects were examined following the proposed sequence: (1) provenance, (2) context of biodeterioration, (3) characteristics of substrate (paper), (4) characteristics of surface, and (5) fungi-stain-formation patterns. The methods and analytical instruments discussed in the preceding chapters were applied. The aim of this case study was to understand whether (or not) papers' composition impacted the formation of fungal stains and what factors could have contributed to the stains' occurrence in the first place. To achieve this goal, an attempt was made to find correlations between fungi growth, and the additives in paper, types of fibers used in paper manufacturing, methods of surface finish such as sizing, its presence or absence and other factors that may have impacted the fungal development and stain formation. Guided by these objectives, three papers were selected that represented different techniques of paper making, two Western-type papers, hand-made and machine-made, and one Oriental-type paper. Therefore, the fiber composition differed, as did the surface finish. In addition, the technique of the artistic impressions on two of the papers represented a woodblock print and a metal engraving. One artwork, printed on the Western-type paper, was a 1920 engraving on commercially produced paper, referred to as machine-made paper. The Japanese 1847 woodblock print was created on the Oriental-type, kozo paper. The third paper, Study Sheet No. 1, did not bear an artwork painted or imprinted. The oldest paper, the 17th-century, hand-made, Western paper, was obtained from the Archives in Mdina, Malta. Although it did not bear an artwork, an inscription in black ink of the word "Fra' and a red "V"-shaped mark, both inscribed on one side of the paper, served as the dating clues. The fungi encountered on all three papers showed great variation in their growth pattern on both surfaces of each sheet (recto and verso) as well as patterns of degradation and staining (Figure 6.1, Figure 6.2 and Figure 6.3).

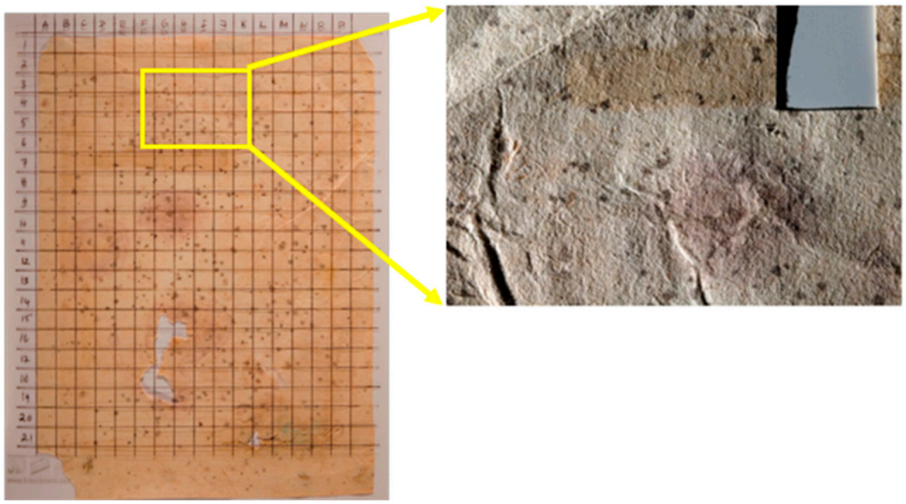
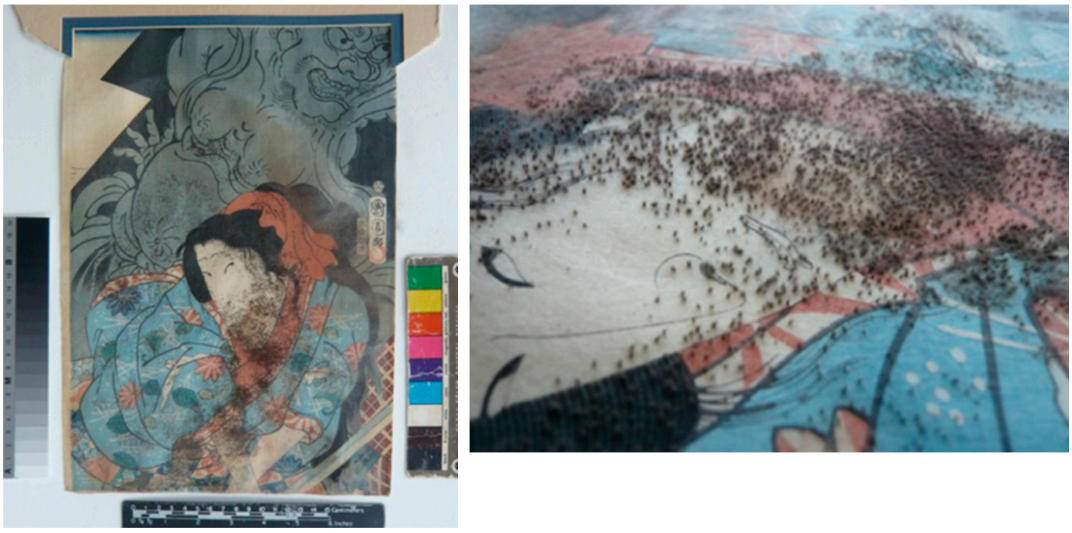


Figure 6.1. (**a**) Case Study No. 1: Study Sheet, 17th century. Dimensions: H: 23.5 cm, W: 17 cm. Texture and surface finish of recto and verso differed; recto was fibrous, verso was smooth. Paper was soft and pliable, off-white. Adhesive splotches were present on both sides. Fungal stains were scattered throughout, more prominent on recto and less visible on verso. (**b**) A detail of the sheet shows variations in fungal stains: scattered, small black stains, brown and purple of various dimensions. Different fungal species produced the pigmentation. The images were taken with a Leica D-lux digital camera, 1:1. Source: Figure by author.



(**a**) (**b**)

Figure 6.2. (**a**) Case Study No. 2: Japanese woodblock print, 1847. Recto of the sheet and verso were identical; both showed well-defined fibers. Paper was soft and pliable, white in color. Dark-brown fungal fruiting structures were located on the surface, on recto and verso. H: 34.3 cm W: 24 cm. (**b**) A detail of recto in raking light (side view) shows protruding dark fungal fruiting structures; image taken with a Leica D-lux digital camera, 1:1. Source: Figure by author.

Figure 6.3. (**a**) Case Study No. 3: a 1920 engraving. Recto and verso of the sheet were identical in appearance; both surfaces were smooth. Paper was crisp, light buff color. Black fungal stains were located along the top on recto and verso. H: 35.2 cm, W: 27.5 cm. 1847, 1:1. (**b**) A detail of the artwork shows black stains along the top edge. The same pattern was observed on verso. Images taken with a Leica D-lux digital camera, 1:1. Source: Figure by author.

The examination protocol of each study case included a description of the artwork's provenance, characterization of paper fibers and any additives that may have had an impact on biodeterioration and bio-staining. The paper topography and morphology of fungal deposits were examined from an interfacial perspective and in relation to the patterns of stain formation. The investigation of each case concluded with establishing patterns of the bio-staining formation. Taking into consideration all these factors, methods of remediation were proposed in Chapter 7.

The examination protocol applied to the case studies can be formalized as:

1 Description of the artwork provenance, art medium, and any markings present
2 Characterization of biodeterioration present
3 Physical characterization of paper fibers and chemical testing of sizing and additives
4 Analysis of fungal deposits and their interfaces with paper matrix
5 Patterns of stain formation.

The analytical techniques used in the characterization of fungi stains and interfaces of fungi and paper included:

1 Transmitted light microscopy (TLM) to characterize paper fibers, fungi identification, and evaluation of the surface characteristics.
2 SEM-VP to study the surface morphology of paper and bio-deposits in paper matrix to determine relationships between fungi and paper. SEM-EDS was used in the elemental analysis of paper additives.

3 Confocal laser scanning microscopy (CLSM) to characterize the surface morphology of biodeteriorated paper and measurements of fungi deposits.
4 White-light confocal scanning profilometer to measure surface topography (WLP) and to determine interfacial relationships between fungi deposited and the surface of paper
5 X-ray microtomography carried out at the European Synchrotron Radiation Facility (ESRF), for the characterization of fungi and paper interfaces in the matrix of paper
6 ToF-SIMS to detect fungus deposits on paper

6.1. The 17th-Century Study Sheet, Maltese Collection, Case Study No. 1

6.1.1. Provenance

A single sheet of paper was acquired from the Archives of the Cathedral Museum in Mdina, Malta, in 1999, for the study of fungi-induced bio-staining; fungi stains were already present at that time. The visual examination of the sheet indicated that it was removed from a book cover, and it appeared to be used as a so-called book-end. These old papers were typically removed by the staff during rebinding at the restoration workshop in Mdina, Malta (Figure 6.4). The conclusion regarding the provenance of this sheet and function it played in the past was reached based on the presence of adhesive splotches on one side of the sheet, which indicate areas of paper attachment to a book cover. The smooth finish of the paper surface was most likely impaired by pressure applied during final assembly of a book.

A fragment of an inscription on one side, 'FRA' in black ink and a red 'V' mark, guided dating of this paper. The elemental analysis of the red mark using SEM-EDS confirmed the presence of lead, the main component of red lead, known as minium, which was used around the 17–18th century (Thompson 1956). The inscription written in ink, which in reverse reads "FRA", indicated a prefix title given to an Italian monk, or friar, pointing to the affiliation of the owners; Malta was one of the territories under Christian monastic influence. Since 1520 Malta was a seat of the Christian Order, Knights of Malta used Italian as their official language. The paper sheet dimensions are: (H) height 233 mm and (W) width 170 mm.

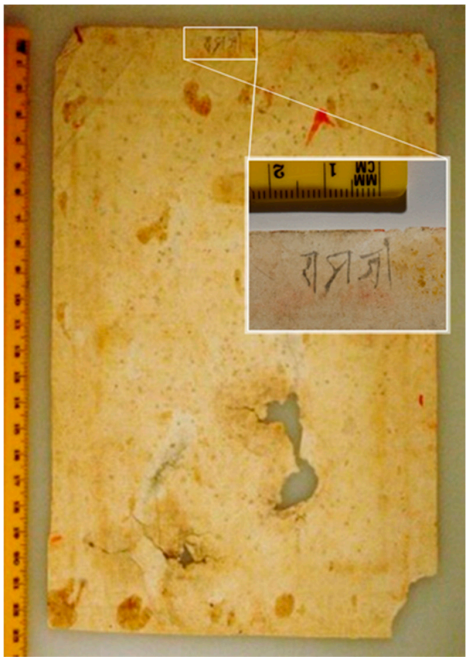


Figure 6.4. (**a**) The verso of the 17th c. Study Sheet shows brown glue splotches in the corners of the sheet. An impression around the perimeter indicated that the sheet was attached to the interior of a book cover. Two markings that aided the dating are at the top portion. (**b**) A detail of Sheet 1, verso, shows the inscription 'FRA' pointing to an Italian monastic provenance of the paper. The chemical composition of the red marking showed the presence of cinnabar. Source: Figure by author.

6.1.2. Characterization of Biodeterioration Present

Biodeterioration was demonstrated as fungal stains and structural deterioration of the paper matrix. The predominant black stains were scattered throughout the paper sheet. The green, pink, and brownish, liquid-like stains were located in the central part; some were associated with structural degradation of the paper matrix (Figure 6.5). The structural degradation, evident as weakened fibers and losses in paper, based on their location in the center of pink and brown stains, indicated that the enzymatic activity of fungi metabolites was most likely the cause. In the area of brown stains, around the paper loss, the presence of fruiting structures of cellulolytic fungi was confirmed through the microscopic analysis. The initial microscopic examination of other stains, pink and green, did not reveal bio-residue; thus they were excluded from further investigation. The black stains were prominent on one side of the paper, which was porous and fibrous with a rough surface; it will be referred to in text as 'recto' (Figure 6.1, and 6.6). On the reverse side of paper, referred to as 'verso', small patches of pinprick-sized black spots were located at the bottom, near the most severely degraded paper, which was also discolored by brown staining (Figure 6.4a).

To ensure repeatability of sampling and the referencing of examined areas, a grid of 10 mm $\times$ 10 mm squares was created as a template to impose on the sheet

(Figure 6.5). The examined areas were located on the sheet by coordinating both a letter and numerical reference; for example, the top left corner of the sheet was A1, the next square below was A2, etc.; when progressing toward the right, B1, C1, etc. This grid system of coordinates was applied to all three study cases, identifying the location of the examined stains with both a letter and a number.

The examination of paper in transmitted light indicated three types of bio-deterioration induced by fungi: 1) small, black scattered stains throughout the entire sheet (stain type A); 2) liquid-like, larger stains, pink, green and brownish, marked green and pink; and 3) localized, pinhole-sized, black inclusions in areas near paper losses, marked yellow (stain type B). All types of stains are marked in Figure 6.5.

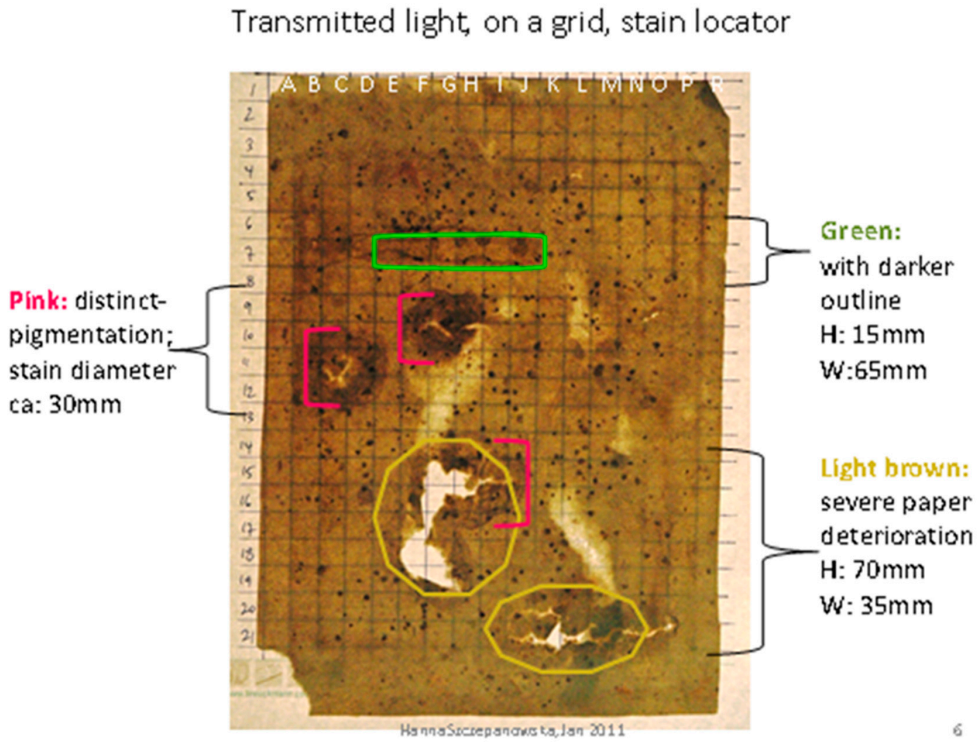


Figure 6.5. The examination of paper in transmitted light shows the location of three distinct types of fungi-induced stains. Source: Figure by author.

6.1.3. Physical Characteristics of Paper Fibers, Sizing, and Additives

The characterization of the physical properties of paper was carried out with focus on features that may have contributed to the fungal growth and pigmentation. This included the morphology and topography of surfaces on each side of the paper sheet (recto and verso), types of fibers used in paper making and sizing. Splotches that appeared to be glue were also tested to assess whether they contributed to bio-staining. The morphology, topography and fiber content were examined and measured using SEM-VP, WLP, and CLSM mapping. The chemical analysis of red pigment in the 'V' mark was carried out with SEM-EDS and indicated lead; minium is

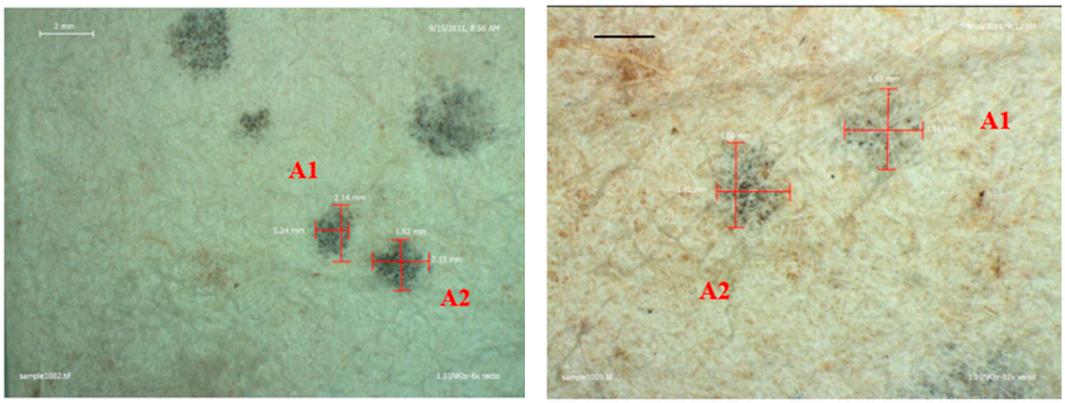
a red, lead-based pigment. The analytical results of the pigment contents contributed to dating of this paper.

The predominant fiber used in this paper was cotton, indicated by luster, smooth surface, and the characteristic twist of fibers. The well-preserved features of fibers also pointed to hand-processing of paper pulp, which is gentler on fibers than mechanical processes, thus enabling the identification of fibers. There was no evidence of internal sizing. The smooth side, however, with adhesive splotches, presumably contained some amount of superficial adhesive if it was removed from a book cover.

The morphology of fungal deposits in bio-stains was characterized using transmitted light microscopy and SEM–VP; spores and other fungal structures were measured using white-light profilometry (WLP) and CLSM. The cross-sections of paper with fungal inclusions in the paper bulk (matrix) were examined with X-ray computed-tomography (Xμ-CT) to recreate a 3D visualization of interfaces of paper fibers and fungi; the data were collected at the European Synchrotron Facility (ERSF) in Grenoble, France (Figures 6.17 and 6.18). The details of the synchrotron analysis on synchrotron beam were reported in a separate publication (Szczepanowska et al. 2015).

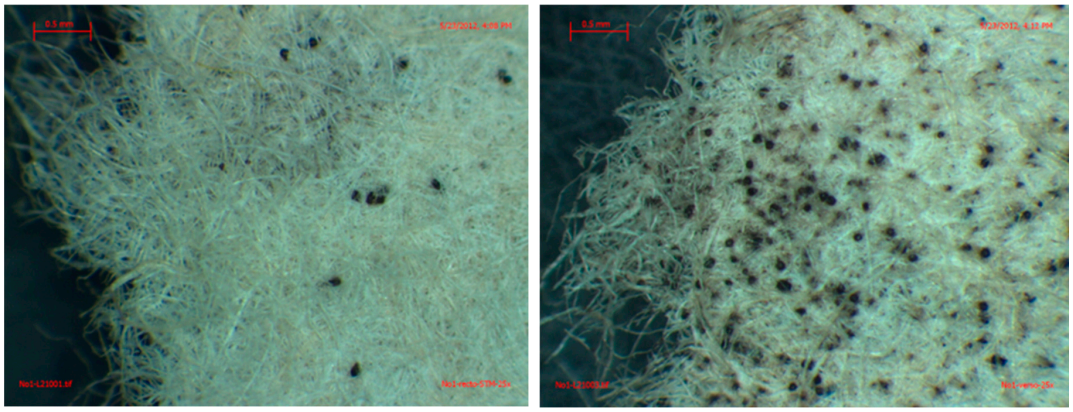
6.1.4. Investigation of Fungal Deposits and Their Interfaces with Paper

Although several types of stains were identified and attributed to fungi activities, the analysis focused on two distinctly different, black-pigmented fungal stains. One type, labelled as type A, was visible as scattered spots with uneven edges on one side of the sheet, and a corresponding smaller footprint on the reverse of the paper (Figure 6.6). The other one, labeled type B, was formed by a congregation of small, needle-prick-sized, well-defined, black inclusions (Figure 6.7). The type A stains were scattered on both sides of the paper, and type B stains were present mainly on the reverse of the paper. The protocol of examination followed the sequence outlined earlier as the proposed systematic approach to the evaluation applicable to biodegraded objects.



(**a**) (**b**)

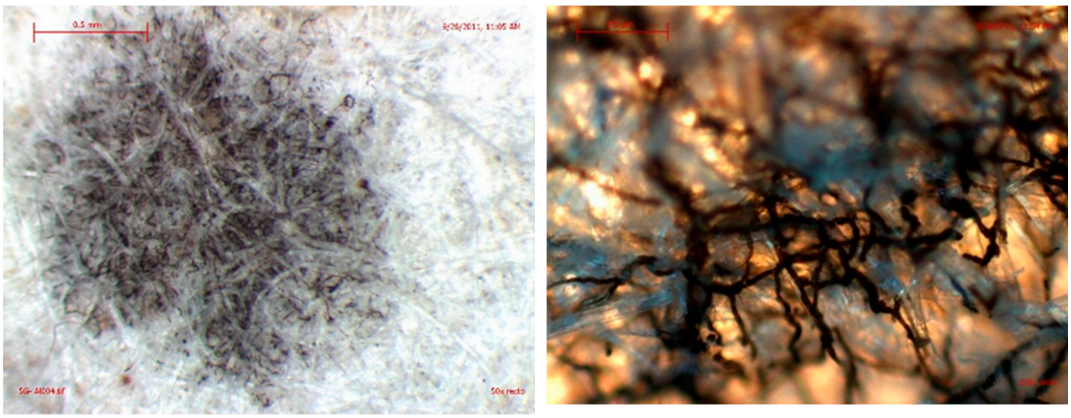
Figure 6.6. (**a**) Type A stains, recto, location N11, dimensions: A1, top stain 1.24 × 2.14 mm, A2, bottom 1.92 × 2.13 mm. Stereo-micrograph, scale 2 mm. (**b**) Stain A; reverse of the same stain (N11); stain dimensions: top 1.41 × 1.60 mm, bottom 1.51 × 1.53 mm. Both stains were smaller on verso and showed lesser amounts of pigmented cells. Stereo-micrograph, scale 0.5 mm. Source: Figure by author.



(**a**) (**b**)

Figure 6.7. (**a**) Type B stains, recto, location of stain, near the main loss of paper, bottom portion of the sheet, L21. Small, black fungal inclusions are less prominent on recto than on verso. Stereo-micrograph, scale 0.5 mm. (**b**) Type B stain, verso of the same area as in (**a**) L21. Fungal fruiting structures are well defined, partially embedded in the paper matrix. Stereo-micrograph, scale 0.5 mm. Source: Figure by author.

In the next phase of the examination, following the stereo-microscopy, the paper samples were analyzed in 3D reflected light microscopy (Figure 6.8), transmitted light optical microscopy and followed with SEM microscopy; the same sequence was applied to the examination of both stain types A (Figures 6.8 and 6.9) and B (Figures 6.10 and 6.11). The size of the paper samples extracted for examination ranged from 1 mm to 2 mm.

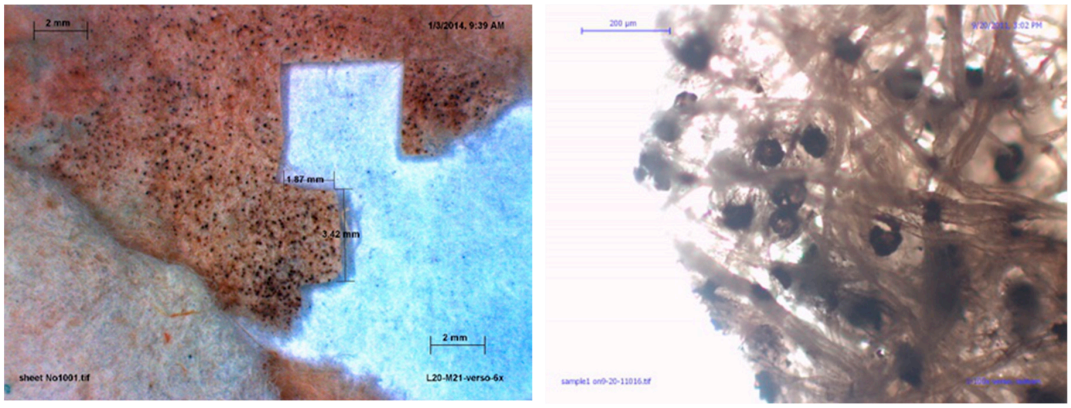


(**a**) (**b**)

Figure 6.8. (**a**) Stereo-micrograph of stains, type A, in location 5 L, on recto. Average size of stains 4.30 mm × 5.17 mm, Scale bar: 2 mm. The black fungal cells, pigmented spores and mycelium accumulated in the niches created by paper fibers, on the surface and just below the surface. Optical surface analysis, stereo-micrograph, scale 0.5 mm. (**b**) Paper fibers were utilized by fungi as physical anchorage. The fungal cells show pigmented walls, melanized, anastomose, partially connected to paper fibers and branching out within the paper matrix. Fungal cell size: 5.7–6.5 µm. Scale 0.1 mm; 400×. Transmitted light micrograph. Source: Figure by author.

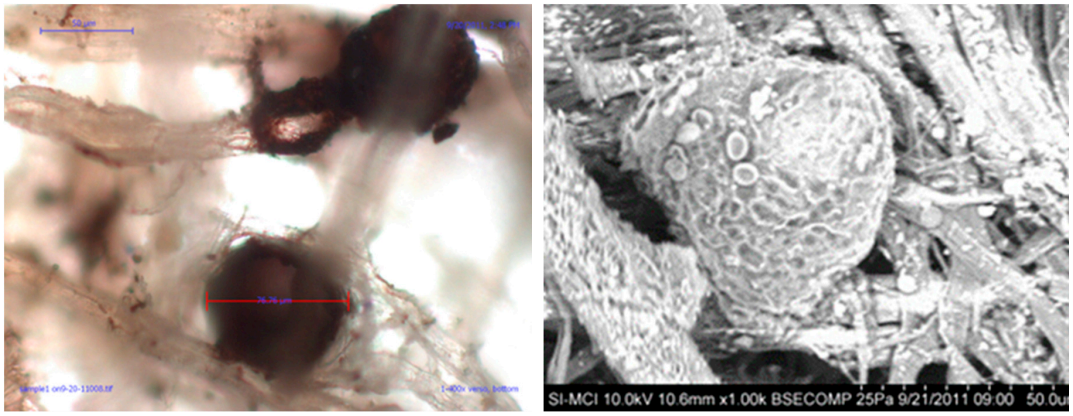
(**a**) (**b**)

Figure 6.9. (**a**) Stain type A. In this characteristic pattern of anastomose cells, fungi showed both partial attachment to the surface of paper fibers and partial branching out towards air pockets, which can be attributed to chemotropism. Transmitted light micrograph, scale 10 µm. (**b**) A detailed examination of bio-deposits in the paper matrix showed congregated, single cells of varied sizes. The noticeable indentations in individual spores occurred most likely due to their desiccation. SEM-VP micrograph, scale 50 µm. Source: Figure by author.



(**a**) (**b**)

Figure 6.10. (**a**) Stain type B.The samples of paper were extracted from the biodeteriorated area near the main loss of paper, bottom portion of Study Sheet No. 1. The size of each sample was 2 mm × 2 mm. Stereo-micrograph, scale 2 mm. (**b**) Transmitted light micrograph shows inclusions of black, fungal fruiting structures. Sample location: L20. Scale: 200 µm. Source: Figure by author.



(**a**) (**b**)

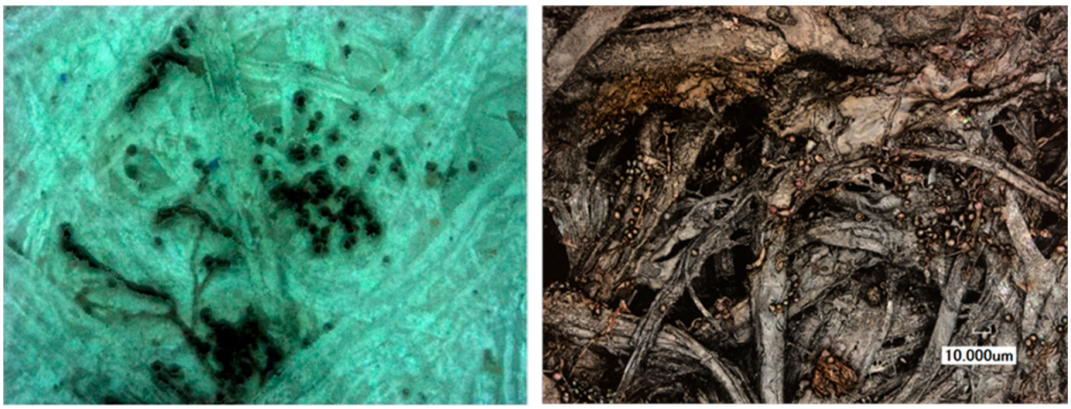
Figure 6.11. (**a**) Stain type B. The fungal fruiting structures were embedded in the paper matrix, which became evident during investigation with transmitted light microscopy. The dimensions of these structures range between 87 × 94 µm and 88 × 91 µm; transmitted light micrograph, scale 50 µm. (**b**) The detail of one of the fruiting structures shows its position in the paper matrix, partially embedded, and attachment to paper fibers. Stain location, L20, SEM-VP micrograph, scale 50 µm. Source: Figure by author.

The optical microscopy of type A stains revealed clusters of cells, trailing along paper fibers, using their surface as anchorage (Figures 6.8b and 6.9). In some areas, in addition to well-defined chains, there were clusters of larger cells, partially attached to the surface of paper fibers.

The examination of type B stains in transmitted light microscopy revealed the morphological features of fungal inclusions, compacted fruiting structures whose size varied on average 87 × 94 µm in diameter (Figure 6.11). These black inclusions were visible to the naked eye (Figure 6.10). Some of the individual structures

were examined with SEM to show the morphology of pycnidia and their surface (Figure 6.11b).

The surface metrology methods relied on confocal laser scanning microscopy (CLSM) and confocal white-light profilometry (CLP) to reveal interrelations of the paper matrix and fungal spores in stain A (Figures 6.12 and 6.13) and depth of fruiting structures embedded in the paper matrix in stain B (Figure 6.14). In stain A, CLSM captured fungal cells as dark particles nested among paper fibers. The distribution of paper fibers in the paper matrix was characterized at the same time, making the surface metrology tools particularly promising in the examination of paper-based artifacts. The configuration of fibers in the matrix correlates with the distribution and clustering of fungal particles, spores, and mycelium. Much greater accumulation was observed along protruding, more-pronounced fibers because they would provide natural protection against the mechanical removal of spores. Figures 6.12–6.14 show cluster stain A in CLSM and WLP.



(**a**) (**b**)

Figure 6.12. (**a**) Stain type A. The stereo-micrograph, 3D view of fungal deposits in paper matrix using confocal laser scanning microscopy (CLSM) showing fungal cells as black particles nested among paper fibers. CLSM captured the image in white light, in addition to a laser image. Figure 6.13. Scale 20 μm. (**b**) An image with 408 nm wavelength lasers and 'pinhole' optics, with a lateral resolution of 0.13 μm to detect micron-scale particles. The VK-9700 CLSM scanned across a target in the x and y directions. Scale 10 μm. Source: Figure by author.

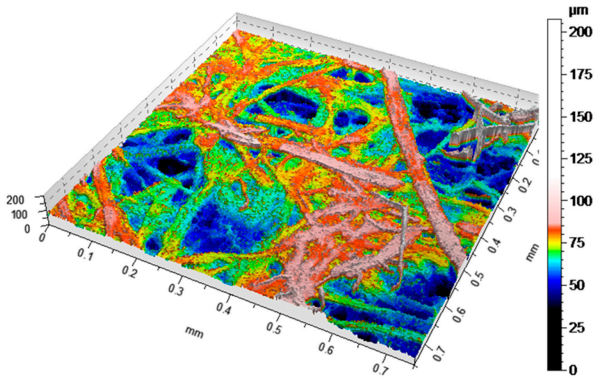


Figure 6.13. Surface topography map generated based on data collected with the white-light profilometer; stain A, stain location N11. The height of fungal deposits along paper fibers reached on average 75 µm in the Z direction, as referenced to the topographic scale on the right. Source: Figure by author.

The image of the area measured with the profilometer is captured by the instrument in white light, serving as a cross-reference correlating the actual appearance of the measured area with the topography map of that area generated by the profilometer (Figure 6.14). It was evident on the 3D topography map that the accumulation of dark-pigmented fungal structures, showing as 'lack of data' (no color-coded topography was recorded), corresponded with the location of fungal cells seen on the white-light image. What was perceived by the instrument as a 'lack of data' provided valuable information as to the location of fungal deposits.

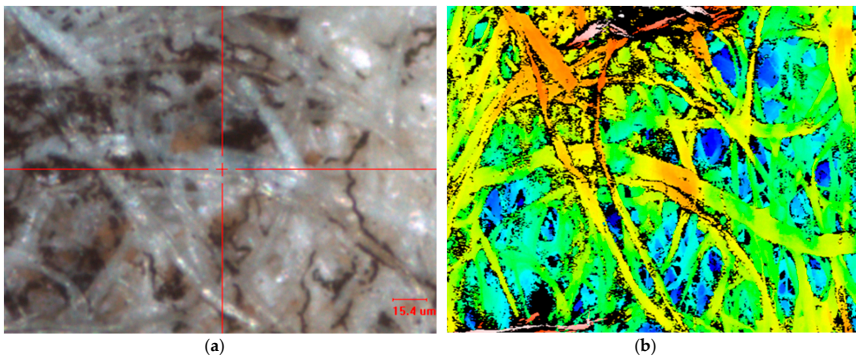
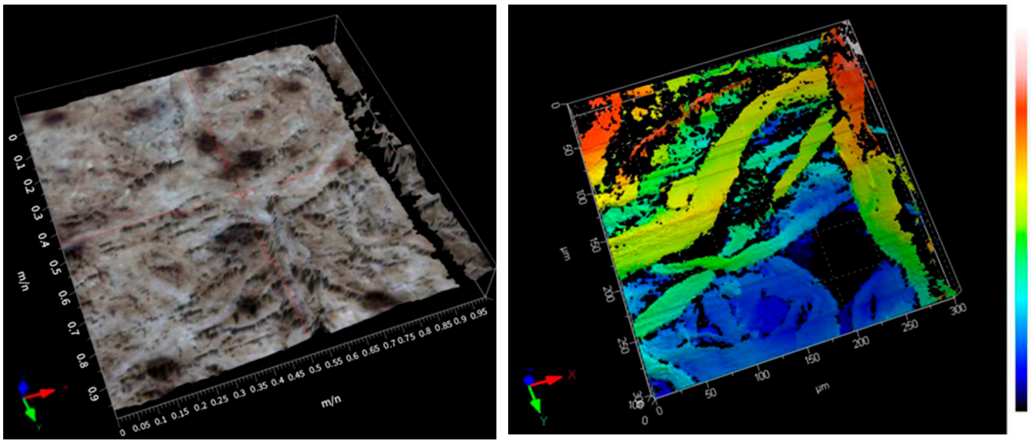


Figure 6.14. (**a**) Type A stain. A view of the measured area captured with the white-light profilometry (WLP). It is used as a reference to correlate the data collected by the profilometer with the actual appearance of the measured area. The dark fungal cells are clustered along paper fibers. It is evident on the 3D topography map, shown in (b). Scale bar: 15 µm. (**b**) A 3D map generated from measurements of the area with fungal deposits, in stain A using optical, non-contact white-light profilometer, Altisurf 500. Black-pigmented fungi absorbed light and thus seen as dark spots along fibers. These dark areas are considered as 'void of data'; they correspond with the location of fungi in the paper matrix. Source: Figure by author.

Mapping the surface of stained paper of both types of stain, A and B, with WLP shed light on the interrelations of fungal deposits with the paper matrix. In stain A, clusters of spores were congregating along paper fibers (Figure 6.14a,b), and in stain B, the spherical fruiting bodies nested in indentations of the paper matrix (Figure 6.15a,b) The indentations and 'valleys' in the paper's surface provided natural protection for the fruiting bodies against physical forces that could potentially remove them from the surface.



(**a**) (**b**)

Figure 6.15. (**a**) Stain type B, viewed as 3D surface topography generated by the non-contact, white-light profilometer. The dark, spherical fruiting inclusions are seen in indentations of the paper matrix. The area measured was 5 mm × 2 mm; sensor tip detects acquired data at 1 µm spacing. (**b**) A 3D topography map of the same area as shown in (**a**) generated by the white-light profilometer, Altisurf 500. The black fruiting structures absorbed light, resulting in 'voids of data'. Their shape corresponded with the shape of the fungal inclusions, both 50–60 µm, and was overlayed with the image obtained in SEM, Figure 6.16. Source: Figure by author.

Furthermore, the WLP generated enough data to plot the profile of inclusions based on the depth of light sensor penetration through the matrix, as seen in Figure 6.16 (Szczepanowska et al. 2013).

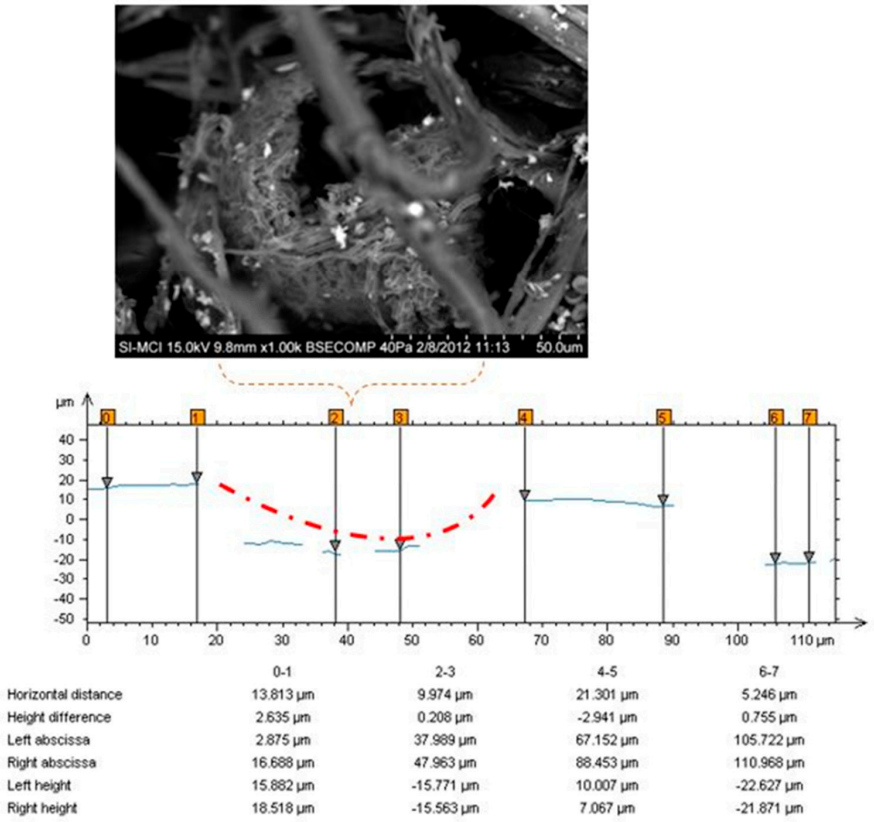


Figure 6.16. A fungal inclusion in stain type B. The SEM-VP micrograph is aligned with a profile generated by non-contact, white-light profilometer; it corresponds with the shape and size of the fruiting structure. The diameter of the embedded part of the fruiting body was 50–60 µm, and the 'void area' indicated by lack of data was near 50 µm. The depth of fruiting body 'immersion' in the paper matrix is ca 25 µm. Source: Reprinted from (Szczepanowska et al. 2013).

6.1.5. The X-ray Microtomography at ERSF, Grenoble

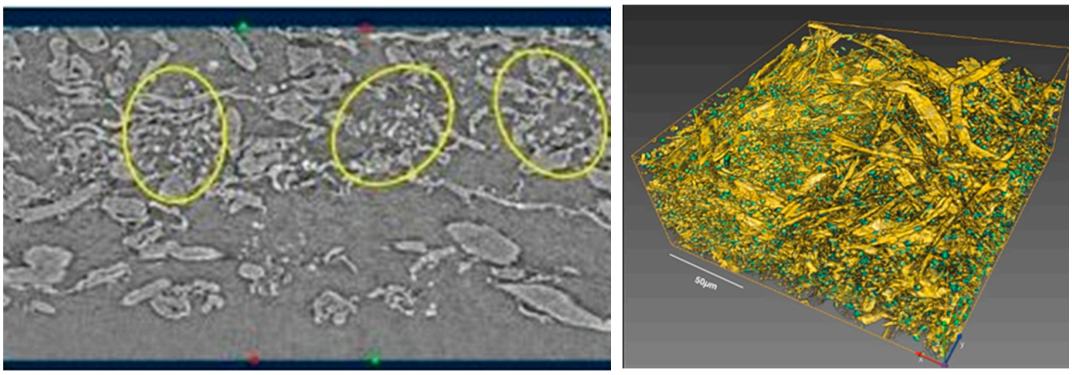
In addition to the traditional microscopy, the type A and B stains were examined using X-ray microtomography on a designated beam line at the European Radiation Synchrotron Facility (ERSF) in Grenoble, France. The beamline ID19 at ERSF described in detail earlier (Chapter 5.3.3) generated data that served as the basis for modeling a 3D visualization. This technique, applied to the analysis of both types of stains, A and B, further elucidated the interfacial relations of paper and fungal structures (Figure 6.17).

Not only were the features of fungi revealed in X-ray microtomography, but some of the microscopic observations of paper were also confirmed, enabling cross-referencing with the results obtained by other modes of examination. This technique also contributed more detailed information about the dimensions of fungal formations, sizes of spores in stain type A, 3–6 µm, and robust fruiting bodies of 60–90 µm in stain B. The morphology of monilioid hyphae, captured in SEM micrographs, showed branched and unbranched chains. This, combined with the morphology of isodiametric enlargements of spores and yeast-like budding

cells, especially clearly visible in stain A, was confirmed and visualized with X-ray microtomography, pointing to Dematiaceae, meristematic fungi.

The interpretation of data generated by X-ray μCT aiming to detect fungi in the paper matrix requires knowledge of both fungal morphology and characteristics of the paper matrix. The size of fungal spores, 3–10 μm, in stain type A, and their shape, mostly spherical to oval, served as a guide to distinguish them in a fibrous paper matrix when interpreting X-ray tomographs. However, differentiation between mycelium, often tubular, fungal filaments and individual paper fibers on gray-monochromatic tomographs is more challenging due to similarities in the diameter and shape of both. X-ray tomography is a powerful tool for the characterization of microstructures that are not accessible with the traditional analytical tools and methods. It is a valuable technique in the characterization of biodegradation, in particular, for defining the interfacial interactions of fungi and the paper substrate; it complements data generated by other instruments, such as SEM-VP and CLSM. The 3D visualization elucidates the intricate interactions between the paper matrix and fungal mycelium, spores, and fruiting body inclusions (Szczepanowska et al. 2015).

The fungal cells in stain A congregated near the surface, among paper fibers, as was illustrated in cross-sections and 3D visualization based on X-ray microtomography data. In Figure 6.17, the cell clusters are marked in circles.

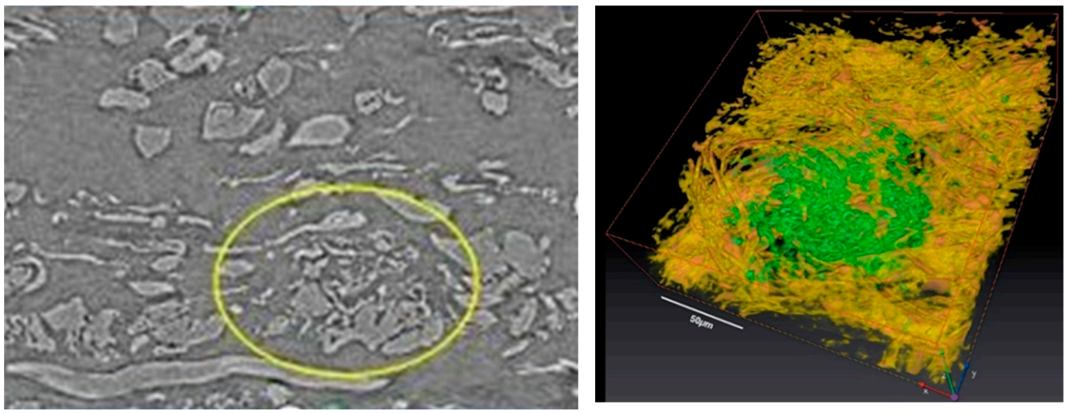


(**a**) (**b**)

Figure 6.17. (**a**) X-ray microtomography of Stain A. One of the 'slices' from approximately 1500 X-ray micro-tomographs (μCT) generated at ESRF shows a cross-section of stain type A. (**b**) The 3D visualization of a black stain type A derived by compiling and processing X-ray micro-tomography data, on beam ID19 at synchrotron ESRF. The green particles are spores dispersed in the paper matrix. Source: Reprinted from (Szczepanowska et al. 2015).

The fruiting structures that form stain B can be seen with the naked eye as pinprick-sized, black inclusions. The stereo-micrographs and transmitted light microscopy showed individual structures in the paper matrix, Based on the microscopic observations and imaging of cross-sections of paper, generated on X-ray

μCT, it was clear that the fruiting structures were located near the surface, partially protruding above the surface, or entirely embedded in the paper matrix (Figure 6.18).



(**a**) (**b**)

Figure 6.18. (**a**) X-ray microtomography of Stain B. One of the X-μCT slices from the type B stain, showing a cross-section of the L20 area with fungal inclusions. The cells are accumulated in round formations Source: Reprinted from (Szczepanowska et al. 2015). (**b**) A 3D visualization of stain B (stain location on the Study Sheet, L20), derived from data obtained as X-ray μCT on beam ID19, at ESRF. Source: Figure by author.

6.1.6. Patterns of Stains A and B Formations on the 17th c. Study Sheet

Diagrammatic illustrations of stain locations in the paper matrix were created as a composite of multi-sensory analytical examination using a broad range of investigative instruments. Each technique used in the investigation of fungal stains and fungal interactions with paper substrate provided supplemental information. However, familiarity with the material that was examined, analyzed, and measured was essential for the proper interpretation of results, especially keeping in mind that filaments of fungi so closely resemble fibers of paper in terms of shape and size.

In Figure 6.19, depositions of pigmented fungal cells in paper, forming black, scattered type A stains, is illustrated. The stains were on the 17th-century Study Sheet, and the pattern of stain formation on both sides of the paper sheet became apparent in the course of the examination. Figure 6.19c shows emerging hyphae in an airy pore of paper on the opposite side of the sheet where the majority of stains were present. Only a small number of mycelial cells penetrated the paper bulk to emerge on the opposite side of the paper.

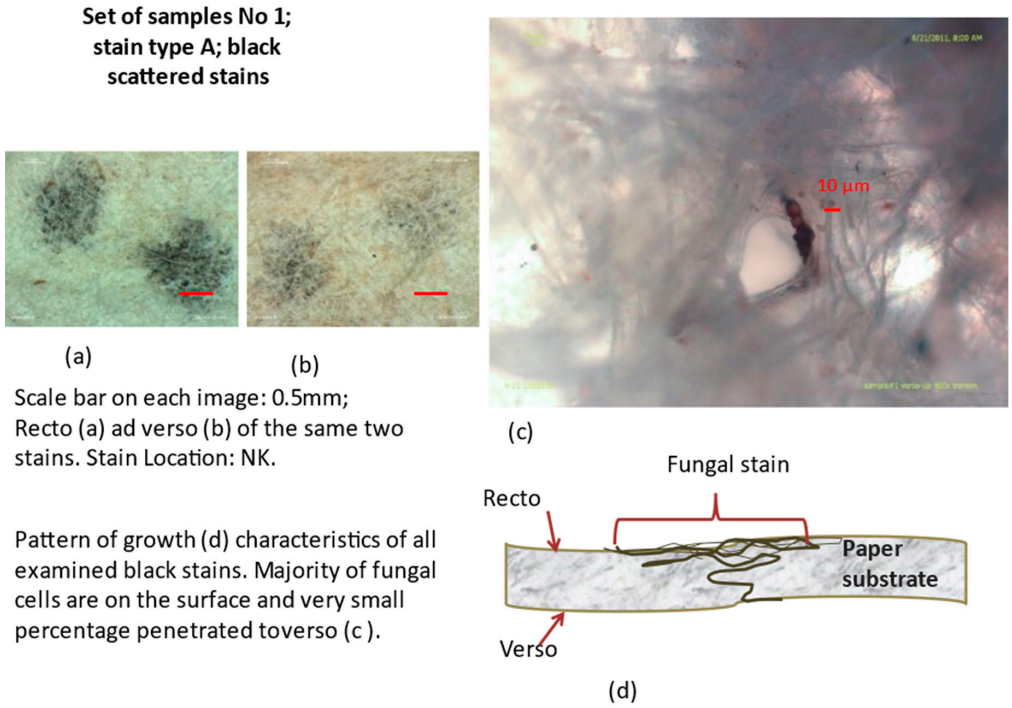


Figure 6.19. A diagrammatic illustration of stain formation in type A stains on the 17th century Study Sheet No. 1. Source: Figure by author.

The pattern of type B stain formation can be defined as a congregation of individual, dark-pigmented fruiting structures in the paper matrix. The clusters of pinprick-sized, black inclusions were captured on a stereo-micrograph Figure 6.20a, in transmitted light micrograph Figure 6.20b, and their pattern of interacting with the paper matrix and location near the surface is illustrated in Figure 6.20c.

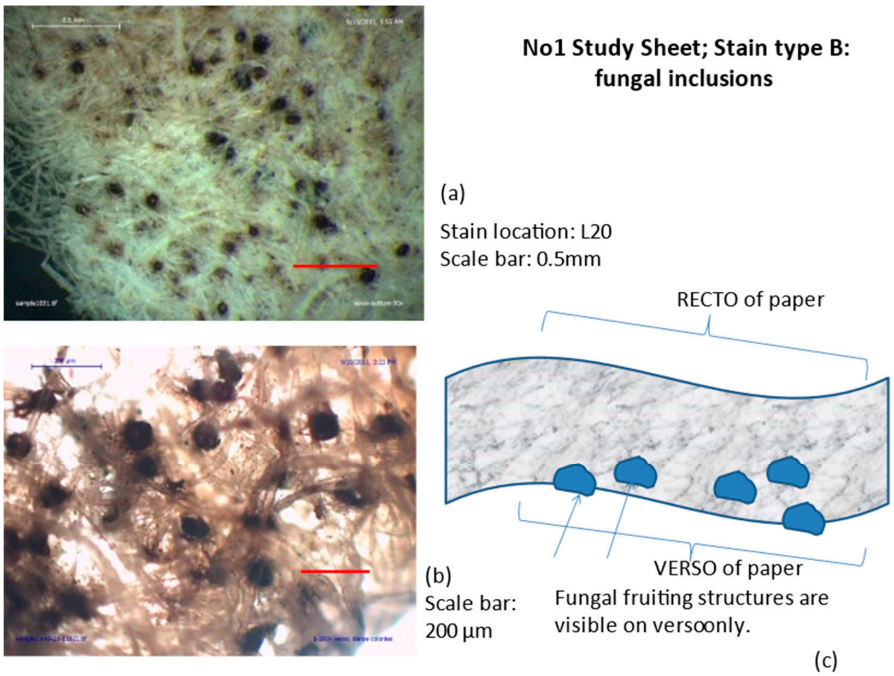


Figure 6.20. A diagrammatic illustration of stain formation, type B stains, Study Sheet No. 1. Source: Figure by author.

Based on the examination of fungal spores, fruiting bodies, and inclusions using various analytical techniques, the patterns of stain formations could be mapped on both sides of the paper sheet. The type A stains were formed by the accumulation of dark-pigmented cells along paper fibers. Some were partially attached to paper fibers; others were branching out into airy pores as chains. As to the type A stain formation pattern, it was apparent that stains were more intense on one side of the paper sheet, due to a dense congregation of cells, than on the opposite side, with a much smaller number of cells. Those on the opposite side of the sheet were located in parallel, symmetrical locations to the larger stains, by migration of mycelium from one side of the paper to the other (Figure 6.19).

In type B stains, the fruiting structures forming stains were partially anchored to the paper fibers with parts protruding out towards the surface (Figure 6.20). This was the side of paper that was presumably attached to a book cover, which would explain the position and growth patterns of perithecia-like structures. Typically, the *Chaetomium* sp. grow in places that are protected from air movement, and when undisturbed, they develop into mature structures.

6.2. Japanese Woodblock Print, 1847, Case Study No. 2

6.2.1. Provenance of the Artwork

The woodblock print, a portrait of an actor printed on Japanese paper, was created by Kunisada, ca 1847; its dimensions are H: 34.3 cm, W: 24.cm. The artwork from a private collection underwent examination and analysis with the owner's permission. The examination followed the protocol as outlined earlier and proposed to apply to all biodeteriorated objects.

The artist, Kunisada (1786–1864), has been at the forefront of *ukiyo-e* artists, print makers, for nearly half a century. Apprenticed to Toyokuni at the age of fifteen, he produced his first independent book illustration in 1807. His images depicted landscapes and scenes of daily life in Japan, presented according to a classical painting style. Kunisada's portraits of actors were superior to others of that genre. Kunisada is believed to have been the most prolific of all print makers, producing thousands of works of art (Mannering 1995).

The art technique is a woodblock print, with a design printed in black and enhanced with colors (blue, red, and blue-green), also printed. Woodblock printing has been practiced in Japan for the past two hundred years, which makes it a relatively 'modern' technique when compared to the centuries-long tradition of ceramics and lacquer paintings. The technique involves transferring the drawing onto a wooden block and cutting away parts that do not produce an impression. The wood that is left on the block will produce an image, typically inked in black. The print is made by pressing the paper onto the cut-away block. Many prints can be made from the same block (Gascoigne 1986). The areas where color is applied are printed in separate blocks; for each color, a new block is prepared. Multicolored

prints have been produced in Japan since 1765 (Mannering 1995). The prints executed in that technique were classified as *ukiyo-e* or 'pictures of the floating world'. Kabuki Theater provided inspiration for the images, immortalizing many actors from that period. The print used in this study, although of unknown title, is of the theater genre; therefore, the assigned title is "The Actor".

The artist used black, three shades of blue, and red printer's ink. Based on the scholarly research of pigments used in prints of the *ukiyo-e* period, blue was most likely indigo and Prussian blue, and red was most likely vermilion. According to the research from that period, the mixture of blue-green was achieved by combining indigo with malachite or another copper-based green; in general, a limited pallet of pigments was used in the *ukiyo-e* period (FitzHugh 2008). An overview of the artist techniques and the paints was provided in the hope to establish any correlation between fungi growth, their pigmentation and the applied pigmented inks. However, there was no correlation observed between the pattern of fungi infestation and pigments used by the artists, as illustrated by the subsequent analysis.

The artwork was printed on Japanese paper, hand-made, with no evidence of sizing. Traditionally, Oriental papers are not sized (Barrett 2005). Long fibers, as was evident during microscopic examination, retained their morphological features, indicating that the paper was made by hand. The fiber size diameter ranged from 8 μm to 14 μm (Figure 6.21).

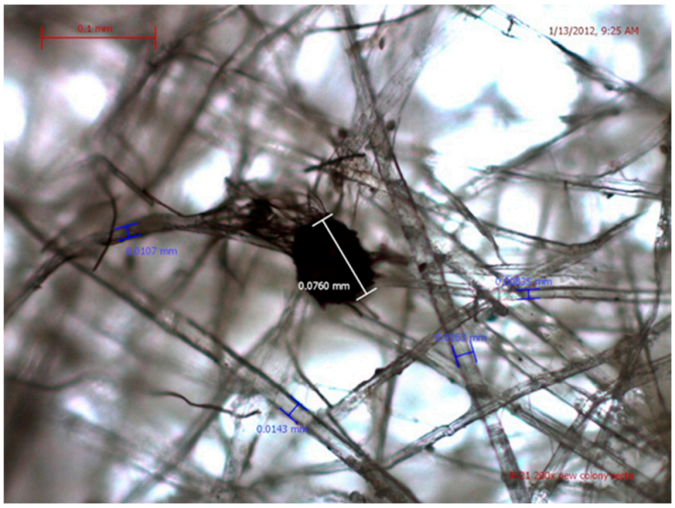


Figure 6.21. Japanese woodblock print, 1847, paper fibers in transmitted light microscope. The fiber diameters ranged from 8 μm to 14 μm; fibers were well preserved, with smooth surfaces. The black inclusion, 0.076 mm long, is most likely a newly formed perithecium. Transmitted light micrograph, scale 0.1 mm. Source: Figure by author.

6.2.2. Context of Biodeterioration

The artwork was covered with fungi, growing on recto and verso (Figure 6.22). According to the artwork's owner, fungi appeared over a two-month period resulting from water sipping through the wall and migrating to the surface from the reverse of

the artwork. A visual and macro-examination indicated three main types of fungi species, based on their color, cells morphology, and density of colonies, as: (1) dark brown, fruiting structures, (2) light-grey, web-like filaments, and (3) brown, powdery aerial growth. A representative area for each of these three types of biomass was analyzed for their unique morphology. The selection and demarcation of areas that were analyzed is shown in Figure 6.23. Considering the focus of this study, the black pigmentation secreted into the substrate and stains associated with the dark fruiting structures were explored in detail.

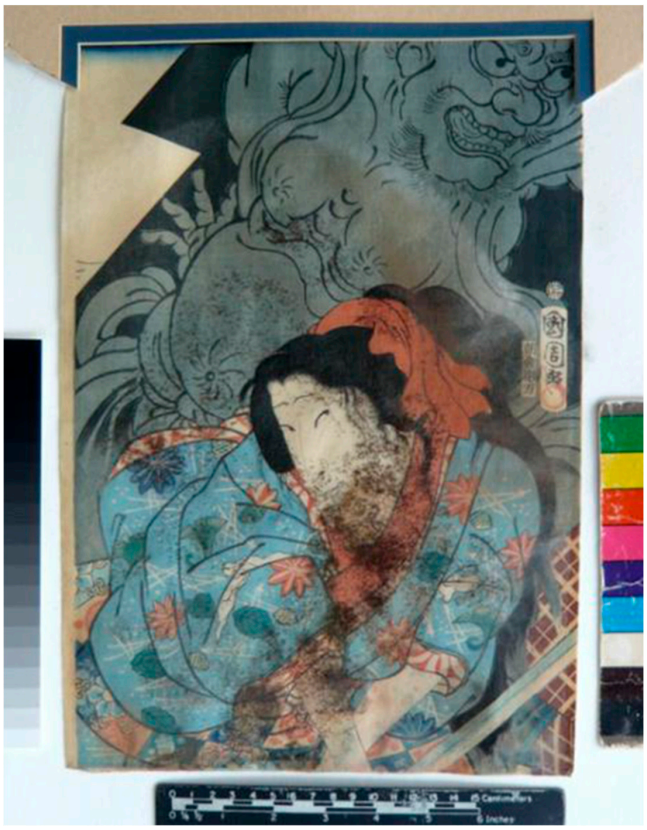


Figure 6.22. An overall view of fungi-infested recto of the Japanese woodblock print, 1847. Several different fungal species can be distinguished based on colonies color and cells' morphology. Further analytical examination revealed the presence of *Cheatomium* sp., *Aspergillum niger* and other, non-identified filamentous fungi. Source: Figure by author.

The first type of fungal species was identified based on dark, fruiting structures, visible as 'hairy', spherical balls, growing on the surface of paper. The 'fuzzy' appearance was due to perithecial hairs that grew on each fruiting body; the morphology of these perithecial hairs of *Chaetomium* sp is discussed in Chapter 2.1.2. The second type of fungi species grew over the perithecia, spreading a web of fine, hyaline mycelial filaments, which terminated in sporangial heads with black spores, most likely pointing to *Aspergillus* sp. In order to examine the perithecia under the web, the aerial growth had to be removed. The third type, brown powdery aerial growth, was also removed as the pigment did not affect the paper, and no

pigmented residue was left in the paper matrix. The color and morphology of the fruiting structures most likely points to *Aspergillus niger*.

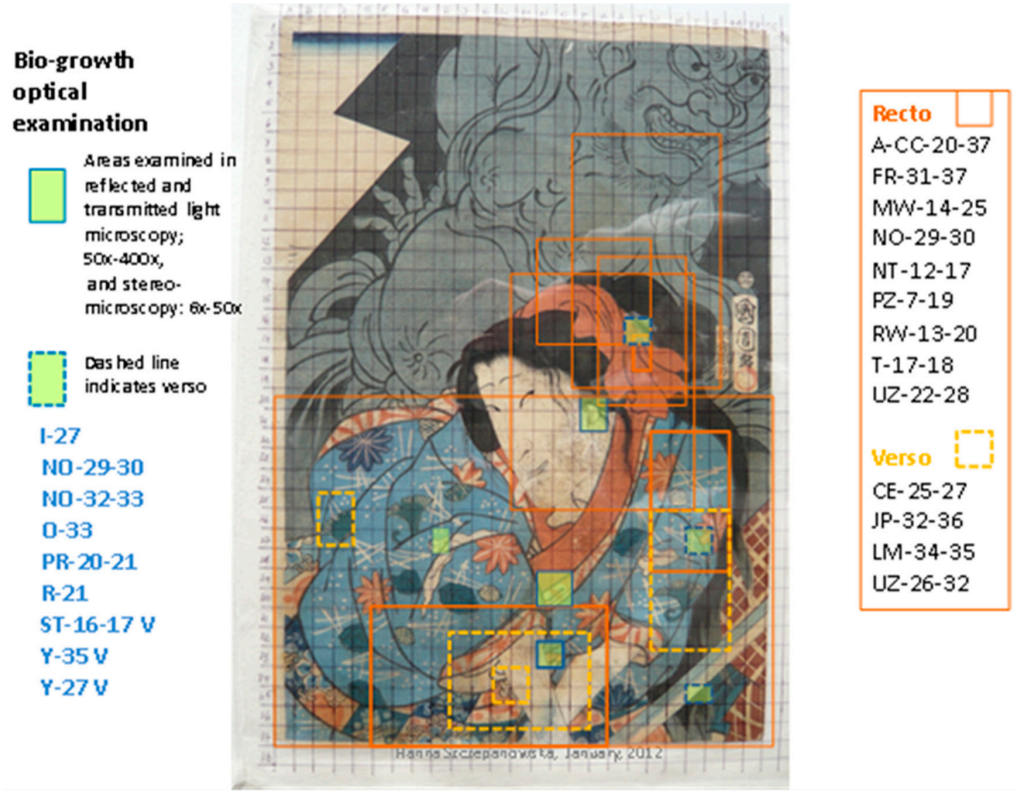


Figure 6.23. A diagram showing demarcation of the areas selected for detailed examination. The grid imposed over the artwork served as a reference for stain location. Solid lines mark the areas on recto; dashed lines demarcate corresponding areas on verso. Fungi grew on both sides of the paper sheet. Source: Figure by author.

The areas selected for the examination represented three different types of fungi species growing on the print, as detailed earlier.

- Dark-brown (nearly black), vertically protruding perithecia, *Chaetomium* sp., located mainly in the center of the print, on recto and verso (Figure 6.24)
- Light, hyaline and white-gray, web-like aerial mycelium appeared to be *Penicillium* sp.; located in the upper part of the print
- Light brown, growing over perithecia, evaluated based on black spores, appeared to be *Aspergillus niger*; located in the bottom, lower portion (Figure 6.25)

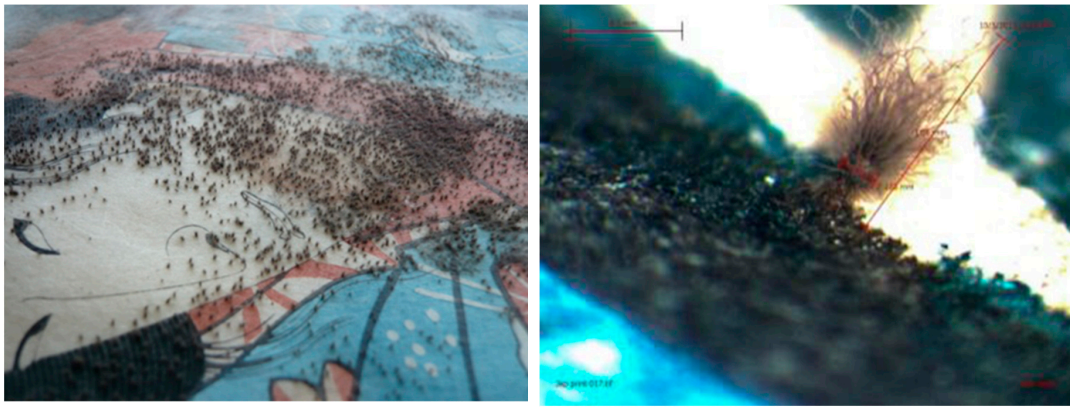
The examination protocol of examining fungal stains followed the methodology applied to all case studies, including physical characterization of the paper morphology, topography of the surface of each side of the sheet, evaluation of the types of fibers used in paper making, and analysis of sizing. The predominant fiber used in this paper was kozo, a common fiber in Oriental papers. The fibers

were long and well preserved, indicating that the paper was hand-made; there was no evidence of internal sizing (Figure 6.21).

6.2.3. Surface Characteristics and Morphology of Fungal Deposits

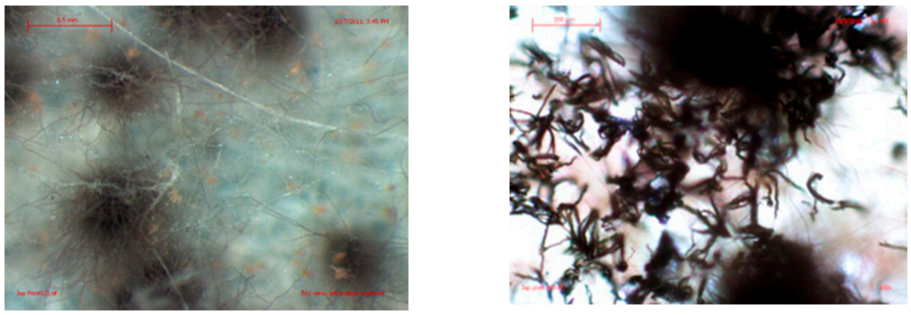
The paper was examined on both recto and verso of the print, showing the strata of fungal formations; some were mycelial web-like structures stretched on top of well-defined fruiting bodies and smaller, pigmented green and dark clusters of brown spores (Figure 6.25). Both sides of the sheet were imaged to determine which areas will be the most representative for further examination. The analysis focused on dark fruiting structures of *Chaetomium* spp.; only in undisturbed conditions are their fruiting bodies as well defined as they appeared on this print. The formations captured in Figure 6.40 on recto and Figure 6.26 on verso provide good examples of individually growing fruiting structures of *Chaetomium* sp.

The dark-pigmented fungal formations, mycelium, and fruiting bodies growing on recto of the artwork are documented in Figures 6.39–6.42 and illustrate the microbial diversity encountered on this artwork.



(**a**) (**b**)

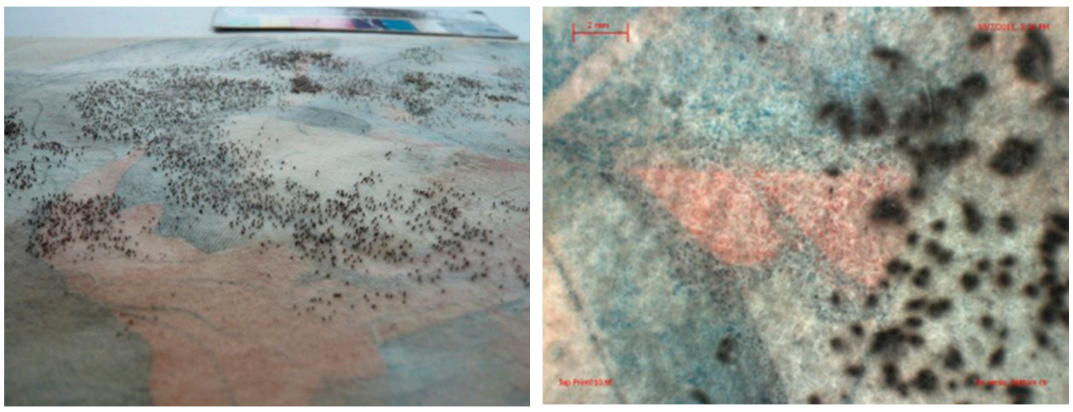
Figure 6.24. (**a**) A side view of the central portion of the artwork, location MR 20 to 25, showing an upward trend of perithecia growth. Macro-photograph, Leica D-lux digital camera, scale 1:1. (**b**) Stereo-micrograph of a single standing, dark-brown fruiting structure of *Chaetomium* sp; average size of perithecia: 120 μm × 20 μm; scale bar: 0.5 mm. Source: Figure by author.



(**a**) (**b**)

Figure 6.25. (**a**) Aerial mycelium of *Aspergillus niger* growing on top of *Chaetomium* fruiting structures. Micrograph, scale 0.5 mm. (**b**) A detail of the area shown in Figure 6.25, black spores of *Aspergillus niger*, a micrograph in transmitted light, scale 200 um. Source: Figure by author.

Figure 6.26 shows black fungal stains on reverse. However, there were no correlations between patterns of growth on recto and verso.



(**a**) (**b**)

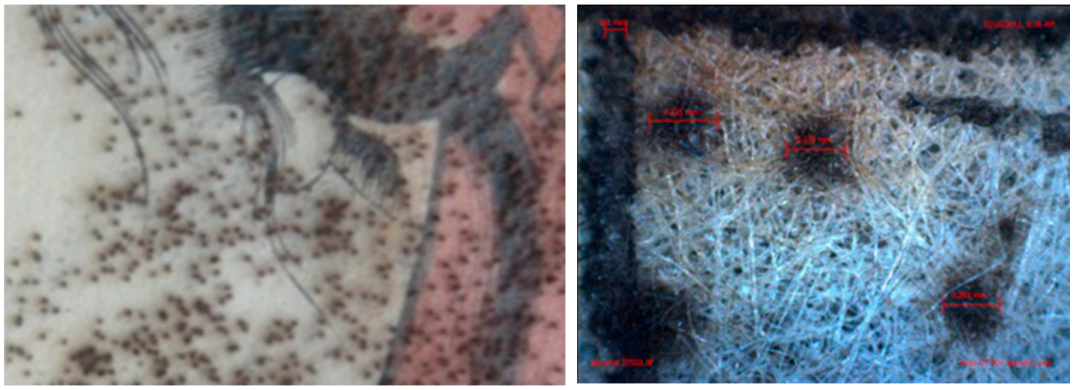
Figure 6.26. (**a**) Reverse side of area shown in Figure 6.24 with Chaetomium perithecia growing on the surface. Location of the examined area on verso, PR-20-21. Macro-photograph, in a side view, of the bio-growth showing the three-dimensionality of perithecia; they are protruding vertically from the surface. Leica D-lux digital camera, 1:1. (**b**) A detail of the reverse side and close-up of individual perithecia that were found on verso of the artwork, a detail of the area shown in Figure 6.26, stereo-micrograph, scale 2 mm. Source: Figure by author.

6.2.4. Surface Morphology of Fungal Stains

The perithecia, fruiting bodies of Chaetomium, were attached to the paper substrate with pigmented rhizoids. This became clear only after the fruiting bodies were removed, and revealed under each perithecium an intense, black stain in the paper. The stains were caused by pigmented filaments of rhizoids and the secretion of extracellular material, which was visible in transmitted light microscopy (Figure 6.28). In this overexposed transmitted light micrograph, the pigmented material shows varied density and how it is dispersed in the paper matrix.

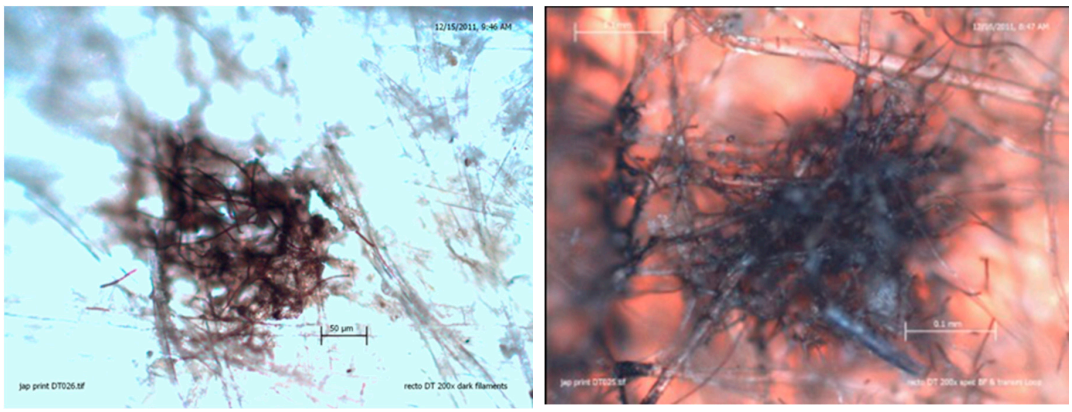
In summary, based on a detailed analysis of black stains induced by Chaetomium, specifically on how the stains were formed, three distinct groups can be seen: (1) superficial, dark clusters of perithecia; (2) black metabolites secreted to the paper matrix; and (3) black mycelial cells intertwined with paper fibers. The first one was the most obvious, visible as a localized congregation of dark perithecia growing on the surface (Figures 6.24, 6.26, 6.27a). The second form, black secretions, as mentioned earlier, became apparent only after the removal of perithecia (Figures 6.27b and 6.28). The stain diameter ranged from 0.28 to 0.41 mm, which was much smaller than the average size of perithecium typically within a range of 1.2×0.5 mm. The third type of staining resulted from a combined effect of pigmented rhizoids and black mycelial filaments penetrating the paper matrix (Figure 6.29). The intensity of black bio-pigmentation was identical to the black ink used in printing the image. This underlines that it is essential to understand the art technique of an examined artwork, or artifact, to differentiate between art media and fungi, especially when analyzing art pigments and bio-pigments.

The attachment and branching of the dark-pigmented mycelium illustrated in Figure 6.29 is an example of fungal tropism: thigmotropism, autotropism, and chemotropism. *Thigmotropism* refers to the detection of the surface texture that fungi utilized as guidance for growth and expansion. *Autotropism*, the natural tendency of mycelium to grow in a straight line, is affected by interactions between neighboring hyphae, partially responsible for spacing of hyphae at the colony margin. *Chemotropism* is the reaction to a present chemical, for example, oxygen or nutrients, governing fungi growth towards or away from the chemical source.



(**a**) (**b**)

Figure 6.27. (**a**) Aerial growth of *Chaetomium* on verso shows individual, well-formed fruiting bodies, perithecia. Leica D-lux optical camera, scale 1:1. (**b**) Black stains were formed under each perithecium, which became evident once the fruiting structures were removed. The stain diameter ranged from 0.28 to 0.41 mm. Optical micrograph, scale 0.5 mm. Source: Figure by author.



(**a**) (**b**)

Figure 6.28. (**a**) The black stain formed under each perithecium. This overexposed image in transmitted light microscopy emphasized the contrast of pigmentation secreted into the substrate and black fungal filaments intertwined with paper fibers. Perithecium that secreted that pigmentation to the substrate has been removed; scale 50 µm. (**b**) In the black stains, pigment was concentrated in fungal filaments. The filaments diameter was on average 2 µm. Close-up of the stained area seen in Figure 6.28; scale bar 0.1 mm. Source: Figure by author.

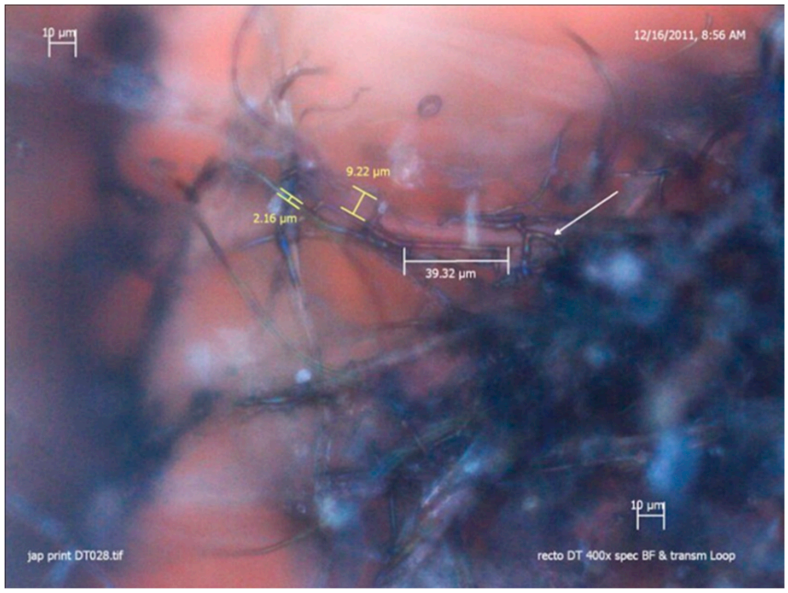


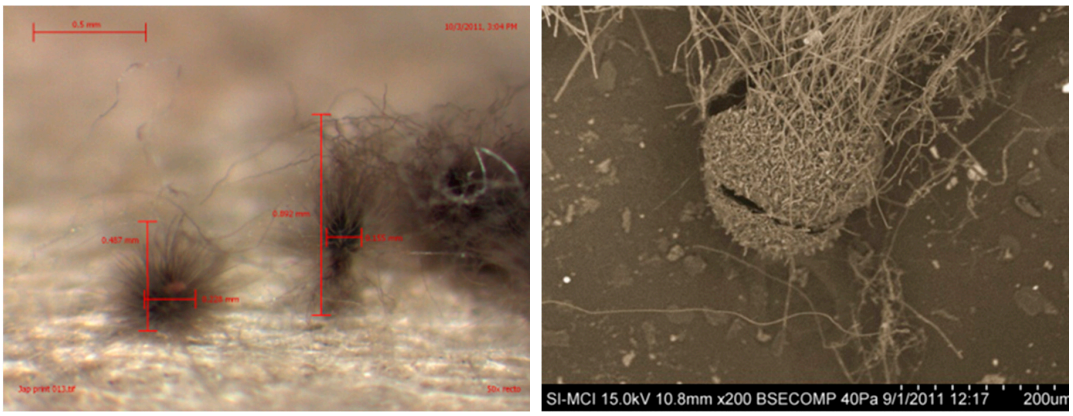
Figure 6.29. The black fungal filaments are shown as trailing along the paper fibers using their surface as an anchorage. A response to surface texture is termed thigmotropism. The diameter of fungal filaments measured around 2 microns while paper fibers were over 9 microns. On this micrograph, the area of fungal filaments attachment extended over 39.32 µm but was longer in other places. There was no evidence that the filaments penetrated the fiber's interior. Transmitted light micrograph in dark field, 400×, scale 10 µm. Source: Figure by author.

6.2.5. Morphology of Microorganisms

On top of the large structures of perithecia, a web of hyaline and gray-color mycelium was stretched over them, thus forming a second tier of fungal deposit

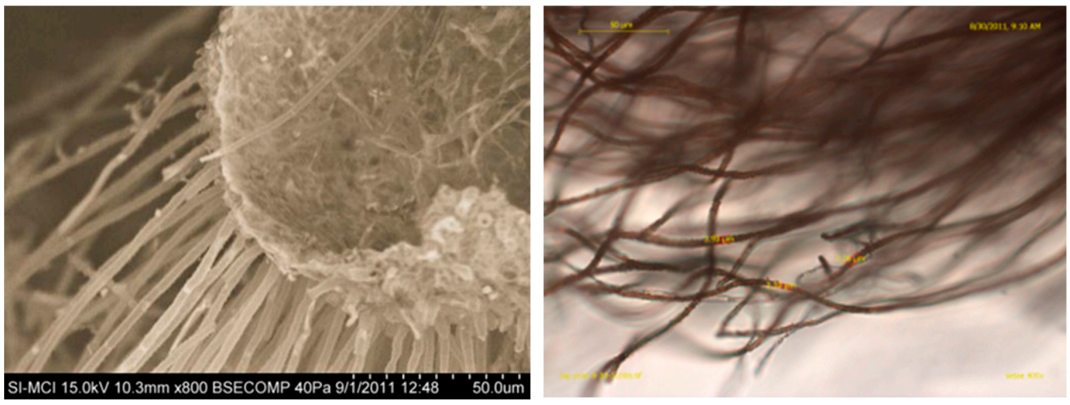
(Figure 6.25a). The mycelium terminated with brown conidiophores and conidia in some places (Figure 6.25b), while in another area, showed green, smaller clusters of powdery spores. The fungi morphology indicated two distinctly different species coexisting in one location, most likely pointing to *Aspergillus niger* (dark brown) and *Penicillium* sp. (green).

The specie of main interest in this study belongs to *Chaetomium* spp. and was identified based on well-developed, individual fruiting structures. A close examination revealed their long, ornamented perithecial hairs (Figures 6.30 and 6.31) and empty shells with conidia attached to the perithecium surface.



(**a**) (**b**)

Figure 6.30. (**a**) A cluster of perithecia, each growing tall, height around 233.29 µm, 254.05 µm, 279.46 µm. Perithecial hairs extended over 800 µm length in all directions. Scale bar: 0.5 mm. (**b**) An isolated perithecium from the same location as in (**a**) shows perithecial hairs and perithecium partially open. SEM-VP micrograph, scale 200 µm. Source: Figure by author.



(**a**) (**b**)

Figure 6.31. (**a**) Morphology of the perithecial shell and hairs shown on SEM micrograph. Sporadic conidia were attached to the shell's exterior; scale 50 µm. (**b**) Ornamentations on the surface of perithecial hairs. The average diameter of filaments ranged from 2.10 µm to 3.60 µm. Transmitted light micrograph, scale 50 µm. Source: Figure by author.

6.2.6. Pattern of Fungal Stain Formation on the 1847 Japanese Woodblock Print

The black secretions underneath each fruiting body on the Japanese print were much more intense than the corresponding stains on the reverse of the paper sheet, indicating that the secreted pigment did not penetrate the bulk of the paper. However, the interconnecting black mycelial filaments penetrated to the reverse, as shown in Figure 6.32a The path of filaments penetrating the bulk and their interconnectedness is marked in Figure 6.32b. The location of stains formed by the black filaments, when examining them on both sides of the paper, indicated their juxtaposition placement in relation to each other, as illustrated in Figure 6.32c.

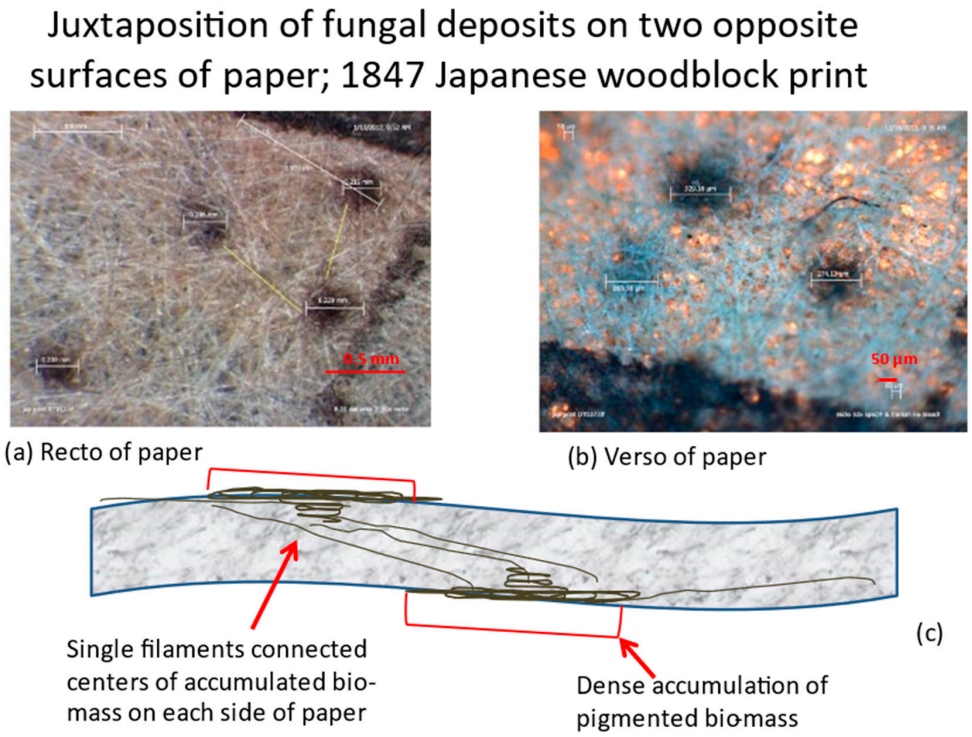


Figure 6.32. A diagrammatic illustration of stain formation under perithecial fruiting bodies of Chaetomium, growing on the Japanese woodblock print. The stains were formed on both sides of the paper sheet, but in juxtaposition one to another. Source: Figure by author.

6.3. Engraving on Paper, 1920, Case Study No. 3

6.3.1. Provenance

The third case study explored black fungal stains on an engraving, titled "Chancery Lane, Annapolis", from a private collection in Annapolis, MD. The engraving was printed on commercially made paper. The date of the engraving, 1920, was signed in pencil. The sheet dimensions are as follows: H: 35.2 cm, W: 27.5 cm (Figure 6.33)

(**a**) (**b**)

Figure 6.33. (**a**) Case Study No. 3, an engraving, "Chancery Lane, Annapolis", by E.P. Metour, in private collection. The recto of the engraving after it was released from the mat. The black fungi stains are located along the top edge. (**b**) The verso of the engraving with black fungi stains accumulated along the top edge, corresponding to the location of stains visible on recto. Source: Figure by author.

6.3.2. Context of Biodeterioration

This engraving was attached to a window mat, four-ply board, along the top edge. The black stains produced by fungi concentrated along that edge and penetrated through all layers of this assemblage, both sides of the artwork paper, the secondary board, backing board of the frame, and penetrated to the front of the window mat. Based on the pattern of growth and its location, it is plausible to assume that the fungal infestation was initiated by the water-based adhesive applied along the top when the artwork was hinged to the backing mat and enclosed in a frame while the adhesive was still wet. A less plausible scenario would be a localized wetting of the upper region of the framed artwork, when the framing was completed. Apart from a greater number of colonies on the artwork itself than on the mat-board, the black fungal stains were identical in their shape and pigment intensity on all affected papers, and on each side of the papers and boards (Figure 6.34).

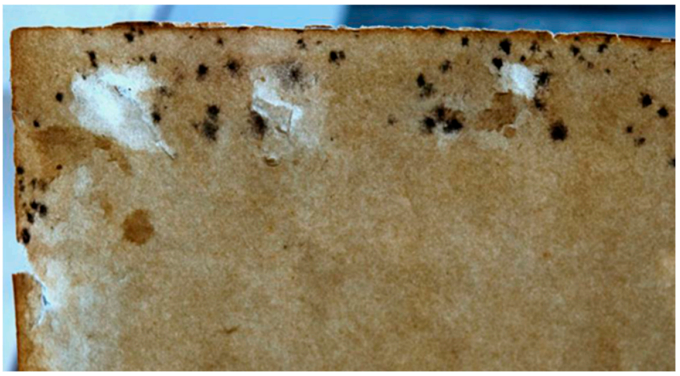


Figure 6.34. A detail of the artwork in transmitted light shows a distribution of black stains in the paper matrix. Stains are on both sides of the paper sheet, on recto and verso. Leica D-lux digital camera, scale 1:1. Source: Figure by author.

6.3.3. Physical Characteristics of Paper Fibers, Sizing, and Additives

The sequence of investigation followed the protocol applied to the other two case studies. It started with the physical characterization of paper, including the morphology and topography of the surfaces of each side of the sheet (recto and verso), type of fibers used in paper making, analysis of sizing, and analysis of adhesive residue.

The predominant fiber used in this paper was cotton; the paper surface was compact and the surface characteristics of both sides of the paper indicated internal sizing; both sides showed similar roughness. The surface along the top edge was tested for adhesive and indicated the presence of starch, thus pointing to the traditional adhesive, paste, that was used for hinging the artwork to the window mat.

6.3.4. Surface Characteristics and Morphology of Fungal Stains

The fungal black stains along the top edge congregated near the surface and migrated to the opposite side of the paper sheet. The stains which were more intense on one side and showed lesser intensity on the opposite side, forming 'ghost stains'. This pattern alternated between both sides of the sheet.

The stains in the upper area of the print underwent detailed investigation. The examined areas were located at the center of the upper edge and near both top corners, as marked on the image (Figure 6.35). The selection was based on the intensity of pigmentation; the most prominent stains supplied sufficient fungal material for analysis.

The intensity of fugal stains, considering the accumulation of pigmentation, was examined visually in corresponding locations on recto and verso. The intensity differed, as mentioned earlier, on each side of the paper sheet. Some stains appeared more intense on recto and others on verso. In all cases, however, the corresponding stains (recto vs. verso) were in juxtaposition to each other, meaning that the stain on one side of the paper, when 'migrating' to the other side, was located in off-location,

not in the same spot as the stain on the opposite side of the sheet. The diagrammatic illustration of that juxtaposition pattern is illustrated in Figure 6.36.

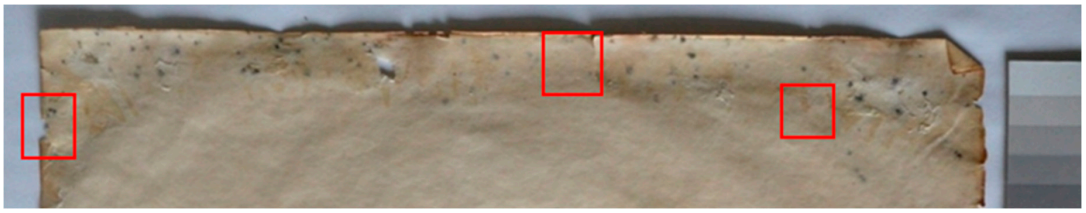
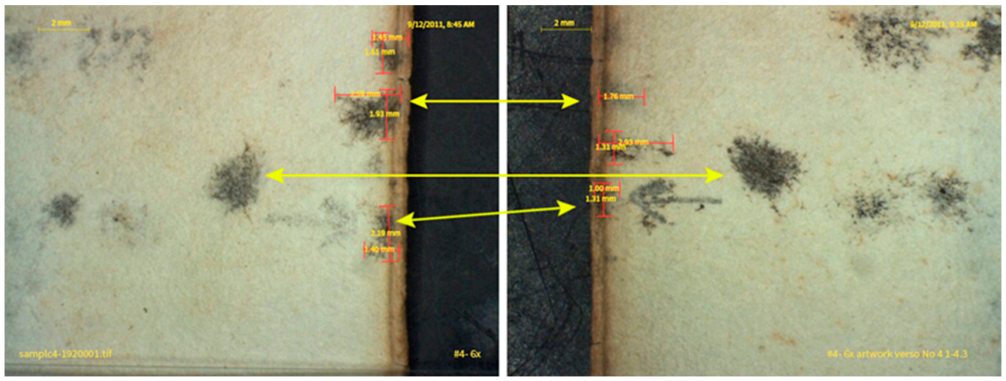


Figure 6.35. The top portion of the engraving with black fungal stains. The intensity of fungi stains varied on recto and verso: in some areas, it was greater on recto, and in others, on verso. The stains in marked areas underwent detailed examination. Image taken with Leica D-lux digital camera, scale 1:1. Source: Figure by author.



(**a**) (**b**)

Figure 6.36. (**a**) A micrograph of stains on recto and corresponding stains on verso. The stains along the edge were larger and showed greater accumulation of pigmentation on recto than stains in corresponding areas on verso (**b**). The dimensions, counting from upper stains downwards, in mm: 1.63 × 2.45, 1.93 × 2.60, 1.40 × 2.19. Scale 2mm. (**b**) The verso with stains corresponding to those seen on (**a**). The dimensions of stains counting from the upper stains downwards in mm: 1.0 × 1.76, 1.31 × 2.93, 1.0 × 1.31, scale: 2 mm. Source: Figure by author.

An interesting pattern of congregation of dark filaments was observed in all stains. The pigmented fungal mycelium appeared growing outwards, from the center, in a radial pattern, which is typical for fungal colony growth and expansion. However, there was no clearly defined central point in any of the analyzed stains, which typically indicates the initial stage of colony development (Figure 6.37). In a microscopic examination, the black cells showed distinct chain formation (Figure 6.38). In addition to the most predominant form, the anastomosed chains (Figure 6.39a), another form was found, clusters of cells nested among paper fibers (Figure 6.39b).

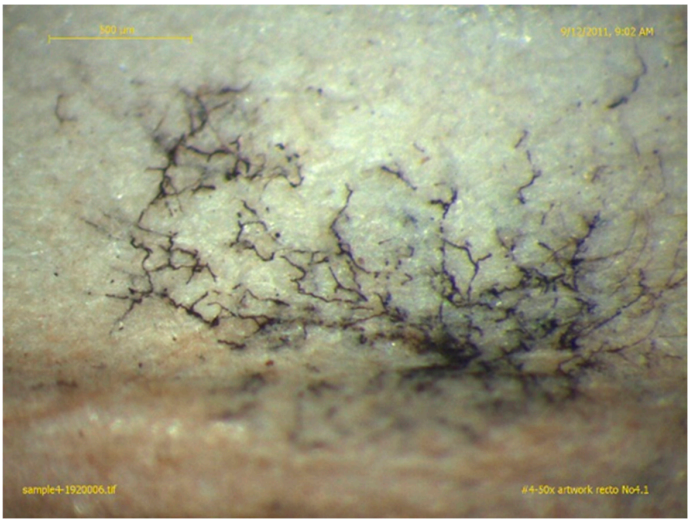
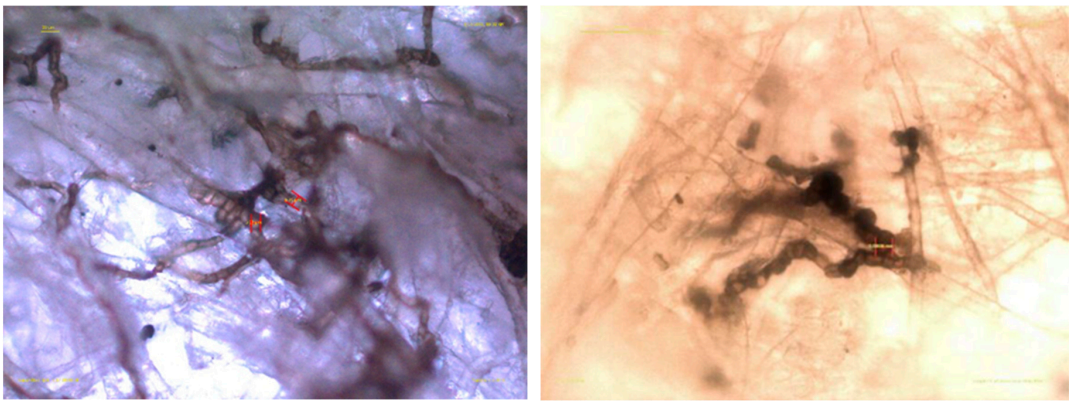


Figure 6.37. Stereo-micrograph of the stain's expansion pattern. The black, fungal filaments appeared to be growing outwards, from the center. However, there was no defined epi-center, which is typical for fungi starting a new colony. The size of this stain measured between extremities was 0.73 mm; scale 0.5 mm. Source: Figure by author.



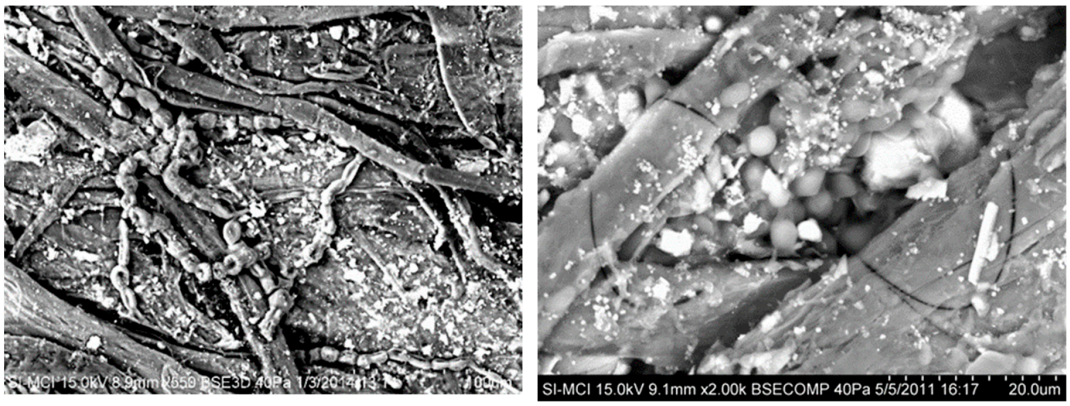
(**a**) (**b**)

Figure 6.38. (**a**) In transmitted light, the dark fungal cells showed semi-transparent cells' interior and thick, dark cell walls. The cells were attached to the surface of paper fibers and anastomosed (forming interconnecting growth patterns). The average cell size was 5.17 µm and the paper fiber diameter varied from 6.91 µm to 22.34 µm. Scale 20 µm. (**b**) A detail of the black pigmented fungal cells intertwined with paper fibers. In transmitted light microscopy, the fungi anchorage to the paper fibers was evident. The trend of growing toward the air pores can be attributed to chemotropism and fungi's need for oxygen. Scale 50 µm. Source: Figure by author.

In transmitted light microscopy and 3D digital microscopy, the dimensions of cells indicated their average size: 6–8 µm. Similarly, to the observed patterns of fungal formations in the 17th century Study Sheet (stain type A), in this case, the fungal cells were also anastomosed and showed chemotropism, branching out towards airy pockets (Figure 6.38b).

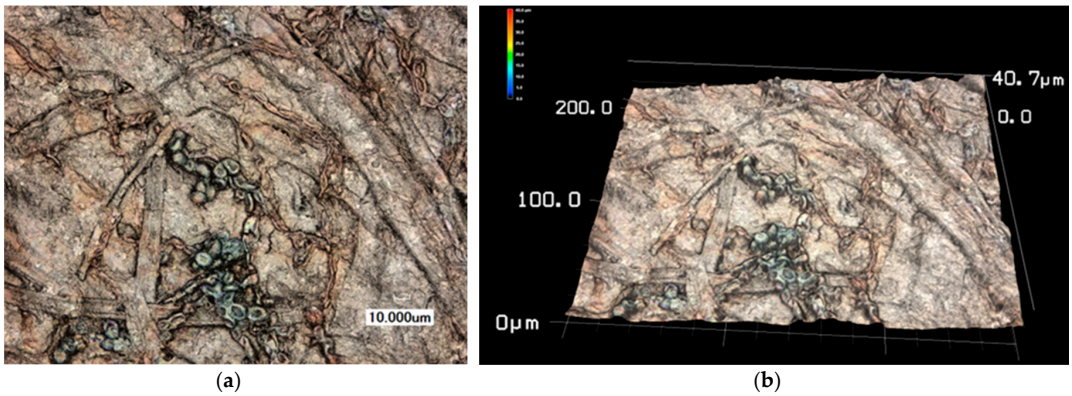
The cell chain-arrangements, well defined in SEM micrographs, contrasted with clusters of various-sized cells compacted on the surface of the paper sheet and under the surface, as seen in Figure 6.39. The average size of individual cells in clusters was greater than those in chains, on average 8–9 μm.

In Figure 6.40, confocal laser scanning microscopy (CLSM) enabled the visualization of those two types of fungal deposits (chains and clusters) and their interactions with paper fibers. The clustered cells visible as bluish in color are larger in size than cells in chains, shown as orange. Additionally, the pigmentation of the cells in clusters appeared more intense than those forming chains.



(**a**) (**b**)

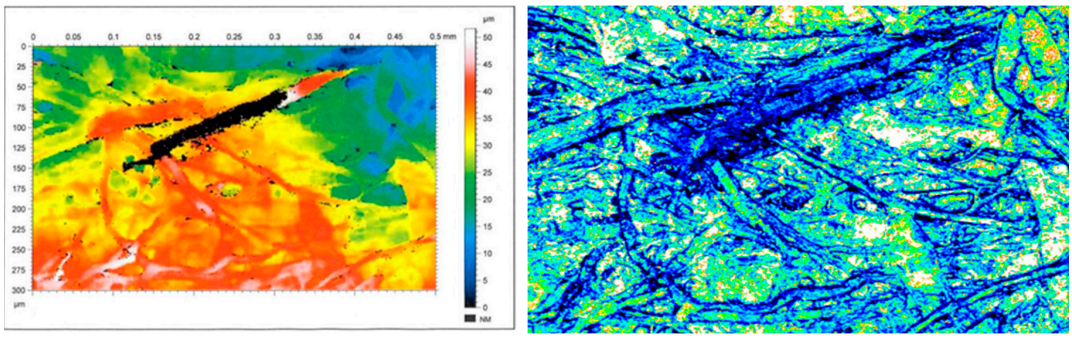
Figure 6.39. (**a**) SEM micrograph illustrates the morphology of microorganisms in the black stains. The cells formed both chains, seen as trailing along paper fibers, and clusters,. Scale 100 μm. (**b**) Another cell formation found in black fungal stains, a congregation of cells in the paper matrix. Their average dimensions were greater than dimensions of cells in chains, ca 8–9 μm. SEM micrograph, scale 20 μm. Source: Figure by author.



(**a**) (**b**)

Figure 6.40. (**a**) CLSM micrograph of paper surface with two different types of fungal formations found in black stains: clusters of cells and chains of cells. The clustered cells are shown in blue, larger in size than cells in chains, seen as orange. Scale 10 um. (**b**) A 3D CLSM micrograph shows the distribution of fungal deposits in the paper matrix and height relationship of deposits versus paper fibers. Source: Figure by author.

The 3D map of fungal cells on the surface of paper generated from the data collected with WLP showed spatial distribution of fungi biomass in the paper matrix and reconfirmed cell attachment to the paper fibers (Figure 6.41).



(**a**) (**b**)

Figure 6.41. (**a**) A 3D topography map generated by MLP shows a 'lack of data' along the fibers between 0.1 mm and 0.3 mm, visible as a black area, where the fungi were located. The black indicates light absorption by fungi in that location. It corresponds with their location shown on the intensity map, as in (**b**). The intensity map indicates the accumulation of biomass along the fibers and corresponds to the dark spots seen in (**a**). Light was absorbed by black bodies of fungal cells and reflected by paper fibers around them. The area measured was 0.5 mm × 300 mm. Source: Figure by author.

The topography maps (Figure 6.41a,b) show the accumulation of fungi mass along fibers as black areas, which typically would be considered as a 'lack of data' by a surface metrologist. Light was absorbed by black fungi in those areas, indicating the presence of black bodies of fungi and defining their location. The microscopy and surface metrology data correlated with the intensity maps, providing a base for the interpretation of surface topography and spatial distribution of fungal deposits.

6.3.5. Engraving 1920, Pattern of Fungal Growth and Stain Formation

Relying on all modes of examination of black stains in the 1920 engraving, it can be summarized that fungi formed stains in juxtaposition to each other on both sides of the paper sheet, as illustrated on the diagram in Figure 6.42 This formation resulted from black fungal filaments penetrating the paper matrix from recto to verso. This combination of juxtaposition pairing of stains was observed on both sides of the paper sheet, meaning that some stains were more intense on one side and corresponding stains on the other side were less intense.

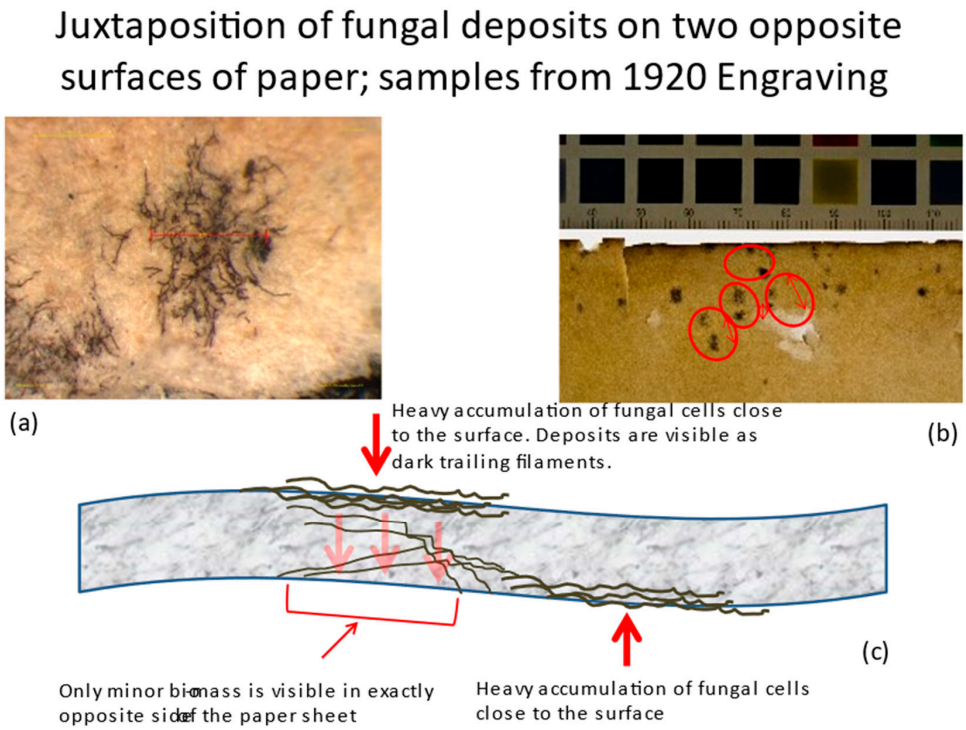


Figure 6.42. A diagrammatic illustration showing how the fungal deposits congregated near the surface and just under the surface in juxtaposition on opposite sides of the paper sheet. (**a**) The micrograph shows the trailing black fungal cells that formed the stains. (**b**) A cluster of stains along the top edge of the 1920 engraving in transmitted light. (**c**) An illustration of the juxtaposition of stains on the opposite sides of the paper sheet, derived from multi-variant analytical examination of stains. Source: Figure by author.

6.4. Summary Points

The different analytical techniques that were used for investigating the case studies provided supplementary information about the nature of fungi interactions with the paper matrix. The protocol of analytical methodology followed the same sequence applied to each case study in an effort to eliminate any additional variables. This protocol seems an optimal and efficient way to examine the substrate, paper, and biological deposits present on/in paper. As such, it is recommended to apply to all types of biodeteriorated cultural objects created on paper sheets.

The results confirmed a variety of species that were imaged, measured, analyzed, and documented on two artworks and one historic paper. The variations between the growth patterns of colonies, formation of pigmented fruiting structures, and stain characteristics indicated that even within a group of black fungi, there are significant differences as to how the stains are formed.

The 17th-centrury Study Sheet was infested by several fungi species; two of which produced black stains. The type 1 stain was caused by black cells clustered in the paper matrix, and the other type was caused by the congregation of black, individual fruiting structures embedded in paper as individual inclusions.

The 19th-century Japanese woodblock print was overgrown by several species that produced varied pigmentation. The black stains were formed by the congregation of large fruiting structures growing on the surface and black mycelial cells anchored to the paper substrates. Even after the removal of the aerial part, the fruiting structures, the stains that were formed under each fruiting structure were prominent.

In the 1920 engraving, the black stains were formed by chains of black, enlarged cells trailing along paper fibers, from one central position in an outward, radial fashion. Two types of black cells were detected, one in chain formations and the other in clusters. The dimensions of cells in chains and clusters differed when measured with various analytical tools, but both types of formations were equally responsible for producing black stains.

In all three cases, the stains were produced on both sides of the paper sheet, recto and verso. The corresponding 'ghost' stains on either side of the paper sheet always differed in size when compared with stains on the opposite side of the paper sheet, meaning that stains were larger on one side of the paper sheet and smaller on the other side, in the corresponding locations. This combination of size variations was documented on both sides of each paper. The penetration pattern of pigmented black hyphae and cells showed the juxtaposition of the stain locations on both sides of the paper sheet; the stains were not in the exact, symmetrical position to one another but slightly shifted away.

The measurements generated during surface topography using white-light profilometry required a repetitious number of measurements to be taken on similar surfaces in order to draw meaningful conclusions. The difficulty of this technique was compounded by the lack of uniformity of the measured surfaces. Paper is highly heterogeneous; therefore, each point that was measured represented slightly but significantly different features, either due to the density of fungal deposits, their different patterns of growth or the thickness of the biomass. The greatest advantage of surface metrology instruments is in their ability to examine ('scan') the entire artwork without the need to sample the original material.

A long-standing belief that sizing in paper provides an additional boost for fungi development proved not to be correct, in both the light experiment and in all three studied cases. Only in one case, in the 1920 engraving, were fungal stains located along the edge where starch adhesive was applied, which indicates a potential connection between additional nutrients and fungal growth. However, that adhesive deposit does not fall under the classification of sizing. Presumably, after exposure to water, the adhesive provided additional nutrients. The proteinaceous, gelatin sizing on Winsor & Newton paper and glue splotches on Study Sheet No 1 did not promote fungal growth. Fungal stains were sparsely produced on gelatin-sized Winsor & Newton. No stains occurred on Sheet No 1 in the areas where glue was present.

6.5. Trends in Fungi and Paper Interactions in the Examined Case Studies

The interfaces of fungi and paper in all three examined cases were investigated using various analytical instruments to explore the interactions between fungi and paper, morphology of fungal inclusions, and characteristics of pigmentation. The observations on a macro- and microscopic level permitted the following preliminary conclusions.

6.5.1. Trends in Macro-Structures

1. Fungi grew in areas where paper was exposed to water. This was supported by evidence of water stains (17th century paper and Japanese print) and the presence of water-based adhesive (1920 engraving).
2. The distribution of fungal mycelium and fruiting structures was random on all papers without any particular pattern or correlation with printers' ink, black or colored. The size of bio-stains on each side of the paper sheets was also random; large and small stains were formed on both sides of each paper sheet.
3. The concentration of fungal deposits was observed on the surface of the recto and verso of paper in all three cases studied, which differed from the patterns of concentration in the paper matrix (bulk).
4. Fungal stains were caused by pigmented cells congregating in clusters or chain formations (17th century paper and 1920 engraving), secretions in paper and mass of dark filaments (1847 Japanese print).

6.5.2. Trends in Microscopic Formations

1. Microscopy of fungal interactions with substrate indicated the tendency of fungi to grow towards air pockets and pores in paper. This supports microorganisms' need for oxygen and areas of expansion. This is an illustration of fungi chemotropism.
2. Fungal elements (single cells, chains of cells or clusters of cells, and filaments) used paper fibers as support for attachment, similar to physical anchorage. The growth directionality on fibers may be associated with fungi thigmotropism, a response to surface topography.
3. Penetration of fungal filaments through the paper bulk led to growth and stain formation on both sides of paper. However, mycelial growth in bulk was less prominent than on the surfaces of paper.
4. Many factors contribute to fungi growth patterns and expansions on substrate. These factors work in concert and impact pigments production and their secretion; light influence in particular, in addition to surface chemistry.

The investigation of such a complex problem as biodeterioration of paper requires a multitude of instruments and a multidisciplinary approach. Each technique and method sheds light on some aspects of fungal interactions with the substrate. Uniform conclusions are not possible due to variations in outcomes resulting from changes in any given variable within that interrelated set of

systems. However, some similarities could be observed in fungal interaction with paper substrates.

Paper fibers in all cases were utilized by fungi as anchorage. All fungal formations, filaments of hyphae and individual cells utilized the paper matrix as a form of protection against external forces. The internal structure of paper fibers and pores in the paper matrix enabled the expansion of hyphae.

7. Removal of Fungal Deposits from Paper

7.1. Review of Current Methods Applied to Fungi Eradication

The available publications report on various aspects of biodeterioration of cultural material; most are primarily focused on describing the observed evidence of biodegradation, but some dive deep into genetic identification of species. A limited number of published articles suggest remediations or practical methods that could be applied to biodegraded paper. This is understandable considering the complexity of the fungi and paper interactions. A selection of the most pertinent publications covering the span of 38 years are listed in Table 2; only the most significant publications are listed.

The focus in most of the listed references is on the study of biodeterioration, describing the visual effects it has on the paper-based cultural heritage collections. The recommendations call for preventive actions such as control of the relative humidity in the environment, which is unquestionably the best preventive strategy. The actual treatments are restricted to mechanical removal of the biomass. The laser removal of fungal stain, which the author previously proposed as a result of collaborative experimental work in 1994 (Szczepanowska and Moomaw 1994), was replicated by Pilch et al. (2003) using the same wavelength irradiation (532 nm) and the same set of microorganisms. The results confirmed the earlier findings. The mechanical and laser irradiation techniques still appear to be the most effective means of eradication methods that can be considered as conservation treatment techniques. The laser removal, however, is complicated mainly because of the need to focus the laser beam on a specific, small target area of application, the need to control its power, and designing the time of exposure, all of which are critical. Too long of an exposure, which measures in fractions of seconds, may adversely affect the paper substrate and show up as burning spots. Conditions of such occurrences were reported by the author in 1994 (Szczepanowska and Moomaw 1994).

Table 2. A review of selected publications dealing with various aspects of biodeterioration of paper-based cultural heritage.

Year	Reference
1980	Kowlik, R., Microbiodeteriration of library materials. Part 2: Microbiodecomposition of basic library materials, *Restaurator* 4: 200–208.
1981	Strzelczyk A.B. and S. Leznicka (1981). The role of fungi and bacteria in the consolidation of books. *Int. Biodeterioration Bul* .I.7(2): 57–67.
1986	Szczepanowska, H. Biodeterioration of art objects on paper. In: *Papers from the 10th Anniversary Conference, 'New Directions in Paper Conservation', Oxford, 14–18 April, 1986.* Part 1. N. Pickwoad, (ed) *Journal of the Inst. of Paper Conservation, The Paper Conservator*, Vol.10:31–40.
1989	Polacheck, I. Salkin, I.F., Schenhav, D., Ofer, L. Maggen, M., and Heines, J.H. Damage to an ancient parchment document by *Aspergillus. Mycopathologia* 106, 89–93.
1992	Szczepanowska, H. and Lovett, C., A study of the removal and prevention of fungal stains on paper" . JAIC (31):147–160.
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2000	Nitterus, M., Fungi in archives and libraries: a literary survey. *Restaurator* 21; 25–40.
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Table 2. *Cont.*

Year	Reference
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2003	*Art, Biology, and Conservation of Works of Art: Biodeterioration of Works of Art*. R. Koestler, V. Koestler, A.E. Charola, F. Nieto-Fernandez (Eds.). Published by the Metropolitan Museum of Art, New York
2003	Szczepanowska, H., Cavaliere, A. R., Drawings, prints and documents–fungi eat them all!. In: *Art, Biology, and Conservation of Works of Art: Biodeterioration of Works of Art*. R. Koestler, V. Koestler, A.E. Charola, F. Nieto-Fernandez (Eds.). Published by the Metropolitan Museum of Art:128–151.
2003	Pilch, E. Pentzien, S. Madebach, H. and Kautek, W. Anti-fungal laser treatment of paper. A model study with laser wavelength of 532 nm. In: Laser in the Conservation of Artworks. LACONA V Proceedings, Osnabruck, Germany, Sept. 15–18: 19–27.
2003	Saiz-Jimenez, C. (ed) *Molecular Biology and Cultural Heritage.* A.A. Balkema Publishers, The Netherlands.
2004	Allsopp, D., Kenneth, S., and Gaylarde, Ch. *Introduction to Biodeterioration*, Second Edition. Cambridge University Press.
2006	Cappitelli, F., Nosanchuk, J. D., Casadevall, A., Toniolo, L., Brusetti, L., Florio, S., Principi, P., Borin, S., and Sorlini, C. Synthetic Consolidants Attacked by Melanin-Producing Fungi: Case Study of the Biodeterioration of Milan (Italy) Cathedral Marble Treated with Acrylics. *Appl Environ Microbiol.* January; 73(1): 271–277.
2006	Pinzari, F., Pasquariello, G., and De Mico. A. Biodeterioration of paper: a SEM study of fungal spoilage reproduced under controlled conditions. *Macromol. Symp.*238: 57–66.
2006	Michaelsen, A., Pinzari, F., Ripka, K., Lubitz, W., and Pinar, G. application of molecular techniques for identification of fungal communities colonizing paper material. *International Biodeterioration and Biodegradation* 58: 133–41.
2007	Choi, S. Foxing on paper: a literary review. *Journal of the American Institute for Conservation* 46 (2): 137–52.
2008	Caneva, G., Nugari, M.P. Salvadori, O. (eds) *Plant Biology for Cultural Heritage. Biodeterioration and Conservation.* The Getty Institute, Los Angeles. (Include post-prints of medical meeting in NJ and HS article)
2010	Cultural Heritage Microbiology. Fundamental Studies in Conservation Science. R. Mitchell and Ch. J. McNamara(eds). ASM Press Washington DC.

Table 2. *Cont.*

Year	Reference
2010	Cappitelli, F., Pasquariello, G., Tarsitani, G., and Sorlini, C'.Scripta manent? Assessing microbial risk to paper heritage. *Trends in Microbiology* 18:538–42.
2011	Principi P., Villa F., Sorlini C., and F. Cappitelli (2011) Molecular studies of microbial community structure on stained pages of Leonardo da Vinci's Atlantic Codex. *Microbial Ecology* 61:214–222.
2013	Michaelsen, A., Pinzari, F., Barbabietola, N. and Pinar, G. Monitoring the effects of different treatments on paper-infecting fungi. International Biodeterioration and Biodegradation 84: 333–41.
2013	Szczepanowska H.M., Mathia T., and P. Belin (2013). Morphology of fungal stains on paper characterized with multi-scale and multi-sensory metrology. *Scanning*, *Journal of Scanning Microscopies*, Wiley Periodicals, Inc. NY.
2015	Szczepanowska H. M., Jha D., and Th. G. Mathia (2015). Morphology and characterization of Dematiaceous fungi on a cellulose paper substrate using X-ray microtomography, electron scanning microscopy and confocal laser scanning microscopy in the context of cultural heritage. *JAAS*, *Royal Society of Chemistry* 30 (3):651–657.
2016	Boudalis, G., Ciechanska, M., Engel, O., Ion, R., Kecskemeti, I., Moussakova, R., Pinzari, F., Schiro, J., and Vodopivec, J., *Mould on Books and Graphic Art*. Horn: Berger Verlag.
2018	Puskarova A., Buckova M., and D. Pangallo (2018). Biodeterioration of photographic and cinematographic materials: methods of investigation. In: Biodeterioration and Preservation in Art, Archaeology and Architecture. (eds) R. Mitchell and J. Clifford. Archetype UK. 57–70.

Source: Table by author.

The focus in most of the listed references is on the study of biodeterioration, describing the visual effects it has on the paper-based cultural heritage collections. The recommendations call for preventive actions such as control of the relative humidity in the environment, which is unquestionably the best preventive strategy. The actual treatments are restricted to mechanical removal of the biomass. The laser removal of fungal stain, which the author previously proposed as a result of collaborative experimental work in 1994 (Szczepanowska and Moomaw 1994), was replicated by Pilch et al. (2003) using the same wavelength irradiation (532 nm) and the same set of microorganisms. The results confirmed the earlier findings. The mechanical and laser irradiation techniques still appear to be the most effective means of eradication methods that can be considered as conservation treatment techniques. The laser removal, however, is complicated mainly because of the need to focus the laser beam on a specific, small target area of application, the need to control its power, and designing the time of exposure, all of which are critical. Too long of an exposure, which measures in fractions of seconds, may adversely affect

the paper substrate and show up as burning spots. Conditions of such occurrences were reported by the author in 1994 (Szczepanowska and Moomaw 1994).

Considering that fungal structures are intricately intertwined with fibers in the paper matrix, their selected mechanical separation from paper fibers is not attainable at this time. Lasers can achieve that, but not a traditional manipulation with surgical scalpels or needles. However, the mechanical removal of aerial mycelium that grows on the surface and does not penetrate the matrix appears to remain the least intrusive option currently used. An illustration of such a treatment is included in the subsequent section. With a better understanding of pigment secretions by fungi and associated secretions of acids, one has to accept the limitations and irreversible damage that accompanies biodeterioration induced by fungi. It is hoped that further studies in the chemical composition of pigments will lead to the development of methods that would eradicate or minimize the fungal stains.

7.2. Mechanical Removal of Fungi

Aerial mycelium growing on the surface of paper can be removed by the application of light pressure and physical forces. Scalpels, tweezers, and fine brushes are suggested as the removal tools to break the weak bonds between fungi and the paper substrate. The effectiveness of this method is illustrated based on the treatment of the 1947 Japanese print, Case Study No. 3, analyzed in Chapter 6.

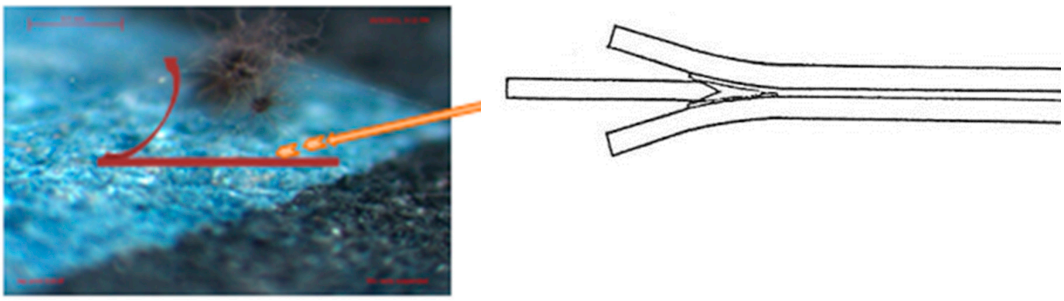
The mechanical removal of fungi (aerial mycelium and perithecia) is illustrated in Figures 7.1–7.4.

Figure 7.1. The Japanese woodblock print, Study Case No. 2, overgrown by Chaetomium perithecia. The yellow demarcation shows the area from which fungi were removed in a process shown in Figure 7.2. Stain location, NO-29-30. Image taken with Leica D-lux optical camera, scale 1:1. Source: Figure by author.

(**a**) (**b**) (**c**)

Figure 7.2. (**a**) The perithecia of Chaetomium are seen as solid particles congregating on the surface as biomass before their removal. Micrograph, scale 2 mm. (**b**) Mechanical removal of fungi residue with tweezers. The objective of mechanical vs. chemical removal is to prevent spores from spreading out on paper and/or smudging pigmented fruiting structures on the surface; therefore, each particle is removed separately, as shown here. (**c**) The same area is shown in Figure 7.2a after removal of aerial perithecia. Underneath each structure, the black pigment was secreted into the paper matrix. Micrograph, scale 1 mm. Source: Figure by author.

Figure 7.3. An illustration of mechanical removal of Chaetomium perithecia from the surface of paper with a fine brush. This image shows verso of the Japanese woodblock print, Case Study No. 2, on which perithecia were prominently growing. Image taken with Leica D-lux digitall camera, scale 1:1. Source: Figure by author.



(**a**) (**b**)

Figure 7.4. (**a**) Perithecia on Case Study No. 3, Japanese woodblock print. The illustration of the mechanics involved in the removal of individual perithecia. The use of a fine brush is one of the techniques applied in the treatment of fungi-infested artworks. (**b**) The Boeing wedge test used for adhesion assessment, adapted after Comyn 1997. The same principle of physical forces involved in the separation of adhered fungi residue can be applied. Source: Figure by author.

The mechanical removal of bio-deposits illustrated by means of separation with a scalpel and brush of the fruiting structures of Chaetomium and aerial mycelium from the Japanese print relies on breaking the forces that hold the microbial structures to the surface of paper.

The objective of a mechanical vs. chemical removal of fungal deposits is to prevent spores from spreading out on paper and/or smudging pigmented fruiting structures on the surface. The mechanical forces involved in the separation of fungal mycelium or fruiting structures can be compared to the Boeing wedge test, used for the assessment of adhesion (Comyn 1997) (Figure 7.4b). The test shows a metal wedge forced between two adherent surfaces. However, the forces applied in microbial separation are incomparably smaller.

8. Current Trends in the Application of Chemoinformatics, Metabolomics, and Genetic Studies of Fungi and Fungal Pigments; A Review

The industrial interest in fungal pigments has exploded in recent decades; fungal pigments are used in agriculture, the food industry, pharmacology, and as natural dyes, among others. Many biotechnological and analytical tools employed in the industry aim to identify and harvest pigmented metabolites, while the study of fungi-infested cultural heritage aims to identify means to remove fungi-induced pigments. The industrial studies towards understanding the fundamentals of the pathway through which pigments are formed, although serving different end-uses, benefit both industry and cultural heritage. Therefore, some of these analytical methods are already employed in the study of fungi-infested cultural heritage materials. A brief review of the most recent techniques for identifying pigmented fungal metabolites is compiled here with the intention to encourage new experimentations.

Chemoinformatics grew out of the pharmaceutical field and refers to computational methods combined with chemistry, in an attempt to elucidate complex chemical processes of pigment formation. Metabolomics is a term that refers to the scientific study of chemical processes involving metabolites that are small, molecular end products of cellular processes, often pigmented. The study reveals unique, chemical fingerprints of the products of these cellular processes, known as the metabolite profile (Daviss 2005).

The typical workflow of metabolomics studies involves sample extraction, preparation, analysis using mass spectroscopy (various methods, such as mass spectrometry (MS) coupled with gas chromatography, or nuclear magnetic resonance (NMR) spectroscopy), data acquisition, analysis, and interpretation. This is essentially a typical workflow of the analysis of any biological matter. For the data processing, an array of specific metabolomic software is utilized.

A comprehensive review of the analytical techniques of pigmented metabolites applied in the dye industry can be found in Kalra et al. (2020), "Fungi as potential source of pigments: harnessing filamentous fungi". This review provides an insightful information about fungal stains, especially when cross-referenced with a survey of pigmentation encountered on paper heritage, compiled by Melo et al. (2018) in "Stains versus Colourants Produced by Fungi Colonising Paper Cultural Heritage: A Review" shows phylogenetic similarities of pigmented metabolites. In addition to a comprehensive list of fungal pigments associated with stains on different paper-based objects, that article also includes detailed references to fungal species associated with specific, colored chemical compounds.

An attempt to replicate the interactions of paper and fungal stains using some of the recent analytical methods was reported in the article by Barrulas et al. (2020), where the authors infused paper with alizarin, an organic dye, meant to simulate polyketide quinones, a compound found in some fungi. The stain applied to paper was removed under various pH conditions and considered by the authors as a model for stain removal. The described experiments, however, exemplify often encountered simplification of the interaction of fungal-induced stains and the paper matrix. Surely, a dye applied on paper will not bond with a paper substrate in the same way as a fungi-induced bio-pigment would; therefore, any 'man-made' stains cannot be considered to resemble bio-induced stains. This, and other similar publications, only reinforce the need for this monograph that illustrates the complexity of fungal and paper interactions.

Another attempt to apply industrial methods, specifically chemoinformatics tools, to the study of pigmented fungal metabolites and the removal of fungal stains was reported by (Liang et al. 2017; Michaelsen et al. 2010, 2013). Liang et al. proposed electrochemical cleaning of paper for the removal of a variety of stains, including fungal stains and foxing. For that purpose, a complex, conductive graphene polyacrylamide montmorillonite hydro-gel was prepared. The concept, although it sounds interesting and the demonstrated results are promising, needs further investigation, especially with respect to the interaction of gel with various paper substrates.

In his article, Nitiu et al. (2020) summarizes the current knowledge on the diversity of pigments with a focus on fungal melanin, including the melanization pathways of fungi growing on paper, the melanin localization in the fungal structures, and their role in the deterioration of paper. A number of research groups engaged in studying paper stains using metabolomic methods are referred to in a paper by Szulc et al. (2018).

The application of modern analytical techniques to both the study of pigmented fungal metabolites and their potential removal is very promising and yet again proves how complex the problem is.

9. Note on the Bio-Hazard of Working with Filamentous Fungi

The majority of filamentous black fungi discussed in this text thrive on non-living substrates. However, some are also recognized as pathogens of plants and humans. The adhesion to surfaces, dispersion of spores, and inhalation of bioaerosols may cause severe human illnesses, especially when facing an abundance of microorganisms in indoor environments. Such extreme situations occur after large-scale disasters caused by storms, floods, and hurricanes, which result in massive outbreaks of fungal infestations, and when cleaning crews, exposed to a plethora of microorganisms, are overwhelmed by the sheer volume of mycotoxins.

Over the past two decades, focused groups of medical professionals held conferences on the hazards of exposure to toxic bioaerosols (Johanning et al. 2012), serving as a resource for governing officials to understand the nature of such exposures and to plan for safe remediation of commercial spaces. The National Academy of Sciences and the Institute of Medicine in 2004 recognized the need to identify connections between biological agents and adverse health effects. Many of their observations and recommendations can be applied to bio-remediation practices in the field of cultural heritage.

Starting with the assessment of a building that houses collections, dampness and poor ventilation is often associated with the propagation and emission of fungal spores, bacteria, and other particles of biological origin, directly affecting not only artifacts but also handlers of these artifacts. The longer such conditions last, the more severe the outbreaks, leading to potential public health problems, along with irreversible degradation of cultural heritage collections. The identified human illnesses associated with fungi respiratory pathway and skin irritation as well as allergic responses.

Environmental microorganisms, fungi and bacteria, are ubiquitously present. Many of them enumerated in Chapter 2, especially soil fungi, are also pathogenic to humans. Among them are *Aspergillus niger, Fusarium*, sp., *Trichoderma* and particularly Dematiaceous fungi, as a group. The black Dematiaceous fungi are particularly resilient and adaptable to all conditions, as pointed out in Chapter 2 Meristematic Fungi, because of the melanin in their cell structure. The illnesses caused by these fungi are therefore difficult to cure. Because this group of fungi is heterogenous, meaning several different phylogenetic groups belong there, the infections are classified based on the type of fungi that caused them, such as phaeohyphomycosis, chromoplastomycosis, or mycotic mycetoma (Pritchard and Muir 1987).

Aspergillus species pose significant risks to immune-compromised hosts (Powers-Fletcher et al. 2016). The wide range of diseases caused by *Aspergillus* species are collectively referred to as aspergillosis (Latge 1999). The first description of aspergillosis was published by Dr. Bennett as early as 1842.

Fusarium spp., known to form red-magenta stains (Szczepanowska and Moomaw 1994; Melo et al. 2018; Powers-Fletcher et al. 2016), are plant pathogens, although some of them are considered opportunistic pathogens in humans, such as F. *oxysporum* and *F. solani*. They cause kidney and heart failure, producing mycotoxins that affect the bloodstream (Lewis et al. 2013). *Mucor* and *Rhizopus* are commonly encountered on paper-based artifacts (Chapter 2), and both produce abundant black spores, the inhalation of which can result in pulmonary diseases (Roden et al. 2005).

Alternaria and some *Cladosporium* species, although not discussed in this text, were reported by other researchers of cultural heritage material as producing black pigmentation (Szczepanowska and Lovett 1992; Szczepanowska and Moomaw 1994; Melo et al. 2018). These fungi are also considered human pathogens, classified as neurotropic, meaning that they may cause cerebral phaeohyphomycosis (Brandt and Warnock 2003; Roche et al. 2005).

Although the types of diseases are severe, filamentous fungi causing illnesses in humans require specific host conditions before this occurs. Therefore, not all exposures result in mycosis. Filamentous mycoses, as they are referred to when caused by filamentous fungi, are often associated with morbidity and mortality; therefore, careful handling of fungi-infested artworks and artifacts is a must. A protective mask, gloves, and a laboratory apron, all discarded after one-time use, need to be worn when fungi remediation is undertaken. When the removal of fungi is planned, their ability to form dormant spores that live for extended periods of time needs to be considered, rather than assuming that the fungal residues 'appear non-vital'.

The fungal abilities to produce adhesives that facilitate their attachment to surfaces have been disused in Chapter 4, Paper and Fungi Interfaces. Their adhesion to surfaces is a prerequisite to any type of pathogenesis (Nicholson and Epstein 1991). Despite advances in fungal genomics, most fungal glues are not well characterized, but it is known that the fungal adhesins are large proteinaceous molecules with abilities to covalently cross-link, thus producing very firm adhesions. Adhesins are also known to bind not only to living hosts but also to plastic (Epstein and Nicholson 2006), indicating that any surface is susceptible to contamination and infection. This knowledge, although still fragmentary, reinforces a cautious approach to fungi remediations from any surface. Finding anti-adhesive strategies applicable to surfaces would not only prevent fungi-caused disease in plants and humans but also biodegradation in cultural heritage material.

10. Conclusions

The biodeterioration of cellular material such as paper is an enormously complex subject. A protocol for the examination process was proposed along with mechanical (non-chemical) treatment of biodeteriorated paper. Some of the phenomena of biodeterioration, with an emphasis on interfaces of fungi and paper, surface modifications caused by fungi, and the role of the environment in these processes, were considered. New investigative techniques adapted from the field of surface metrology were explored as potential tools that may contribute additional data towards a better understanding of the surface phenomena of biodeteriorated paper.

The format of the investigation protocol, research results, and observations presented in this study were part of the doctoral work carried out at the University of Lyon, France. The research was designed to advance the understanding of biodeterioration induced by black fungi and how they affect paper and other cellulose-based materials. In spite of the known deleterious impact of biodeterioration on cultural heritage material, no such investigation has been reported in the literature. Therefore, this monograph is considered to be an initial attempt at mapping areas of further research that will lead to the prevention and/or treatment of biodeterioration. In conclusion, the most relevant points are summarized below:

- The interdisciplinary nature of investigating biodegradation is the key towards achieving a better understanding of this complex phenomena, specifically fungal staining. Among the main disciplines that lend their expertise are mycology, physics, biological physics and optics, tribology, biochemistry, and engineering, especially mechanical engineering.
- Multiscale and multivariant types of the biodegradation investigation provide complementary information regarding the responses of microorganisms to external stimuli, the characteristics of materials upon which they grow, and the interactions between them.
- Traceology is a novel approach in the investigation of living systems on paper. It reveals interdependences between surface topography and microbial responses, such as patterns of growth and pigment deposition and secretion.
- New techniques involved in this monograph include: confocal laser scanning microscopy, white light confocal profilometry, and X-ray computed microtomography. Each one supplements the traditional techniques with more accurate and repeatable measurements of fungal interactions with the paper substrate. None of these techniques have previously been reported as being used for the investigation of the biodeterioration of cultural heritage materials.
- All factors involved in biodeterioration work in synergy, the environment, types of microorganisms, and characteristics of substrates undergoing biodeterioration. Alteration of a single environmental, or nutritional factor, changes the pattern of fungal growth as they all govern microbial responses, some of which are manifested by the production of varied pigments.

- The monolayer of liquid water is the single essential factor that initiates new life of microorganisms and, consequently, the biodeterioration of artifacts.
- The presence of sizing in paper plays a much lesser role in supporting fungal growth than has been reported in the literature. In some cases, it prevents fungal growth rather than supports it, as some bibliographic references have claimed.
- Interfacial interactions between paper and fungi revealed fungal tropism, growth towards or away from environmental of chemical stimuli. The following examples were found: thigmotropism, a response of fungal hyphae to topography of surfaces; phototropism, a response to light; and chemotropism, growth towards airy pores in the paper structure. These fungal behavioral trends attributed to tropism were recognized in historic papers and on cultured species. No reference has been found so far in the literature regarding tropism of fungal growth in the context of cultural heritage material.

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