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Titre:

Living systems on heterogeneous cellular substrate: contribution to a better understanding of dynamic interfaces of fungal pigmentation and paper in biodeterioration of cultural heritage.

École Doctorale Matériaux de Lyon

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Preface

Biodeterioration of cultural heritage irreversibly damages precious historic and artistic works. Paper, heterogeneous cellular material which serves for centuries as carrier of human thoughts, and a base on which artworks were created, is particularly bio-susceptible material. The complex interactions between materials and diverse microbial communities involve among others, physico-chemical forces of microbial adhesion, extracellular material secreted by microbes, topography of surfaces on which microbes grow and parameters of the environment: light, temperature and relative humidity.

The broad array of factors involved in biodeterioration required multi-scale and multi-sensor approach to study structural characterization of materials and bio-mass, to analyze the morphology and physiology of fungi and to evaluate the role of paper's surface topography. Tracing the surface topography with white light confocal profilometer and confocal laser scanning microscopy supplemented scanning electron microscopy and transmitted light microscopy utilized both in surface analysis and examination of biological specimens. The synchrotron X-ray electron beam tomography revealed distribution and interacti0on of fungi with the three-dimensional structure of paper fibers. All efforts were aiming towards a better understanding of the complex interactions at the interfaces of microbes and substrate.

The findings that have the greatest impact on designing the preservation strategy of cultural material include the following:

1. Liquid water as a monolayer (not chemically bond in materials) was essential to initiate the new life cycle of fungi, thus the beginning of biodeterioration processes. The evidence of that was provided in cultures that grew rapidly on paper which absorbed water and in the presence of condensed water.
2. Fibrous paper surface was a favorable environment in which fungi grew (providing water was present). Once the attachments were formed, niches in the paper matrix shielded the spore against physical forces such as air movement or efforts to remove fungi with a brush. That also is one of the reasons explaining the difficulties of removing fungal bio-deposits from paper; only those deposits which are not embedded in the paper matrix but residing on the very surface can be removed successfully by applying mechanical actions.

3. Structure of cellular materials impacted the patterns of fungal growth:
    a. airy pores in paper matrix influenced chemotropism of fungi
    b. surface of paper fibers affected thigmotropism (growing fungi followed the directionality of paper fibers utilizing their surface texture).
4. Presence of sizing in paper did not support fungal growth, which contradicts frequent citations in most of the bibliographic sources. Water alone, as sated earlier, was the decisive element initiating and supporting fugal growth.
5. Exposure to light had multi-faceted effect on fungi. It governed the pigmentation patterns , secretion of enzymes (leading to structural deterioration of paper) and alteration of chemical composition of papers with filers (producing calcium oxalates from calcium carbonate) among others. Two distinct patterns of calcium oxalate crystals formations were detected in both cultures grown in daylight and dark in papers with calcium carbonate filler.

The above conclusions were drawn from the investigation of carefully selected study materials which represented a cross-section of different papers and environmental conditions conducive to fungal growth. One group of biodeteriorated papers consisted of the original artworks executed on Western and Oriental papers. The other group of two papers, Whatman 4 (unsized pure cellulose) and Winsor&Newton, artist paper (sized with fillers) was used in bio-simulation inducing black fungal stains.

Continuous changes of living systems, by the shear fact of their growth, relatively little understood mechanisms of water transport in paper and scarce diagnostic information about black meristematic fungi made this research particularly challenging. The listed findings contributed to a better understating not only of the dynamics of interfacial interactions but more significantly to the complexity and synergy between all elements that play various roles in biodeterioration processes, thus mapping the new lines of research.

Abstract

Biodeterioration of cultural materials is one of the most complex types of deteriorations that cultural materials are subjected to mainly, because it involves living organisms and synergy of many factors. There are different forms of biodeterioration, stains of substrate caused by pigmented fungi is one of them.

Multitude of events occurs at interfaces between substrate and fungi, from the moment of spores' first contact with surfaces, next fugal growth and their responses to the environment. Multiscale and multisensory analysis of interfaces between black pigmented fungi and paper substrate was the subject of these theses. Two types of black fungal pigmentations were analyzed; one that occurred on the original artworks the other one was induced in biosymulation on known papers in controlled environment.

Paper characteristics, such as surface topography and structure, morphology of fungi and patterns of their pigmented bio-mass deposition as well as fungal growth were examined with an array of analytical instruments and methods: transmitted light microscopy, scanning electron microscopy in variable pressure, confocal laser scanning microscopy, white light confocal profilometer and X-ray microtomography.

The ultimate goal was to develop a preservation strategy for biodeteriorated cultural heritage material; therefore the choice of the analytical methods and instruments was dictated by real-life protocols that limit sampling of cultural materials.

This works is the first attempt towards a better understanding of interfacial forces in fungal stains on paper.

Résumé

La bio-détérioration des documents culturels compte parmi les types de détériorations les plus complexes que sont amenés à subir ces objets; et ce, parce qu'elle implique des organismes vivants ainsi que la conjonction de nombreux facteurs. Il existe différentes formes de bio-détérioration; les taches de substrat (support?) engendrées par des champignons pigmentés en sont un exemple.

Une multitude d'actions se développent aux "interfaces" entre le substrat (support?) et les champignons, depuis le premier contact avec les spores, en passant par la prolifération fongique, jusqu'aux interactions avec l'environnement. L'analyse multi-échelle et multisensorielle de l'interface entre les moisissures pigmentées noires et le support papier est le sujet de ces thèses. Deux types de pigmentations fongiques noires ont fait l'objet d'une analyse; la première apparaissait spontanément sur les oeuvres d'art , la deuxième résultait d'une pigmentation déclenchée en biosimulation, sur des papiers connus, dans un environnement maîtrisé.

Les caractéristiques des papiers telles que le relief et la structure de la surface, la morphologie des champignons, les processus de dépôt de la biomasse pigmentée, ainsi que la prolifération fongique, ont fait l'objet de multiples examens, tant en termes d'instruments que de méthodes : microscopie à lumière transmise, microscopie électronique à balayage (MEB) dans une chambre à pression variable, microscopie confocale à balayage laser, profilomètre confocal à lumière blanche, microtomographie aux rayons X.

L'objectif ultime étant de développer une stratégie de préservation des objets du patrimoine culturel bio-détériorés, le choix des instruments et des méthodes d'analyse était dicté par un souci pratique qui limitait l'échantillonage des éléments analysés.

Ce travail constitue une première tentative afin de mieux comprendre les forces en présence au niveau des "interfaces", dans le cas des taches sur le papier dues aux champignons.

Acknowledgements

I would like to express my gratitude to Prof. Dr. Thomas G. Mathia, Director of the Theses for his creative guidance and numerous discussions through the course of this work.

I am indebted to Mr. Serge Carras, CEO of Altimet, for his generosity in permitting access to the surface measuring instrumentations. Special thanks are due to Prof. Dr. J.F Bloch for sharing his insights in paper technology and especially for enabling me access to the European Synchrotron Radiation Facility (ESRF) in Grenoble.

I am grateful to Dr. M. Mecklenburg of Museum Conservation Institute (MCI) of the Smithsonian Institution for sharing his knowledge on intricacies of museum environment and his support during experimental, laboratory work.

Discussions with Dr. R. Cavaliere were always enlightening and brought humor in the midst of arduous biological experiments.

I am grateful to the directors of the Museum Conservation Institute for permitting me the access to the analytical instrumentation in the course of this work.

Support and encouragement of my colleagues and friends, Dr. C. Neves, S. Dhami and H. Kotwicka made this journey easier at times of doubt and hesitations. Their kind words carried this project through and always will be part of the final outcome.

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Introduction

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 finish of surface. Deposits and debris in archaeological surfaces reveal objects' history and ways of living of the cultures that created them. Thus, surfaces of cultural objects are an important source of information about our past, aesthetics and technology. Accuracy of information about these artworks and artifacts relies on the quality of data that we extract form their analysis. Therefore, qualitative and quantitative data obtained with classical optical microscopy and scanning electron microscopy was complemented with the surface metrological instruments used in traceology. These methods included white-light profilometry and confocal scanning laser microscopy with data generated by electron beam microtomography to ensure repeatability of the investigated phenomena. Traceology, a branch of tribology (science of measuring surfaces) applied for the first time to the study of surface topography of living systems offered a promising set of methods and instruments contributing to a better understanding of objects' biodeterioration.

The study of surface, its modifications caused by fungi, and chemical and physical alterations that accompany those changes requires a multidisciplinary approach combining the knowledge of experts from diverse areas of science. Such expertise was available at the Tribology Department at the Central School in Lyon where study of surfaces is the main focus.

Biodeterioration of our cultural heritage is particularly damaging because it alters, often irreversibly, the surface and matrix of materials. It is also one of the most challenging forms of deterioration to study and prevent, because it involves living organisms, fungi and bacteria, and unpredicted ways in which they respond to the changes of the environment and modifications of materials on which they grow. 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 formation of biofilms are associated with pathogenesis of animals and plants, and were therefore studied extensively in the respected fields. However, there is no reported research on interfaces of microbes and cultural heritage material. In fact, that deficiency has been noted by Orio Ciferri, Chairman of the Scientific Committee, during the 1999 International Conference on Microbiology and Conservation (ICMC) in Florence, that was exclusively devoted to the relationships between microorganisms and cultural heritage: "...although enough data is 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, Tiano, Mastromei 2000)

This work is the first attempt to look at a multitude of phenomena occurring at interfaces of bio-deposits and paper-based materials with the purpose of contributing to the preservation of cultural heritage.

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. The complexity of each involved system and the phenomena occurring at their interfaces allow only fragmentary understanding of those interactions.

Awareness of this inherent, enormous complexity of the subject guided the selection of the study area, focusing on the formation of dark-pigmented fungal stains on cellulose paper substrates. Paper is a heterogeneous cellular material produced from fibers and additives. The interaction of those two entities, the microorganisms and paper, opens the door to yet little know and studied area of science. The resulting outcome contributes to the understanding of the interactions of fungi with paper and their growth and stain formation. Recommendations for the conservation and treatment of cultural material infested with fungi are suggested in parallel with projected areas of further research.

Two main parts of these theses deal with the theoretical background of biodeterioration of cultural heritage, as a whole (first part) and experimental work focused on investigating the interfaces of black fungal stains on paper(second part). The theoretical part defines the terms used in the text laying the foundation for the understanding of materials used in the experiment and choices that were made in their selection. Fungi Imperfecti (Class Deuteromycetes) which are the most prevalent on cultural, paper-based material, are introduced in the first part, which also includes a critical bibliographical review of the conservation literature dealing with biodeterioration. Some parallels are drawn between the biodeterioration of cultural heritage material and selected aspects of fungal pathogenecity in agriculture and medical field, such as microbial adhesion to surfaces and discussion on microbial cells hydrophobicity. The first part serves as an introduction to the second part that details the experimental work.

The experimental part consists of two sub-divisions. The first deals with the influence of light on fungal pigmentation and how that pigmentation modifies paper surface and affects the paper matrix. The data were collected by culturing *Aspergillus niger* on two types of paper (Whatman 4 and Winsor & Newton, watercolor paper), in dark and daylight conditions. The second sub-division deals with interfaces of dark pigmented fungi with paper on three actual artworks. The selected artworks exemplified different types of papers and fungal biodeterioration, such as staining of paper surface by pigmented aerial deposits and secretion of colored deposits into paper matrix. Correlations were made between substrate's composition, the environment and resulting microbial adhesions to surfaces.

The experimental part concludes with the review of methods and instruments typically applied to the investigation of microorganisms and paper comparing them with the novel techniques proposed in this study, such as surface tracelogy approach. Introduction of surface metrology techniques to the study of biodeterioration are evaluated in the context of cultural heritage.

In conclusion, removal of fungal deposits is proposed as one of the forms of cleaning the fungal residue. Handling strategies of infested objects is proposed as a practical outcome of this study. Recommendations for further research are outlined, based on identifying the areas which would most benefit the advancement of cultural heritage preservation.

I. Part I: BIODETERIORATION OF PAPER BASED MATERIAL

1. Definitions: Cultural Heritage, Paper and Biodeterioration

Two diverse entities participate in the dynamic processes occurring at their interface – fungi and heterogonous cellular material. The interactions of those two entities occur in varied environments and change over time in sequence with the life cycle of the fungi. Effects of these interactions leading to biodeterioration of all types of substrates are explored in the context of cultural heritage materials. Each entity, paper, fungi, cultural heritage and biodeterioration represents an enormous area of study therefore brief definitions are offered here for clarity in the further discussions.

Cultural heritage is the legacy of humanity passed on from previous generations. It encompasses tangible, physical artifacts, and intangible ones, which are attributes of 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, and artifacts, and intangible heritage refers to non-physical culture such as folklore, traditions, language, and music. Smaller objects such as artworks and other cultural masterpieces are collected in museums and art galleries.

Cultural heritage, unique and irreplaceable, needs to be preserved. **Conservation** is one of the forms of preservation, aiming to stabilize and restore the 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 nearly as possible in an unchanging state" (International Committee of Museums, ICOM, 2004).

Fungi is a term encompassing thousands of species of heterotrophic organisms. This text will focus on a few select examples of fungi which are most prevalent in the bio-deterioration of paper-based cultural material. More specifically, so called Fungi Imperfecti, which are capable of producing black pigments are of particular interest in these studies. The substrate upon which these fungi grow and interact is paper, a **heterogeneous cellular** material. Selected examples of microorganisms found on paper are discussed in chapter I.2.1; patterns of their growth in the context of induced biodeterioration in chapter II.8; and fungal formations encountered on artworks on paper in chapter II.9.

Paper has been used for centuries; its invention is attributed to a 105 AD Chinese scripter (Hunter, 1995) yet its properties are still tirelessly studied, rediscovered, engineered and manipulated. Fungi thriving on paper- based cultural materials, such as art on paper and documents, diminish their artistic experience, depreciate their monetary value and often degrade their structural integrity. The concept of bio-degradation and bio-deterioration has been studied for number of years mainly in the field of agriculture, medicine and industry, but not in the context of cultural heritage. Thus, the mechanisms governing the bio-deterioration of cultural heritage material remain largely unknown, consequently that lack of knowledge does not provide sufficient base for remediation solutions.

Exploring some of the dynamics of interfaces in the interactions of fungi with cellular material will contribute towards better understanding of forces at play leading to advancing the techniques which can be employed for preservation of paper-based cultural material.

1.1 Definitions of biodeterioration

Biodeterioration and biodegradation are often used exchangeably referring to processes of materials' alteration resulting from microbial activities. Even though these processes exist as long as the living world, the word biodeterioration has been in use for about 40 years only (Allsopp, et al 2004). Furthermore, no formal definition has been established and the processes of biodeterioration are not fully understood.

The definition of biodeterioration proposed by Hueck (1965, 1968 cited in Allsopp et al. 2004) applicable to cultural heritage, refers to biodeterioration as "any undesirable change in the properties of a material caused by the vital activities of organisms". Biodegradation on the other hand emphasizes positive aspect of the process that is decaying abilities of organisms to render a waste material more useful (Cavaliere 2003).

Although classifying biodeterioration is artificial, yet has been attempted in many areas of science. Some researchers, such as Allsopp, (Allsopp et al. 2004) classify biodeterioration in general as mechanical and aesthetic. By "mechanical" the authors understand distortion of materials by growth or movement of microorganisms but not the use of the substrate as their food source. As support of that description, the authors cite examples of expansion of microbial masses causing rock cracking and erosion of underground pipes. By "aesthetic biodeterioration" (fouling or soiling) the same authors (Allsopp et al.2004) understand "presence of an organism or its dead body, excreta, or metabolic products" on any substrate. These two above distinctions are not clear, because in both cases aesthetic change of the material occurs and in both cases matrix of the material is affected.

Another classification of biodeterioration proposed by the same researchers (Allsopp et al 2004) is "assimilatory" and "dissimilatory". Utilization of the substrate as a food source is the basis of the first one. Chemical damage caused by secretion of waste products such as pigments or acidic compounds is the basis of the second type. Again, one may argue that utilization of the substrate as food source involves enzymatic breakdown, which in turn causes secretion of products of metabolism, thus the division is not clear.

Other researches in the field of study of cultural materials focus on studying single phenomena, such as for example DNA sequencing rather than classification of the deteriorations 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) alteration of the surface and (2) alterations of the matrix, thus encompassing all forms of microbial interaction with materials.

1.2 Forms of biodeterioration of paper-based cultural heritage

The objects of cultural heritage are composed of all imaginable types of materials which support equally diverse microbial communities. Fungi do not discriminate and cultural materials, like any other, are no exemption to microbial biodegradation. It is occurring in all types of environments. Outdoor monuments, underwater archaeological sites and underground hypogeum can all be infested by microorganisms specific to their ecological and nutritional requirements; mural paintings, in the tomb of the Egyptian desert was overgrown with fungi (Szczepanowska and Cavaliere 2005). Underwater sites and hypogeum provide 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 alteration of the surface first, next degradation of the structure.

Following the above suggested definition, biodeterioration of paper can be discussed in two main categories, as biodeterioration phenomena occurring on the **surface** and in **bulk.** Each category can be subdivided into physical and chemical form of biodeterioration, however all are interdependent.

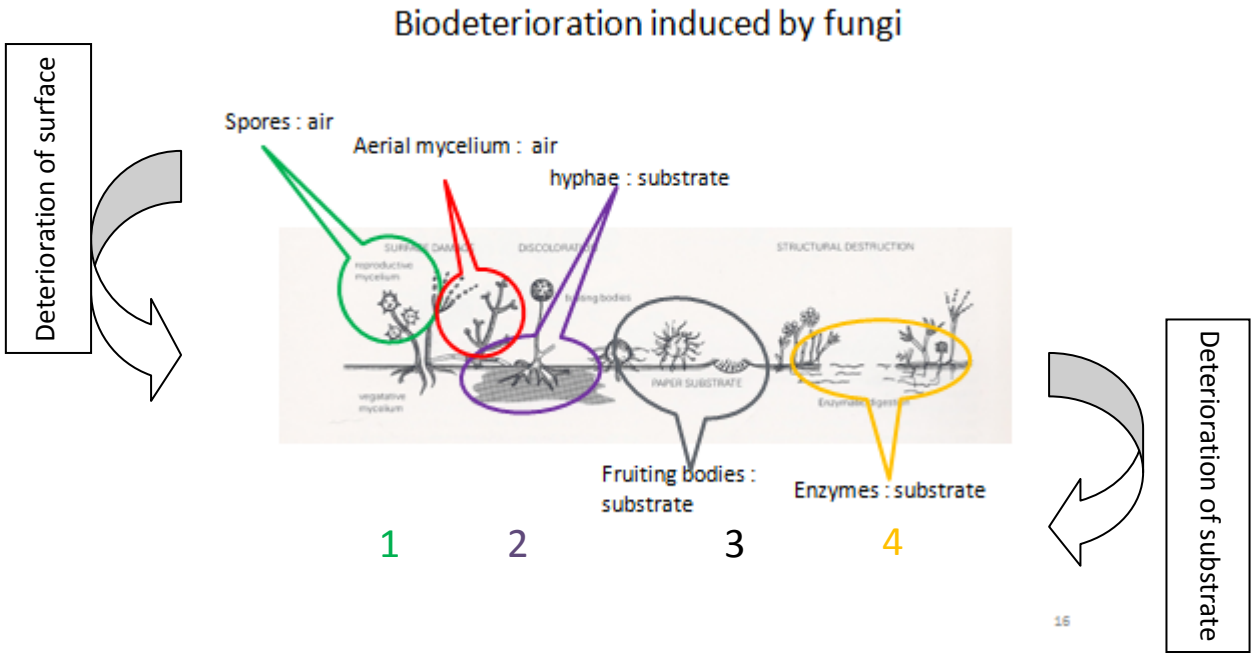
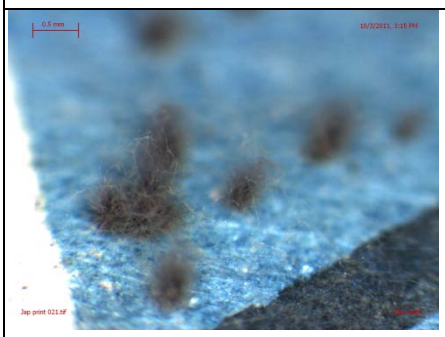


Fig. 1.1 Visualization of biodeterioration of paper substrate caused by fungi. (Diagram expanded based on fig.1, p. 31, Szczepanowska 1986).

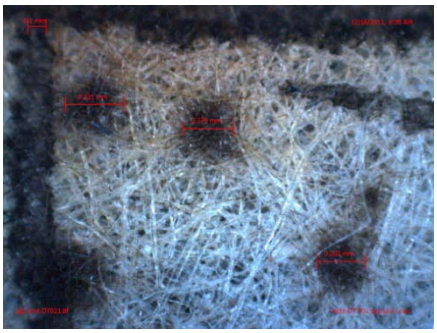
1: pigmented spores in aerial mycelium
2: A- pigmentation secreted into substrate; B -stains caused by deposits of pigmented cells in substrate's matrix
3: fruiting bodies embedded into substrate
4: enzymatic digestion of material

Each type of the biodeterioration is exemplified in selected actual artworks and study papers with fungal deposits.

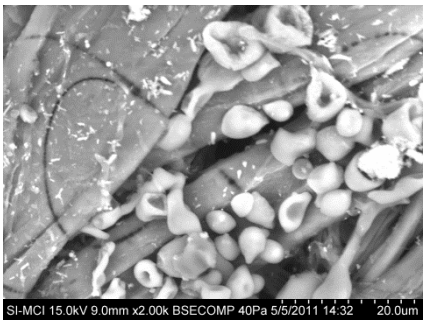
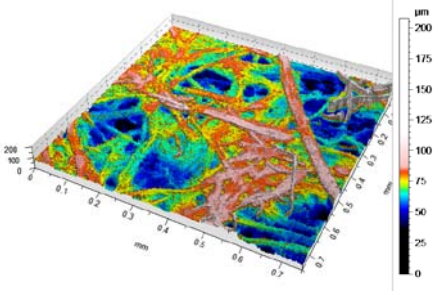
1. Pigmented spores in aerial mycelium

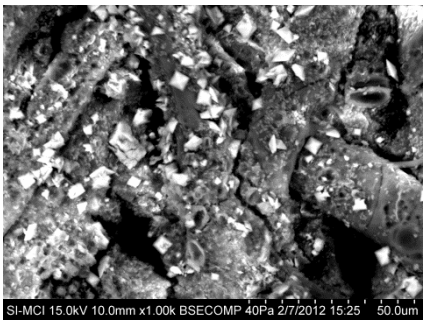
Biodeterioration of paper surface	Symptoms and assessment
	**Fig. 1.2 The19th century Japanese woodblock print covered with mycelium and fruiting structures.** Image is obscured by mycelium and fruiting structures extending into the air. Although the growth occurs on the surface, bulk of the substrate is also affected by secretion of metabolites. The image is obscured by aerial growth, the substrate stained and the paper's structure is weakened. Macro-photography served as a documentation of the biodeterioration and provided an overview of intensity and characteristics of fungal deposits. Leica Delux-2C.
	**Fig. 1.3 Aerial growth of peritecia chracteristic for Chaetemium sp.; detail of fig.1.2.** *Chaetomium* sp. utilizes cellulose as a source of nutrient. Enzymatic secretions produced by these species chemically degrade cellulose into simpler units to make it suitable as nutrient. Stereomicroscopy was applied in the surface analysis, using Wild M8 Heerburgg binocular microscope.

2. Pigmentation secreted into substrate (A)

	**Fig.14 Detail of the 19th century Japanese woodblock print.** Staining of the surface caused by fungi. Pigmented metabolites are secreted into the substrate causing stains. Stereomicroscopy was applied in the surface analysis using Wild M8 Heerburgg binocular microscope.

Staining caused by deposits of pigmented cells in substrate's matrix

 **Fig.1.5 Fungal cells attached to the surface of paper fibers.**	Unidentified black fungal specie produced cells with features pointing towards meristematic fungi. Scanning electron microscopy in variable pressure (SEM-VP) was utilized in this characterization of the paper surface. Instrument used: SEM-VP Hitachi S 3700N.
 **Fig.1.6 Morphology of surface with fungal deposits reveals spatial distribution of fungal cells; 17th c. paper.**	Spatial distribution of fungal cells, visible here as small, spherical particles on the surface of paper were characterized with confocal laser scanning microscopy (CLSM). Fungal formations penetrated the paper matrix near the surface. Instrument used: Keyence color 3D Laser Scanning Microscope, VK-9700.
 **Fig.1.7 3D topography of fungal residue on paper surface; 17c paper.**	Fungal residue on paper can be characterized with white light confocal profilometer. Fungal deposits, visible as 'orange' residue along paper fibers. 'Orange' indicates the dimensions of deposits on fibers in relation to the paper surface topography. Instrument used: AltiSurf500, white light, confocal profilometer (CLA)

Biodeterioration of paper matrix	Symptoms and assessment techniques
 **Fig.1.8 Black pigmented fungal cells partially anchored to the paper fibers surface.**	Aerial macelial growth often penetrates into the substrate leaving pigmented deposits, one of the modes of stains foramtion. Transmitted light microscopy was used to investigate interactions of microorganisms with the substrate. The instrument used: transmitted light microscopy Leica DMLM.
 **Fig. 1.9 Biogenic re-mineralization of calcium carbonate paper filler to calcium oxalate by enzymatic activities of fungi.**	Chemical alteration alkaline filler in paper caused by fungal growing on paper lead to the formation of calcium oxalate; crystalline deposits on paper are of calcium oxalate. Scanning electron microscopy in variable pressure (SEM-VP) was utilized in the characterization of paper matrix. SEM-EDS indentified calcium oxalates. Instrument used: SEM-VP Hitachi S 3700N.
 **Fig.1.10 Bio-deposits in paper matrix; deterioration of paper bulk revealed on a cross -section of paper embedded in resin.**	The extent of microbial penetration as visible on this cross-section was examined first in transmitted light microscope which followed examination with SEM-VP. Here, black deposits are located near the surface. Transmitted light microscopy(Leica DMLM) was utilized to examine the depth of pemnetration of microorganisms into the substrate's matrix.

3. Enzymatic digestion of material

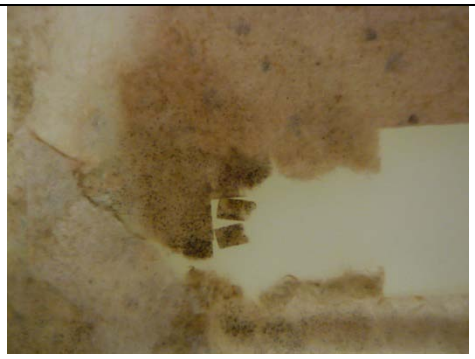


Fig.1.11 Cellulolitic fungi were identified in the area where paper structure was deteriorated.

Stereo-microscopy transmitted light microscopy and surface topography confocal scanning profilometry were used to characterize fungal structures embedded in paper. Interactions of fungal inclusions with paper matrix were observed in X-ray tomography, see fig. I.11 and 12.

Cameras used: Leica Delux-2 and Nikon D3100 with exchangeable macro-lens Tamron SP Di, 90mm1:2.8 macro 1:1 with UV filter.

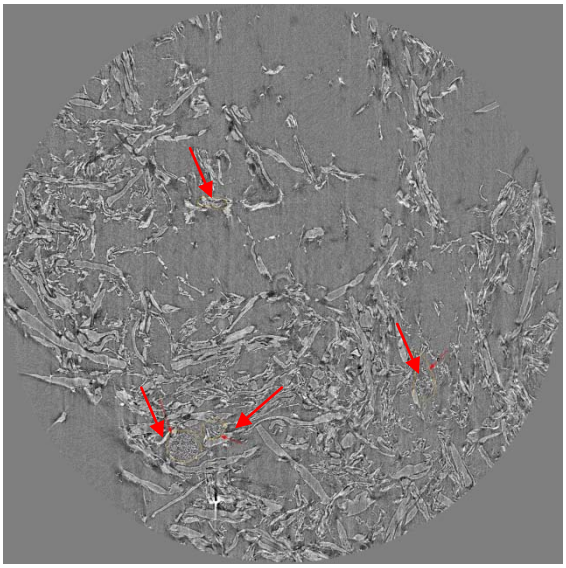


Fig. 1.12 Tomograph of a biodeteriorated, the 17th century Study Sheet procured from microtomography at the European Synchrotron facility, Grenoble, designated beam line ID 9.

Although fungal structures are visible, the full extension of their interaction with the substrate matrix can be achieved after 3D visualization of stacking all image-slices.
Red arrows re pointing to spherical fruiting structures.

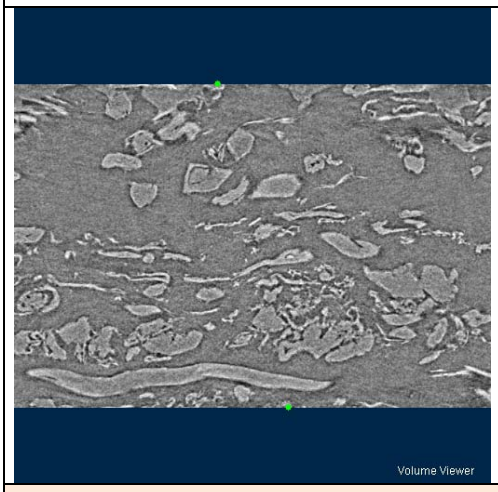


Fig.1.13 Cross-section obtained in X-ray tomography synchrotron. In addition to the analysis of fungal structures in the paper matrix, characteristics of the paper fibers were revealed.

Complexity of each component, paper, fungi and their interactions leading to biodeterioration are discussed in the following chapters in the context of the environment, time, and characteristics of the substrate.

2. Elements involved in bio-deterioration

2.1 Fungi, an overview in the context of cultural heritage

Fungi are microorganisms essential to many of our life functions. They degrade and decompose most organic materials as a means of attaining nutrients. That distinctive nutritional strategy is one of the defining characteristics of the entire fungal kingdom. These organisms secrete acids and enzymes into the surrounding environment which 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 consortia of organisms from various kingdoms. In addition, fungi are extremely important for the existence of human life. Some the species of *Penicillium* and *Aspergillus* produce antibiotics such as penicillin and griseofulvin, which are widely used in medicine. Moreover, molds play an important role in cheese production which enhances our dietary choices and tantalize our palette. Mushrooms, higher fungi, are culinary delicacies and a play a significant role as a food source in many cultures. Many positive attributes of fungi make them an important component of our life (Cavaliere, 2003).

Fungi make up a kingdom of heterotrophic single-celled, uninucleate, multinucleate, or multicellular organisms which include yeasts, molds, and mushrooms. Recent DNA evidence suggests that fungi are more closely related to animals than to plants. Because of that fungi are used as experimental organisms which attempt to solve human problems, particularly cell divisions, growth patterns and metabolic pathways. Lack of chlorophyll profoundly affects the life style of fungi, permitting them to dwell in dark habitats. Production of exoenzymes allows them to invade substrates with absorptive filaments. These exoenzymes are the most important reason why fungi are so successful. Fungi secret them at the tips of their hyphae into the substrate, and break the substrate into simpler molecules. The species which gain nutrients from dead organisms are termed **saprophytes,** and the ones feeding of off living organisms are termed parasites.

Fungi reproduce by sexual and asexual means. The asexual state is common for a group of fungi commonly called ‘mold’ and at times referred to as the anamorphic or mitosporic state. The sexual state is known as the telemorphic or meiosporic 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, Deuteromycetes (or Fungi Imperfecti), for which there is no known sexual state, are affiliated with at least three phyla, mostly to Ascomycota. Deuteromycetes, therefore, is not a valid taxonomic name, but a loose assemblage of organisms that do not belong to any specific phyla. Once a sexual form of reproduction is found, the fungus is moved to an appropriate taxonomic group. The advent of phylogenetic analysis of fungi using DNA-sequencing now allows describing and naming fungi which previously had no established association and taxonomic placement (Redhead, 2007).

Many fungi encountered on cellular heterogeneous material of cultural importance belong to the Deuteromycetes. They are sometimes referred to as Soil Fungi, because many of them live in soil, playing an important ecological role decomposing plant and animal remains. The Deuteromycetes include approximately 30, 000 widely distributed species (The Columbia Electronic Encyclopedia, 2007). Another name is Fungi Imperfecti, referring to the lack of sexual stage of reproduction. Those fungi in which a sexual stage is discovered are moved to a new taxonomic group based on morphology of the taxonomic form. However, to avoid the confusion, in 2013 a new regulation will be implement by the Department of Agriculture that will classify fungi based on their genetic makeup. For now, the nomenclature Fungi Imperfecti or Deuteromycota even though not a formally accepted taxonomic name will be used here because it is referred to in many texts dealing

with deterioration of cellulose-based cultural heritage collections. At the time of this writing (late 2011) a Google search revealed 204,000 hits for Fungi Imperfecti and 85,200 for Deuteromycota indicating that the nomenclature is still very much in use.

Fungi identified on cellulose -based materials have well developed, branching, multicellular mycelia. The term mycelium refers to the total mass of **hyphae.** (fig.2.1, 2.2) A hypha is a branching, tubular structure from 2-10 µm in diameter and usually divided into cell-like units by crosswalls called septa. The portion of the mycelium that anchors the mold and absorbs nutrients is called the **vegetative mycelium;** the portion that produces asexual reproductive spores is termed the **aerial mycelium.** The appearance of the hyphae and mycelium, along with the forms of **spores/conidia** provide the main criteria for identifying and classifying molds.

In their quest for finding the most suitable surface to attach to and begin 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 and overview of their growth and expansion, propagation, pigment formation, and some environmental and nutritional requirements are reviewed.

2.1.1 Mechanism of fungal growth

A new life cycle of a fungus begins with spore germination. For that to happen, a spore must come in contact with a suitable surface and get attached to a digestible substrate; suitable environmental conditions need to be met. That initial moment is essential and involves physical and chemical forces aiding adhesion (discussed in detail in chapter I.4. Paper and fungal 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.

Hyphae are polar and grow only at one end, forming a new wall that is tubular despite the presence of a high internal hydrostatic pressure (turgor). The fungal colony expands by hyphae extending and branching. A constant ratio that is maintained between the total length of hypahe and the number of tips is known as “hyphal growth unit” (Wessels 1997). (fig.2.1)

Hyphal growth

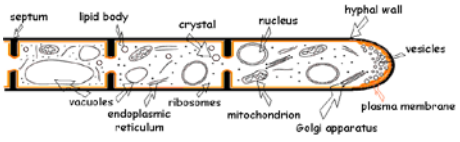
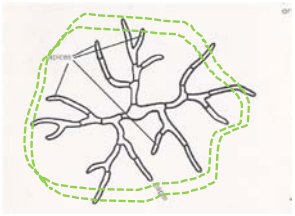


diagram: www.Fungionline.org.uk



H.Szczepanowska, JPC 1986

Peripheral growth zone of a colony

Hyphae are highly polar and grow only at one end. A new wall is deposited keeping the hyphal tubular shape, despite the presence of a high internal hydrostatic pressure (turgor). Enzymes are secreted at the apex. (Wessels 1997)

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 ‘hyphal growth unit’.

Fig.2.1 Schematic illustration of a hyphal tip and radial growth of hyphae; not to scale.

Hyphae are designed to transport water, nutrients and cytoplasmic components, through pores in septa. If hyphae lack septations, cytoplasm flows within the plasmatic interior. Hyphae secret enzymes at the apex which makes them particularly suitable to penetrate into solid organic substrates degrading the constituent polymers from within; examples of enzymatic digestion of paper substrate, see fig. 1.11. Enzymes are often pigmented, therefore one of the sources of stains in substrates. In the absence of nutrients, hyphal growth can be sustained by the transport of water and nutrients contained in cytoplasm, until a patch of nutrient is found. Hypahe can expand over large areas that contain only isolated patches of nutrients. The colony grows radially at its periphery where apical extension of each hyphal filament forms branches.

Emergent growth of hyphae, extending into the air as felt-like mats of mycelium, may be a manifestation of hyphal adaptation to lack of nutrients (fig.2.2). Aerial hyphae also bear fruiting structures which break at their apices dispersing spores. That is part of the vegetative, asexual stage of reproduction of fungi (fig. 2.3-2.8). Spores are then dispersed and land on a suitable substrate ensuring continuation of their life cycle. Many of them are pigmented, and produce colored colonies on materials on which they grow, as illustrated in different types of biodeterioration in chapter I.1.Definitions.

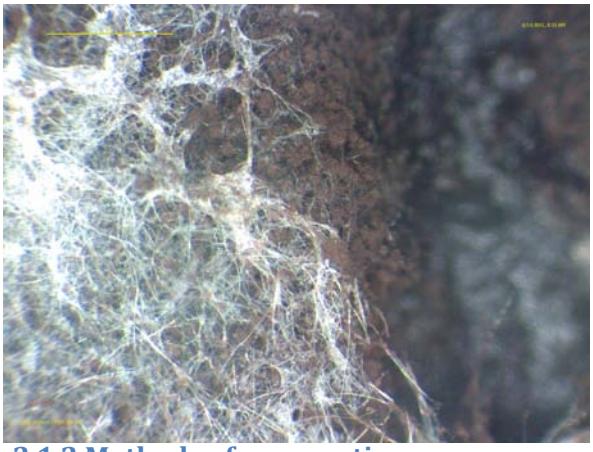


Fig. 2.2 Mycelium of *Aspergillus niger* growing in culture. White, filamentous growth bears dark brown or black fruiting structures. Stereo-surface analysis microscope, WILD, magnification 12x. Scale bar: 500µm

2.1.2 Methods of propagation

Fungi Imperfecti propagate by the production of asexual spores (conidia) or 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 fusion of cells. 1) sporangiospores, 2) conidiospores, 3) arthrospores, and 4) pycnidiospores are some of the most common forms of asexual spores. Their features are used in the identification of species. Sporangiospores are borne internally within a terminal sac (sporangium). Conidiospores are borne externally in chains on aerial hyphae called conidiophores (fig.2.3-2.7). Arthrospores are produced by division of hyphae. Pycnidiospores are produced in a fruiting structure called a pycnidium.

1. **Conidiospores**

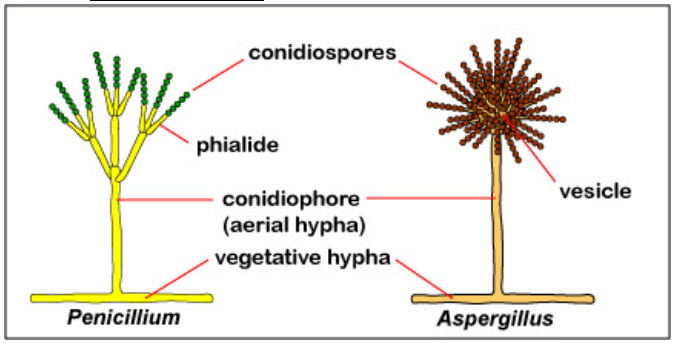


Fig.2.3 A schematic illustration of conidiospores growing on conidiophores (Kaiser, 2008). (not to scale).

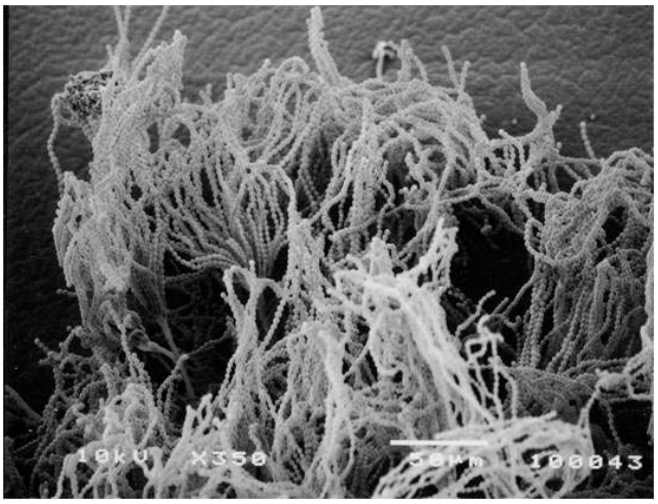


Fig. 2.4 Chains of conidiospores of Penicilium sp. (Szczepanowska & Cavaliere, 2003) ScalChains of conidiospores of Penicilium sp. (Szczepanowska & Cavaliere, 2003) Scale bar: 50µm

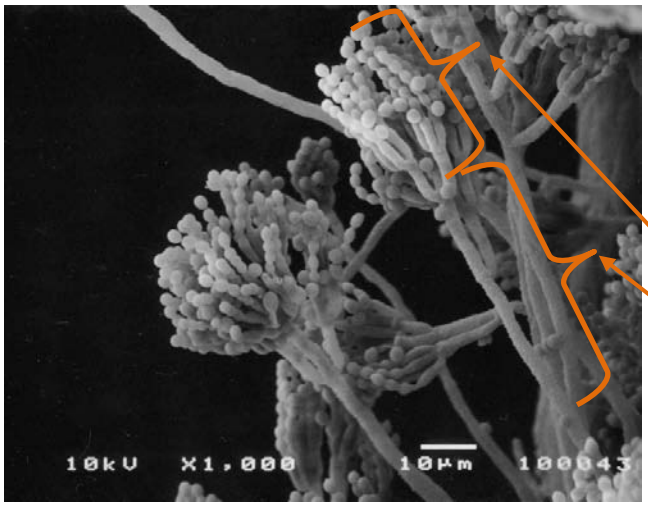
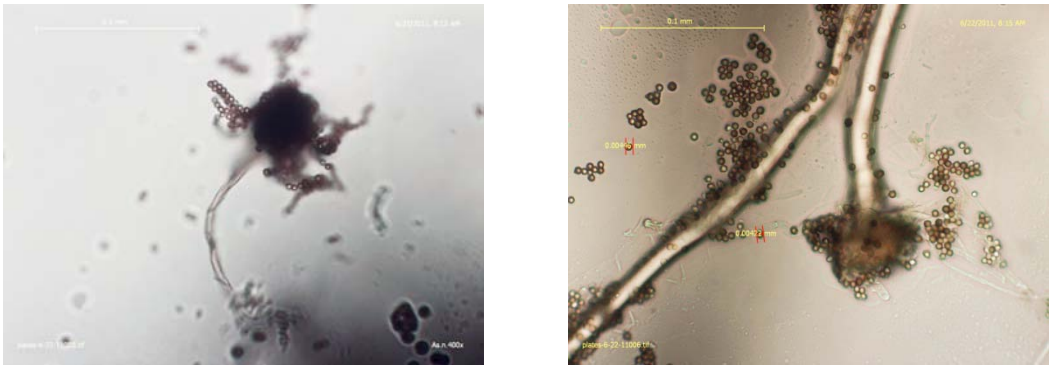


Fig. 2.5 Conidiophores and conidia on phialides. The conidiospores of Penicillium, usually grey, green, or blue are produced in chains on finger-like projections called phialides growing from the conidiophore (Szczepanowska & Cavaliere, 2003).



(a) (b)

Fig.2.6 (a) Aspergillus niger, conidiospores growing on a culture plate. After the spores are dispersed, a well defined vesicle is visible (b); transmitted light, 400x. Scale bar: 50µm.

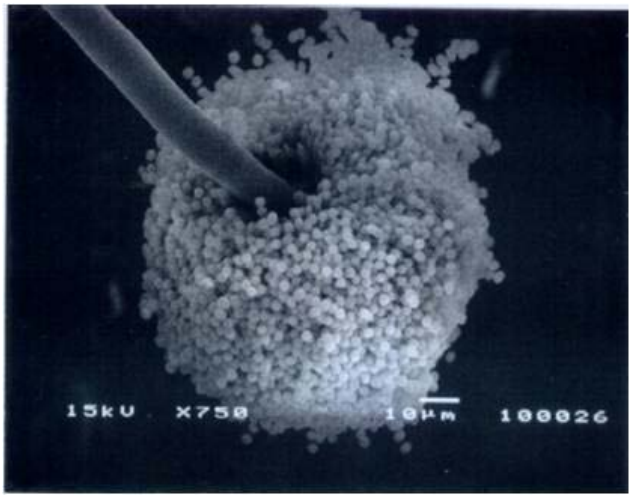


Fig. 2.7 Aspergillus niger, well expressed conidiospores on a conidial head; 750x. (Szczepanowska & Cavaliere, 2005).Scale bar: 10µm.

2. Sporangiospores

Sporangiospores can also be 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 a sac-like structure called a sporangium (fig.2.8).

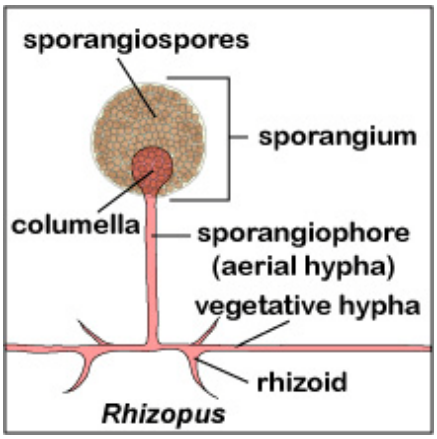


Fig. 2.8 A schematic illustration of sporangiospores within a sporangium.. (Kaiser; 2008) (not to scale)

Rhizopus is an example of a mold that produces sporangiospores. The sporangiospores, typically brown or black, are produced within the sporangium (see Fig. 2.8 and 2. 9). Anchoring structures called rhizoids are also produced on the vegetative hyphae. *Rhizopus* has been identified by various researchers on paper-based cultural material. It was also found on the study plates that infested the test papers.

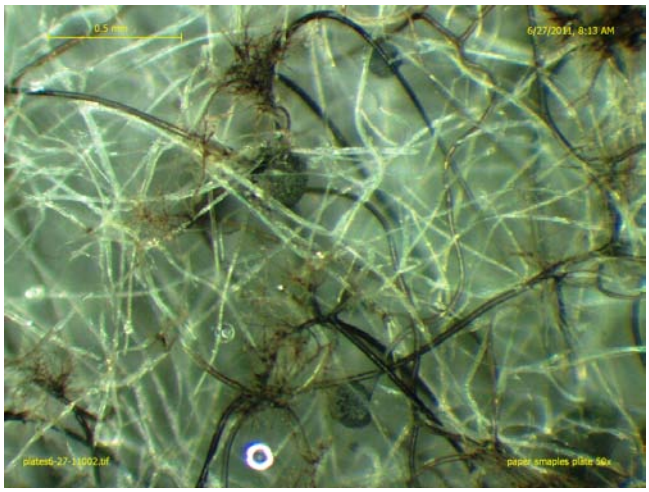
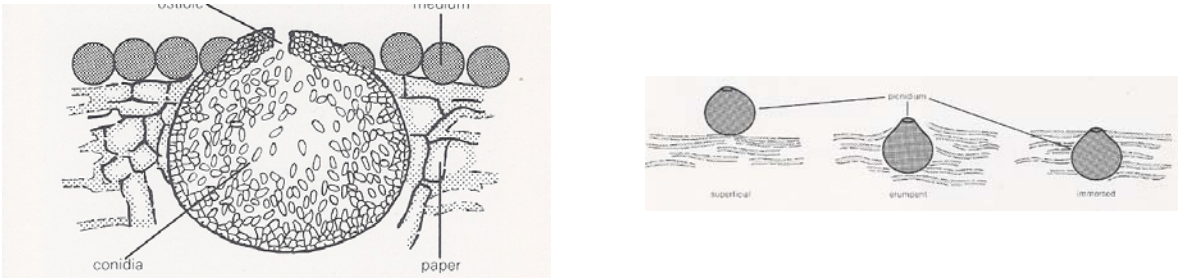


Fig. 2.9 Rhizopus stolonifer, with dark sporangiophores and rhizoids. One of the functions of rhizoids is to attach the organism to a substrate. Their pigmented filaments cause staining of substrates. Stereo-surface analysis microscopy, culture plate, 50x, scale 500 µm.

Sporangiospores and conidiospores are disseminated by air, water or animal, and once in a suitable environment, germinate and produce new hyphae. As dispersal units, spores may travel great distances. Arthrospores and some conidia may be long-lived. Viability of conidia is reduced rapidly in the presence of UV radiation, and in dry conditions.

3 Pycnidiospores Pycnidiospore (or pycniospores) form in the fungal tissue itself (a pycnidium), which is shaped like a bulging vase. 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 submerged in it and open by a pore, (ostiole), releasing spores (fig.2.10-2.12).



(a) (b)

Fig. 2.10 A schematic illustration of pycnidium with an open ostiole, containing pycnidiospores. (Szczepanowska, 1986; fig. 14,p.34.) (not to scale).

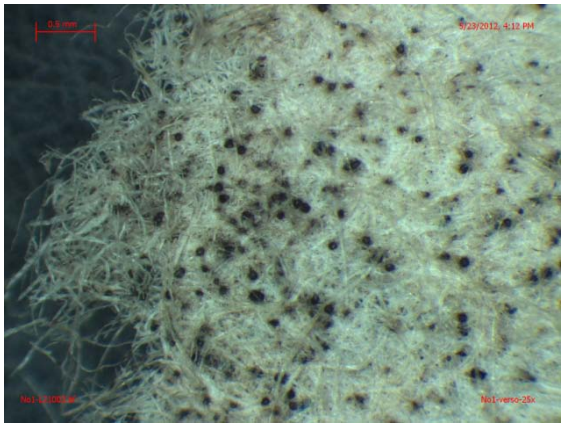


Fig. 2.11 (a) Various locations of pycnidia in the substrate: superficial, partially buried (erumpent) and inside the substrate matrix (immersed). (Szczepanowska1986, fig.9 p.33).

(b) Erumpent pycnidia on No1 Study Sheet; details see ch.9.1

Conidia germinate by germ tube extension. In a few cases however, conidia germinate to form yeast cells, or to form a further conidium. 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.

4. Ascospores produced as a result of sexual reproduction.

Sexually produced spores are formed within flask-shaped structures (**ascocarps**) formed by aggregation of hyphae surrounding spherical or club-shaped **asci.** Sexual **ascospores** are produced within asci. The various types of ascocarps are cleistothecia, perithecia and apothecia. Ascocarp structure is a major criterion used in separating the Ascomycota into classes. (fig.2.12)

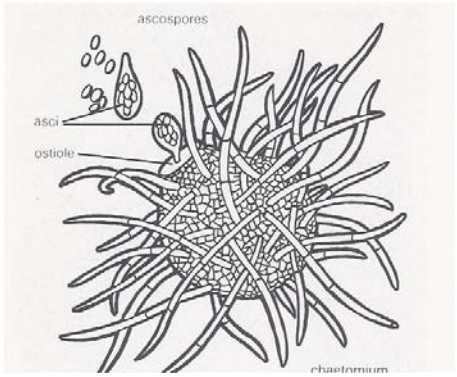


Fig. 2.12 A schematic drawing of an ascocarp (perithecium) of Chaetomium , showing seta (perithecial hairs) and asci being released via the ostiole. Each ascus contains 8 ascospores. (Szczepanowska, 1986, fig.12, p.34). (not to scale)

Fig. 2.13 *Chaetomium globosum*, perithecium with seta, perithecial hairs, growing in all directions. The species was growing on deteriorated paper pamphlets. (Szczepanowska & Cavaliere, 2000)

2.1.3 *Chaetomium globosum*

Chaetomium sp has been identified on the Japanese print as the predominant microorganism and on many other paper-based artifacts therefore a brief discussion of its characteristics is offered here.

Chaetomium is one of the largest genuses of saprophytic Ascomycetes, and as a cellulolytic fungus is frequently encountered on paper substrates. For some species cellulose is required to ensure good growth and fructification. Since its first description in 1817, over 300 species have been described (Arx et al. 1986). *Chaetomium* species are characterized by producing superficial perithecia usually covered with hairs (setae) (fig.2.13 and 2.14) and are attached to substrates by rhizoidal hyphae. The ascospores develop in asci, are brown or gray-olive when mature, but never opaque or black (Arx et al. 1986). Optimal temperature for species of *Chaetomium* is 25-35^{o}C and dark cavities are favored.

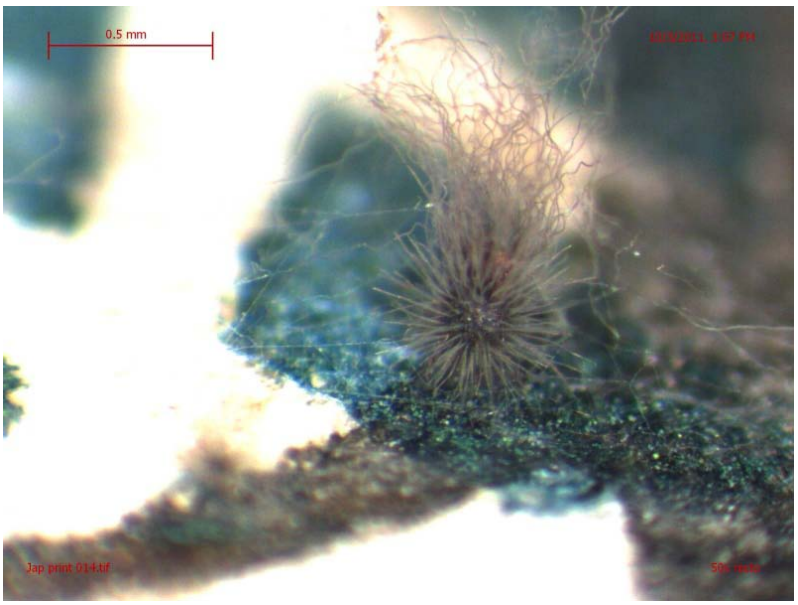


Fig. 2.14 A single perithecium of *Chaetomium globosum* on the surface of a Japanese woodblock print. Perithecial hairs extend from the walls of perithecium. Attachment of a single perithecium on the substrate can be attributed to electrical charges between the surface of the substrate and the electron dense hyphae and perithecial hairs. Stereo-micrograph, scale bar: 0.5mm

Characteristics of the perithecial walls are often used in species identification; some of its features are discussed here in the context of biodeterioration. The perithecium is composed of densely intermingled and branched hyphae. The cells have an inner electron transparent wall layer, bound

together by an outer interhyphal wall which is slightly more electron dense than the inner layer. Cells facing the exterior are surrounded by an electron dense outer wall. Actively growing hyphal tips, surrounded only by a single wall layer are present on the entire perithecial surface.

In further development of peridium the cells shaping the outer regions of 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.*) Electrons fill the outer cells of growing hyphae throughout the development of perihtecium until its maturity (Froeyen, 1980). Cells of the terminal perithecial hairs are bound by a thin wall also made up of an outer electron-dense melanized zones (Ellis 1981). Therefore it can be deduce that the attachment of single perithecium on the substrate is attributed to electrical charges between the surface of substrate and the electron dense hyphae and perithecial hairs.

2.1.4 *Aspergillus niger*

Aspergillus niger was selected for the production of black pigmentation in the Light Experiment (see chapter II.8.); therefore, a brief characterization of the fungus is discussed here.

Aspergillus Niger is a member of one of the oldest genus of fungi, *Aspergillus which* received its name from Micheli in 1729 (Bennet 2010). It contains several hundred species. It is a common member of the microbial communities found in soils. However, in 1854, early in the scientific history of this genus, deBary became the first person to notice that an *Aspergillus* mycelium, specifically *A. glaucum* produces cleistothecia, which is another form of reproduction, and characterizes different reproductive phase of the same organism. It is also named differently, *Eurotium herborarium.* That observation was a beginning of a dual naming of the same organism, based on its stages of development.

A. niger is an important industrial fungus that is widely used for the production of enzymes and metabolites, such as citric acid, one of the most widely used food ingredient and utilized also in the pharmaceutical and cosmetic industries. The first patented citric acid process occurred nearly 90 years ago.
http://www.broadinstitute.org/annotation/genome/aspergillus_group/GenomeDescriptions.html

Furthermore, *Aspergillus niger* has been used to study fungal protein secretion, proteolysis, to understand cell wall biosynthesis and degradation of cell wall in plants. As a soil saprobe it produces a wide array of hydrolytic and oxidative enzymes which are involved in the breakdown of plant lignocelluloses, therefore it is a subject to intense studies in agriculture. *A. niger* has been an important model fungus for the study of eukaryotic protein secretion in general, 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. Source:
(http://www.broadinstitute.org/annotation/genome/aspergillus_group/GenomeDescriptions.html)

Species of *Aspergillus propagate* asexually via spores that drift on air currents, dispersing themselves both short and long distances, depending on 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, pectins and other sugar polymers. Maximum decomposition occurs when there is sufficient nitrogen, phosphorus and other essential inorganic nutrients (Bennet

2010). Both paper and textiles (cotton, jute, and linen) because of the content (cellulose and starches) are vulnerable to *Aspergillus* degradation in all three realms.

Identification

The defining characteristic of the genus *Aspergillus* is the aspergillum-like spore-bearing structure. Fig. 1.7. It is the most important microscopic feature used in *Aspergillus* taxonomy. Black spores are characteristic for *A. niger;* other species in the genera maybe green or yellow. Another factor used in species' identification is the color of the colony, the growth rate and thermotolerance. In some case, in the identification process, morphological features are supplemented with biochemical characteristics such as secondary metabolite production or ultramicroscopic traits such as spore ornamentation (Kozakiewicz, 1989).

2.1.5 Meristematic fungi

Morphological features of black fungal stains studied on the historic paper (II.9.1 and 9.2) resulted from the growth of meristematic fungi a group of fungi that slowly expand by reproducing via isodiamteric enlargement of subdividing cells. (fig. 2.15, 2.16) Dark pigmentation of their cell walls is associated with the production of melanin (Sterflinger 1999). Meristematic fungi can exist in demanding environments of extreme temperatures, with scarcity of nutrient and limited water availability, UV radiation and oxygenic action. Their presence was reported on rocks and stone in diverse localities from the deserts of Arizona to Antarctic, from the Ivory Coast, to the Mediterranean countries. Those found on marble and granite monuments were responsible for their deterioration by disrupting the coherence between crystals (Sterflinger 1999). Only two references noted presence of meristematic fungi on paper (Strzelczyk, 1981; Urzi, 2002).

Phylogenetically meristematic fungi belong to or have close affinities to at least three different orders of the fungal Kingdom: *Chaetothyriales, Dothideales* and *Pleosporales* (Sterflinger 1998). Their identification is difficult because they have the ability of conversion from meristematic to filamentous to yeast-like growth (Sterflinger 1999). In addition to black fungal cells, an often local clump of extracellular material was present.

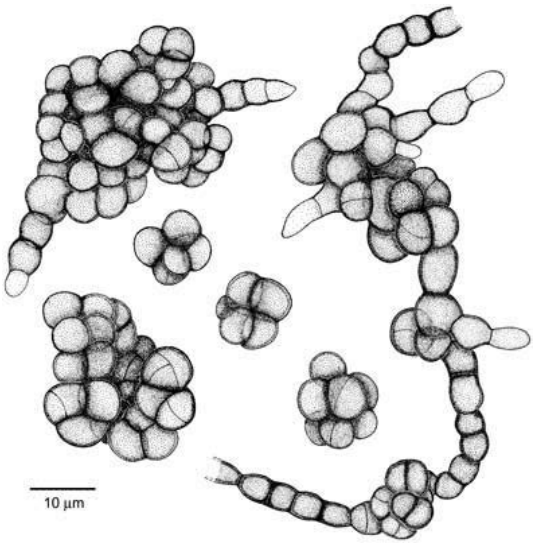


Fig. 2.15 Phaeosclera dematioides, UAMH 4265, clumps of meristematic cells on hyphae. (Sigler at al 1981; original: Hoog, G.S. de 2000, Atlas of clinical fungi, ed. 2: 1-1126. source: www.mycobank.org)

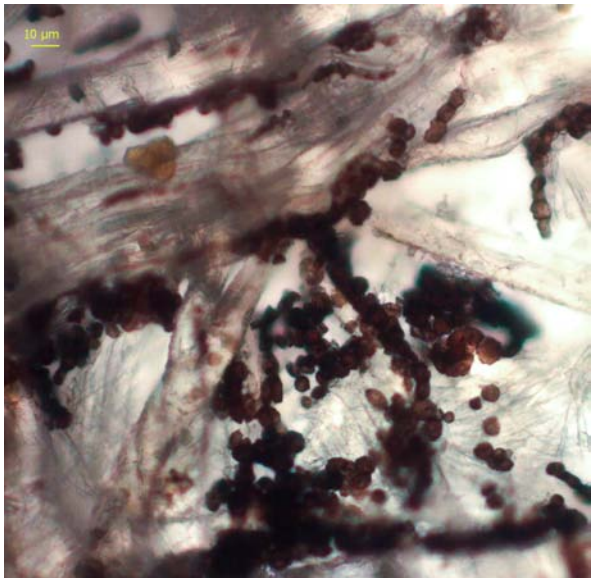


Fig. 2.16 Dark pigmented fungal cells in black stains on No Study Sheet 17th c. Scale bar: 10 µm; details see II.9.1.

2.1.6 Summary points

1. The criterion used in the selection of the specific types of fungi for discussion in this chapter was their frequency of occurrence on cultural heritage material. Most of them are filamentous fungi that belong to Fungi Imprefecti. 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.

2. Two of the species, *Chaetomium* sp and *Aspergillus niger* were discussed in more detail because both are commonly encountered on paper-based cultural material (*Chaetomium* was found as the predominant fungus on the Japanese 19th c. woodblock print, II.9.3, while *Aspergillus* was used in the Light Experiment II. 8).

3. Meristematic fungi pose particular challenge because even though they have been recognized as present on cultural heritage objects they have not been studied extensively, thus leaving that area open for further research.

2.2 Paper, surface and bulk characteristics

2.2.1 Paper, an overview

Paper is a web of fibers and fillers assembled with a great degree of heterogeneity in make-up, shape and chemical composition. Paper 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) However, the scientific attempts to characterize paper structure and link it with paper properties are relatively recent, beginning in the 1960s (Bristow 1987).

Paper has been used for many applications, from a material on which art was created and documents written, to clothing and umbrellas, to balloons that carried incendiary bombs during WWII. Each application required different optical, physical and chemical properties of paper to meet such diverse purposes. Properties of paper rely on properties of fibers that compose them, process which fibers undergo and characteristics of additives.

Paper is a two-phase system, a three-dimensional structure of fibers and voids between them. Voids play an important role in water sorption and consequently in bio-susceptibility of paper. Surface of paper has different properties than bulk. Surface energy plays a role in microbial attachment, as pointed out in chapter I.4. Smoothness or roughness of the paper surface are imparted by application of **sizing** (or its absence) and different methods of flattening, while paper pliability, rigidity, density or weight, are engineered during paper pulp processing. Because of the importance of sizing on paper characteristics, papers are described in two broad categories as sized and unsized. Synergy of all these features affect microbial interaction with paper to various degrees as demonstrated in the experimental part of this work. (See part II Experimental Work).

There are many different plants that supply **fibers for making paper**. Commercial papers use primarily wood-origin fibers, while artists' papers are made from cotton and linen (flex) fibers derived from rag. Thus such paper is often referred to as 'rag paper'. The main difference 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 1986). Paper made in the Far East, Oriental paper, use mitsumata or mulberry-paper (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 where it was produced and the characteristics of the raw materials used as paper fibers.

The **paper making processes** serve as one of the broad criteria in describing paper, as hand or machine made. Examples of artists' (cotton) Western hand and machine made paper and one Oriental paper were used in this study in an attempt to discern any correlation between the papers' structure, fibers type or surface finish and biodeterioration. The discussion is confined to cotton and mulberry-paper fibers selected as predominant fibers of papers selected for these study papers.

2.2.2 Characterization of paper

Paper sheet

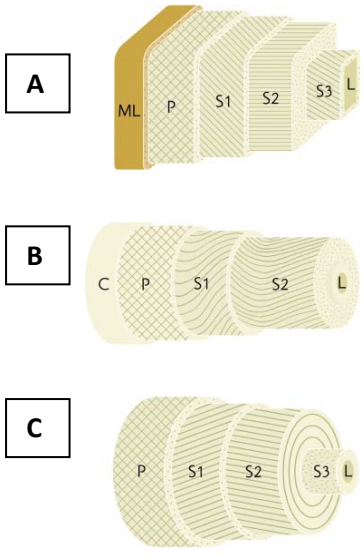
Fibers form **paper sheet** which is characterized using different criteria: grammage, fiber distribution in a sheet, fillers distribution in the paper, fibers anisotropy and rigidity or pliability are some examples. The descriptors reflect the purpose of paper. Grammage is the term for the mass of a unit area of paper recommended by ISO 4046-1978 as replacement for "basic weight" or "substance". It is expressed in SI units kg/m $^{2.}$. Within a paper sheet there are always variations in **grammage.** Uniformity of paper fibers distribution in a sheet is referred to as formation. A uniform sheet has a low formation number. However, nothing can be concluded from the formation number alone (Norman 1986). The grammage and fiber distribution in the sheet have impact on paper biodeterioration, with respect to presence of pores or open, airy space in paper, as supported by the experimental investigation of biodeteriorated papers. (see part II, Light Experiment)

Paper fibers structure

The basic unit of paper, a plant fiber, serves as support onto which fungi are attached in biodeteriorated papers. Fiber structure has impact on water adsorption; therefore fiber structure and surface morphology are discussed in more detail, emphasizing those features that play a role in biodeterioration of paper.

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. (fig 2.17)

Fig. 2.17 Cell wall structure of paper making fibers: A- native wood pulp; B- cotton; C- flax.



The layers are distinguished by differences in the orientation of cellulose microfibrils indicated here as lines. Their configuration has impact on water adsorption, as elaborated in the subsequent chapter (I.2.3 Environment)

P-primary wall
S1-2 – several layers of secondary walls
L-lumen
ML-middle lamella in wood fiber
C-cuticle layer that covers cotton fiber

(Image source; Fig. 4.26. p. 102 paper and Water, G. Banik and Irene Bruckle 2011; reprinted from: Smook (1989), Krassig (1993))

Cellulose morphology

Cellulose microfibrils are the basic reinforcing element embedded in a stress –transferring matrix of amorphous polymers in a fiber (Bristow 1987). Cellulose exists in different phases, as crystalline and non-crystalline regions (Kolseth and de Ruvo, 1986). Each region reacts to temperature changes, water and acidic conditions differently, and that aspect is most closely responsible for the fibers susceptibility to microbiological infestation.

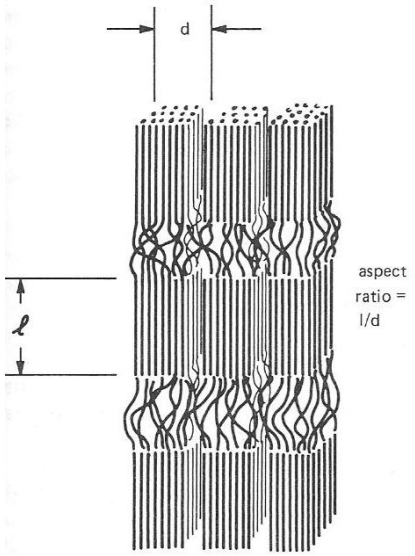


Fig. 2.18 Model of amorphous and crystalline regions in cellulose fibril according to Hess, Mah and Gutter (1957). Parallel lines indicate crystalline structure and flexing lines, amorphous regions; d-is the diameter of crystalline region and l- its length. (fig.1.p53 Salmen 1986)

Exposure to dilute acid hydrolyzes amorphous regions, while the crystalline remain intact (Kolseth and de Ruvo, 1987 citing O.A. Battista, 1975). The disordered regions in cellulose are more accessible to water than crystalline, thus, one may assume, more prone to microbial colonization. The phenomenon of **imbibitions** by paper fibers is complex and presents an area of extensive studies in the physics of paper (Alava and Niskanem 2006), some aspects of which are discussed in the subsequent sections.

Cellulose chemistry

The high content of hydroxyl groups in cellulose provides many possibilities for hydrogen bonding. (fig.2.19) Hydrogen bonding, covalent bonding and van de Walls forces which are considered in discussions of various cellulose models play a role in microbial adhesion to fibers' surfaces (as reviewed in chapter II.3 Microbial adhesion to surfaces).

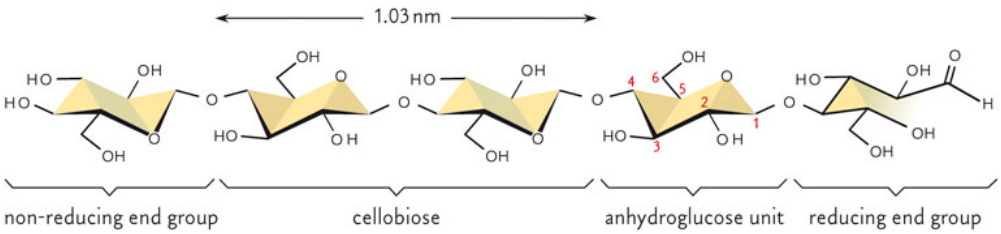


Fig. 2.19 Cellulose molecule. Covalently bonded anhydroglucose units make up the cellulose chain. Each unit features three hydroxyl groups. (Banik, Bruckle 2011; fig. 4.2 p. 83reprinted from: Klemm et al. 1998).

Paper fiber content

Cotton fibers are the seed hairs of the plant *Gossypium.* Each fiber is 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, looking like a twisted ribbon or a collapsed and twisted tube. These twists are called convolutions. (fig.2.20)

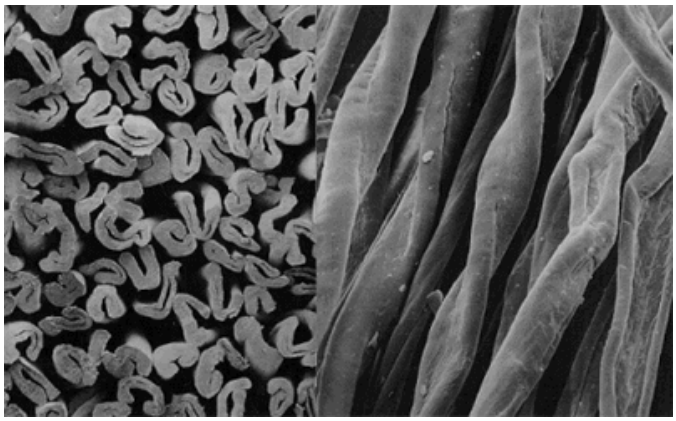


Fig. 2.20 Morphology and cross-section of cotton fibers were used as a guide in the analysis of paper fiber content. Image source: http://www.freewebs.com/textile-technology/fibrescience.htm); accessed: 4/13/2012)

Cotton used for paper making is derived from new textile cuttings, old rags and linters. Linters are the shortest fibers in the harvested lot, generally 12-64mm long and 20 µm wide (Emerton 1980). 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.

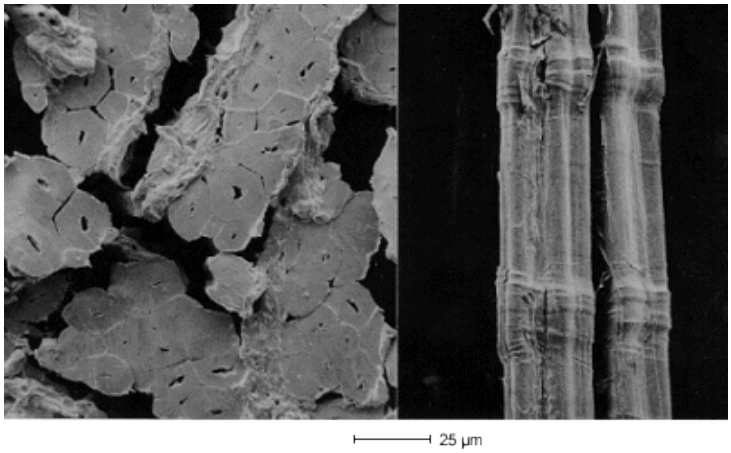


Fig. 2.21 Flax (linen) fibers are often used in artist's paper, are seen here on SEM micrograph. The morphological features of their surface (prominent nodules) and their cross-sections were used in the comparative analysis of paper fiber content in the experimental papers.

Image source: http://www.freewebs.com/textile-technology/fibrescience.htm); accessed: 4/13/2012)

Flax is the bast fiber used in textile and in fine paper (rag papers). The fibers occur 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). (fig.5) Chemically, the flax polymer is the same as the cotton polymer; both are cellulose polymers. Physically, the flax polymer differs from the cotton polymer in the degree of polymerization. (Source: http://www.freewebs.com/textile-technology/fibrescience.htm).

Comparative analysis of the morphological features was applied in the evaluation of the fiber content in all studied papers (see Part II, chapter 6.2.4).

Oriental paper fibers

Fibers of mulberry-paper (common name: kozo) and .mitsumata are bast fibers, and similar to flax derive from the inner bark of plants or shrubs. These fibers are used in Oriental handmade papers producing strong and permanent papers. Besides showing thick-walls, the fibers do not have very distinctive morphological features, therefore, are not easily identified microscopically. Paper-mulberry may show a transparent membrane in some places along the fiber wall. Mitsumata has a broader portion in the central part of the fibers (Ilvessalo-Pfaffli, 1995). Only some of these features were identified on the fibers in paper used in this study. (see Part II, chapter 6.1 and Table IV: Oriental Paper Fibers).

Fibers dimensions are one of the features guiding their identification and also serve in differentiating fungal filaments from paper fibers. Fig.2.22.

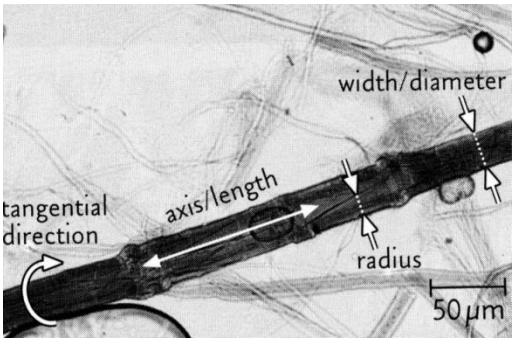


Fig. 2.22 The measurements of paper fibers, length, width and tangential direction is illustrated on a fiber. (Banik and Bruckle, 2011; fig. 4.24, p. 101; adapted from D. Meyer).

2.2.3 Paper structure

Formation of paper sheets

Handmade paper produces superior material which is 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 mould). Fiber flocks are almost completely avoided. (note: **flocculation** is a process in which particles aggregate into small lumps). Scale of measuring the flock size is important in the proper assessment of the forming property (Norman 1986, Alava and Niskanen 2006).

Although evenness of fibers distribution enhances quality of paper (mechanical properties and suitability for printing) it did not seem to have an effect on the fungal growth or fungal pigment distribution. The 17th century paper (Sheet No.1) and the Oriental paper, both handmade papers, with very evenly dispersed fibers were randomly stained by fungal pigmentation. The staining was characteristic for the type of fungal specie and most likely water availability rather than related to fibers distribution in the paper sheets. Similarly, no pattern of fungal growth or pigmentation was observed in machine made paper of the 1920 Engraving or on the Winsor and Newtown watercolor paper. One common factor in the examination on a microscopic level was partial attachment of fungal cells (mycelium and swollen hyphae) to fibers and growth towards pores in paper, airy and open spaces within the matrix, as discussed in detail in the subsequent section of this chapter. Fig. 2.28-2.30.

Thickness and density

In classical mechanics 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, there is a density distribution in the thickness direction (Feller et al. 1986). Density is a structural characteristic that is one of the primary parameters determining the mechanical properties of paper (therefore one of the descriptors of paper's structure). A specific density can be achieved in the manufacture process, using different methods of fiber beating, wet processing, or finishing, such as calendaring. Density along with surface finish appears to have impact on the fungal growth, pigment deposition and stain formation.

Paper sizing

Gelatin and starch were historically used as paper sizing. Gelatin is made of animal skin, therefore of protenacious base. Starch is a polysaccharide derived from plants. It is estimated that on a commercial level starch sizing was abandoned ca 14th century in favor of gelatin (Clapp cited in James 1978). However, today both are being used in individual, small production paper mills.

Watercolor papers are traditionally sized, meaning treated with a substance to reduce the paper sheet absorbency and making it hydrophobic (that is repelling water). Internal sizing is added to the paper pulp after rinsing and before it is cast in the paper mould; external sizing is applied to the paper surface after the paper has dried. The traditional sizing has been starch, gelatin, gum Arabic or rosin (Hunter 1978; Emerton 1980). These substances bond with cellulose via hydrogen bonding and have film-forming propensity on the surface of fibers, thus modifying the paper surface. Because the presence of sizing has impact on paper-water relationship it affects biodeterioration of paper as elaborated in chapter 2.3 The Environment.

2.2.4 Paper surface

Forces of suction and compression that are applied during the paper making process conform the fibers of the surface to the topography of the support on which paper is formed. It could be a felt fabric or metal wire depending on the process of the sheet formation. Surface is irregular, and these irregularities and depressions may be seen as an extension of the pore system in the matrix of fibers.

Surface topography, or roughness, is defined in terms of reference plane brought into contact with paper under a defined pressure. Fig. 2.23

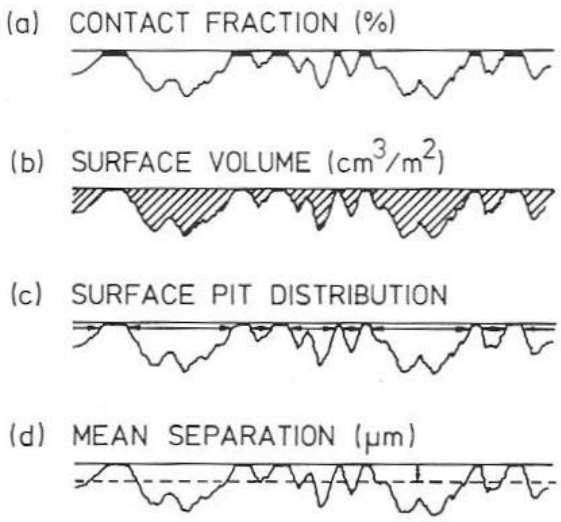


Fig. 2.23 There are many different ways in which the surface topography of paper can be defined. (A.J. Bristow, 1986; p.172 fig.1)

Surface topography can be measured with many different techniques depending on the purpose of the measurement. Earlier methods employing profilers that traversed the surface have been replaced by non-contact optical instruments that reflect light at different angels from the surface. A comprehensive review of surface analysis and measurement techniques is included in J. Preston's, Surface Analysis of Paper (Preston 2009).

Other methods characterize the roughness in terms of the voids volume 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 continuous with the pore network.

In practice, paper roughness is commonly determined by measuring the air flow between the paper surface and a reference plane under specific conditions. The values are expressed in air resistance (min/ml) or as air flow units. There are several instruments measuring air flow, the earliest on is Parker-Print-Surf. The 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 and 1971 cited by Bristow, 1986).

It is an essential feature of paper that both paper itself and the surface are compressible, due to the porosity of the structure. However, because no general relationship can be established, density and surface roughness ought to be considered as independent properties. Furthermore, the surfaces of both sides of paper often differ from each other, and that appears to have impact on fungal stain formation. Fig. 2.24-2.27. (details see Part II, chapter6 and II.9.1). Because those two parameters are important specifically for the printing industry mathematical formulas were developed for measurements of the surface compressibility. They are not included here, because even though there is a correlation between surface roughness, there is also randomness in location of fungal deposits.

Fig. 2.24 Rough surface of paper with scattered fungal deposits. 17th c. p Study Sheet No1.

Fig. 2.25 Verso of the same paper, smooth, with minimal number of fungal deposits.

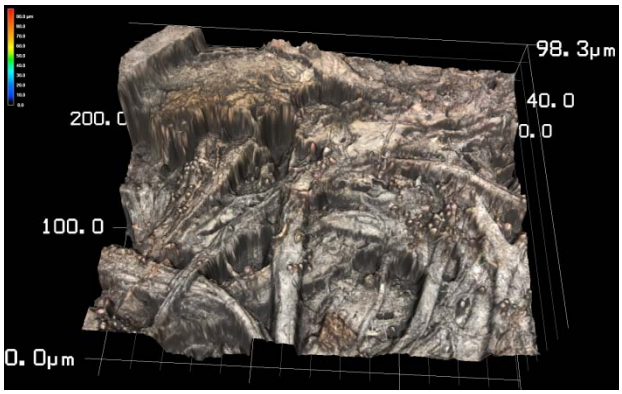


Fig. 2.26 3D topography of the same paper, rough side, showing numerous fungal cells. Confocal Laser Scanning Microscopy, (CLSM).

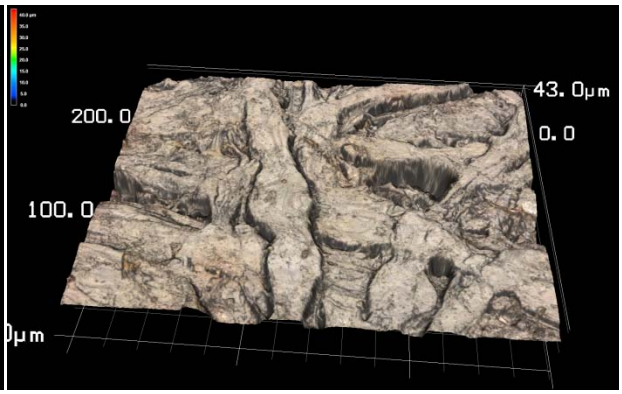


Fig. 2.27 3D topography of the reverse of the same paper; no fungal cells are detected. CLSM visualization.

Another aspect of paper structure that has impact on fungal growth and pigment deposition is the openness of the fibers' matrix, more precisely, the fungal access to air in the course of their development. In the microscopic observations of the biodeteriorated study papers, fungi showed chemotropism, directional expansion towards oxygen [1]in open pores, once they established firm attachment to the substrate. (fig. 2.28 and 2.29). That pattern of growth can be explained by the fact that fungi are aerobic and require oxygen for their development.

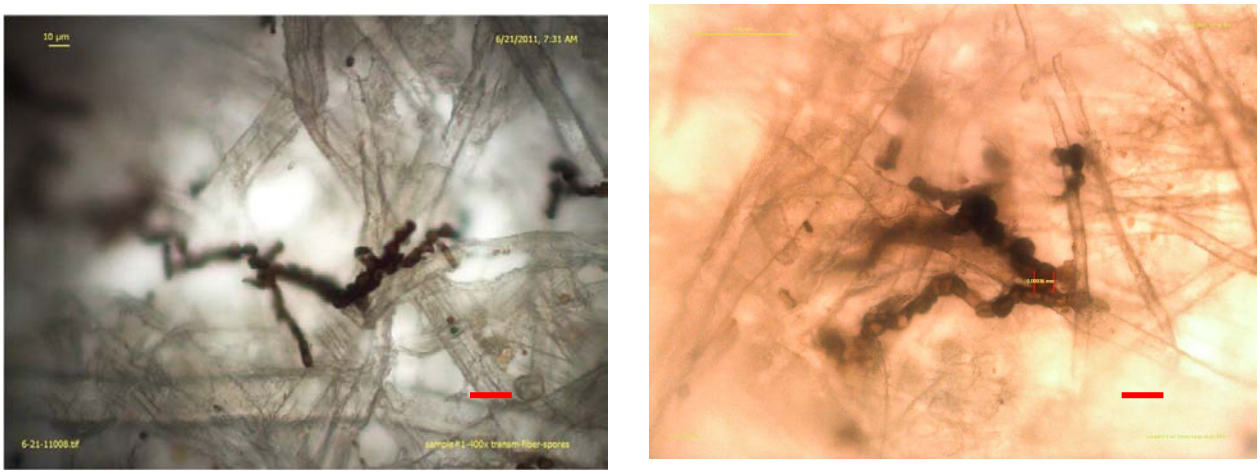


Figure 2.28 Two micrographs of fungal chemotropism in the area of black stains. (2.28) Paper no1, 17th century paper, (2.29) paper No4, 1940 Etching. Scale bar on both micrographs: 20 µm.

[1] Tropism (frequently as a suffix). A turning or growth in response to an environmental stimulus. Dictionary of the Fungi. 2008.

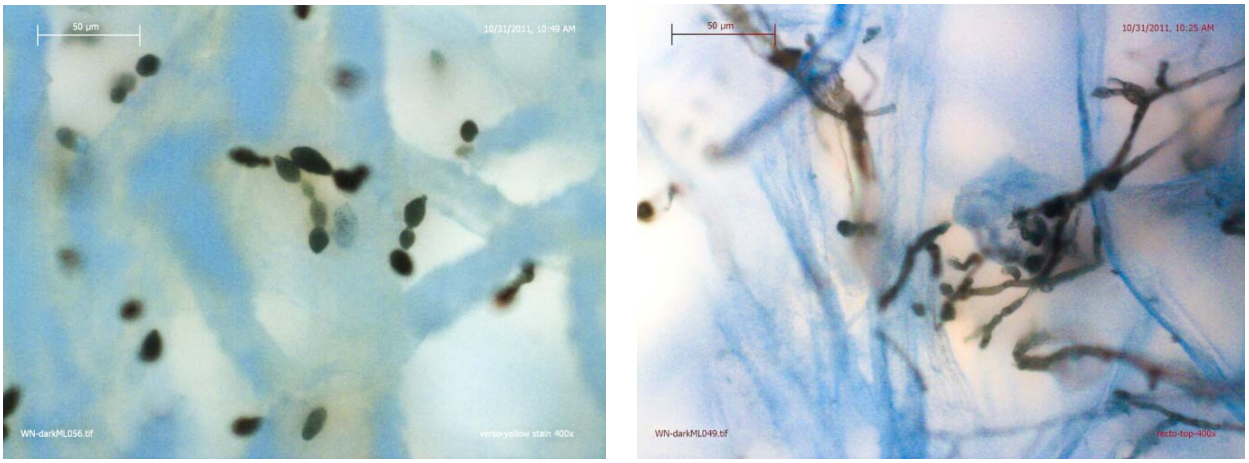


Fig. 2.30 Two micrographs of fungal chemotropism observed in fungi cultured on paper samples, in dark condition. Two types of microorganism grew on W&N paper (experiment No1, June 2011). 2.31. In both cases directionality of growth is towards air pockets in the paper matrix.

2.2.5 The pores' structure and the sorption of liquids.

Paper is a network of fibers embracing a network of pores, qualifying paper as an aerogel (Bristow 1986) or, a two-system structure of solid and gas. The word pore is used here to denote the macropores of interfiber spaces. Experiments by other researchers showed that virtually all pores are accessible to a liquid, and that influence on sorption of liquids is an important feature of pores. In most cases lumen of the fibers is collapsed. If the fibers are not collapsed, the lumen is accessible through the cell wall pits. That information may indicate that fungi not only search for air in pores but also presence of humidity which may promote their growth.

Paper is a two-phase system in which both phases are potentially absorbent, therefore the sorptivity of paper is not only associated with the porosity of the material. In other words, it is not only pores between the fibers which can take up liquid. The fibers can also take up liquid if the liquid has the ability to cause the fibers components to swell (Bristow, 1986; Alava and Niskanem 2006).

The sorption of water into paper fibers follows a complex diffusion process, where vapor phase diffusion in the pores, surface diffusion along the fibers, and bulk diffusion through the cellulosic material all play a part, and where the diffusion through the cellulosic material path is dependent on the network structure. The fact that both capillary flow in the pores and between fibers, and diffusion into fibers cell walls lead to a sorption, indicates that it is difficult to distinguish between them. These two types of sorption are at times referred to as interfiber and intrafiber penetration.

Based on experimental work of other researchers, dependence of swelling (volume increase) on water sorption into fibers and into pores can be plotted as illustrated in fig. 2.32

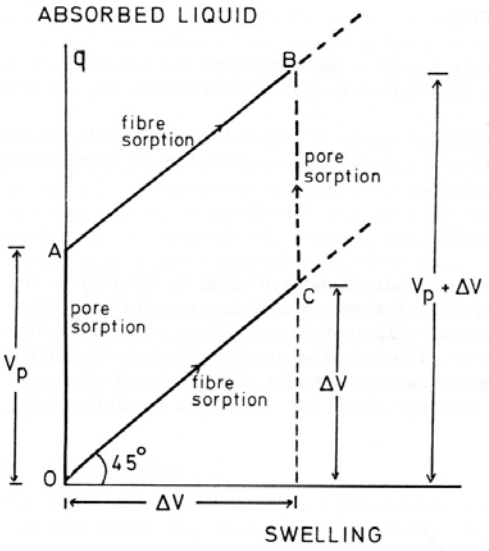


Fig. 2.32 A simple schematic parallelogram indicating relationship between volume increase and liquid sorption. The route OAB is the expected path if pore sorption precedes fiber sorption and OCB the expected path if fiber sorption occurs first. (Image source: Bristow, 1986 p.191 fig.4)

The water transporting properties of paper are one of the relatively little explored areas of the paper's physics (Alava and Niskanen 2006).

The path of absorbency appears to be in direct correlation with fungal growth and intensity of pigmentation on paper. Absorbency of water by Whatman4 translated into abandon and rapid growth of cultured *Aspergillus niger*. Water resistance of Winsor& Newtown paper was associated with a much smaller and slower fungal development and growth. (fig. 2.33 and 2.34) Detail analysis of induced pigmentation by culturing *Aspergillus niger,* see Part II chapter 8.2, characterization of paper surface modified by induced fungal stains.

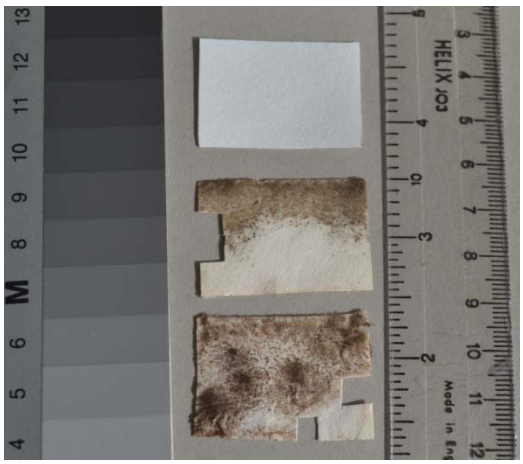


Fig. 2.33 and 2.34 Whatman4 (W4) paper on the left, with fungal pigmentation produced by Aspergillus niger cultured in light and dark conditions.

Whatman is an unsized, very absorbent paper. Note the intensity and abundance of dark pigmentation. Winsor& Newtown (W&N) paper on fig. 2.33, with fungal deposit produced by *Aspergillus niger* in light and dark conditions, showing difference in fungal growth and pigmentation by the same species cultured on hydrophobic paper. (Dark stain on one side and yellow discoloration

are caused by microorganism that infested the culture plate, and not by the organism of choice (*Aspergillus niger*).

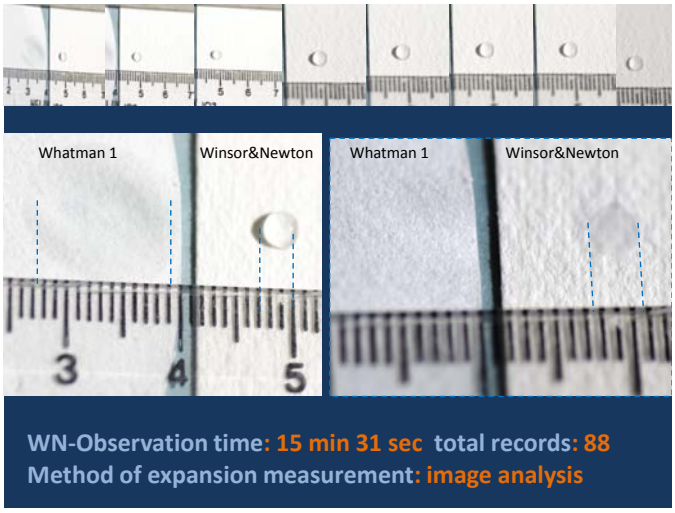


Fig. 2.35 Water absorption by Whatman paper and Winsor&Newton had impact on fungal growth. More absorbent paper (Whatman4) supported rapid growth abundant pigmentation, as seen on previous figure, 2.33 and 2.34.

The above absorbency is directly related to the presence of sizing in paper; its presence or absence is one of the broad categories used in describing papers. Experimental work carried out by Bristow (1986) was concerned with swelling as a function of water sorption. In conclusion of his experiment the unsized paper (in our case Whatman4 paper), there was a simultaneously sorption of water by pores and fibers. Furthermore, a more rapid rather than extensive swelling occurred in the early stage of exposure to water.

This situation in sized papers is different in that water cannot enter the pore system of a sized paper and that the only sorption occurring is the one associated with diffusion into and swelling of the fiber system. It has been hypothesized that in this case the intrafiber diffusion leads to a swelling not only of the fibers but also of the pores, and that in the sized paper water is unable to enter the pore space. Sorption, therefore, is taking place not by capillary action but by a diffusion process in a swelling medium. Sizing effectively prevents the entry of water into the pores and the uptake of water is solely due to fiber sorption.

The above statements are hypothesis only, and it is not clear which type of sorption predominates. There are many variables in the process of swelling and water sorption occurring simultaneously and no analytical data has been presented to support these processes (Bristow 1986, Alava 2006).

2. 2.6 Summary points

1. Paper characteristics, such as surface topography (roughness) and porosity appear to have direct impact on how fungi grow and deposit pigmented cells.
2. Rough, fibrous surface of paper provides a more conducive environment for fungi to grow, most likely due to fungal mechanisms of survival, ensuring that spores will not be removed by physical forces, and will germinate when water become available.
3. Paper with open pores provides better environment for fungi. It may be explained by fungal physiology and their requirement for oxygen during their development.

2.3 The Environment

2.3.1 The environment, an overview

Relative humidity, light and temperature, the three main elements of the environment, work in synergy in biodeterioration processes of cultural material. Each one of these factors contributes to the various stages of fungal development leaving different imprints on cultural objects. In that context, the porous, heterogeneous surface of paper provides a natural, yet specific habitat for fungi, see fig. 2.36.

Gomaa Abdel-Maksoud, (2011) recently discussed paper surface morphology in the context to biodeterioration. The extent to which fungi interact with that substrate largely depends on the conditions of the environment. Alteration of one environmental factor triggers different fungal responses within the same species, expressed in varied growth patterns, colonization or pigmentation. This fact was evident in the responses of *Aspergillus niger* cultured in daylight and in dark conditions. (For details see chapter **8 Light Experiment**)

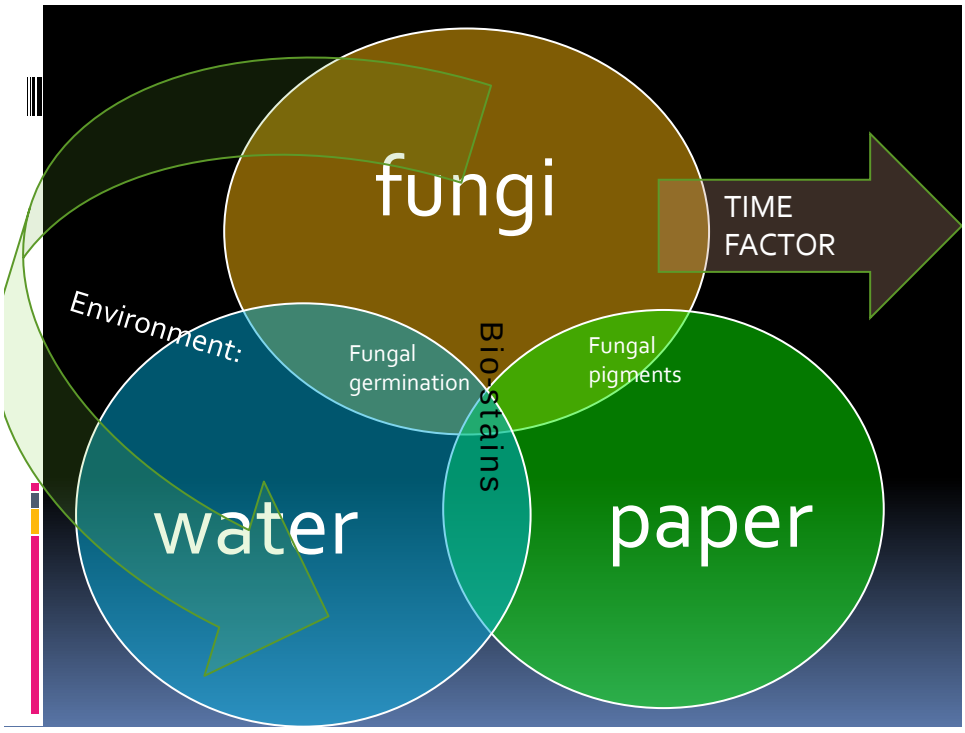


Fig. 2.36 Interrelations of environment upon fungal growth (relative humidity, temperature and light) presence of water, paper characteristics, and time factor (©Szczepanowska 2011).

In addition to environmental factors, chemical characteristics of the substrate, such as acidity and nutritional content often are brought up as contributing factors to fungal growth. Authors who discuss biodeterioration of paper-based cultural heritage associate bioreceptivity of paper with the presence of sizing (Florian 2002). The experimental work on light and pigmentation (chapter 8) and analysis of existing fungal stains on historic papers (chapter 9) indicated however that it was not the

sizing that promoted growth on paper, but the ability of the substrate to absorb water. Analysis of fungal stains on historic paper showed no correlation of fungal stains formation in the presence of sizing or adhesive residue. Furthermore, in case of induced fungal stains, they were more prominent on the side of paper that showed less sizing (Winsor&Newton paper).

Many hypotheses have been proposed as to the role 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 factor. Recent advancements in molecular technologies permitting in-situ studies of microbes in their natural environment revealed that our knowledge is fragmentary and much more is there to learn about microbial diversity and responses to the environment. A brief review of the relative humidity, light and temperature in the subsequent sections emphasizes their role in fungal staining of paper substrate.

This study showed also revealed biogenic re-mineralization of calcium carbonate filler in paper (Winsor&Newton). The chemical analysis with SEM-EDS indicated chemical configuration of calcium carbonate to calcium oxalate. That phenomenon has been widely studied from a mineralogical point of view (Pinzari et al. 2010; Khan 1995; Gadd 1999, 2007). Re-mineralization of the filler on Winsor&Newton paper has been reported here to illustrate different responses of fungi to varied light conditions. Light was selected as one environmental factor to explore further in the experimental production of fungal pigmentation because it is known to be directly involved in fungal life cycles and production of secondary metabolites, which often are pigmented. The impact of light on pigmentation can be observed once the spores begin germination and hyphae begins to expand into mycelium. The presence of liquid water is essential for that 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 for the impact of light upon fungal pigmentation.

The environment and characteristics of the substrate work in concert contributing or prohibiting fungal growth and development, therefore none can be discussed as an isolated factor.

2.3.2 Relative humidity, water and dew point

The definitions of relative humidity, water activity and dew point are simplified here as each one being a complex concept is discussed elsewhere**. 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 percent of saturation humidity, generally calculated in relation to saturated vapor density. (http://hyperphysics.phy-astr.gsu.edu/hbase/kinetic/relhum.html; accessed 6/19/2012)

Relative Humidity (RH) = partial water vapour pressure : saturated water vapour pressure x100%

A common definition states that relative humidity is the amount of moisture in the air compared to what the air can "hold" at that temperature. When the air cannot "hold" all the moisture, then it condenses as dew. Relative humidity is expressed in percentage. **Molecular water** is referred to as a monolayer of water that condenses on the surface when material reaches dew point. That occurs during rapid change of temperature in the environment and when a particular material's moisture equilibrium is not reached.

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 in the air will saturate the air, is called the dew point. If the air is cooled further, some of the moisture will condense. (fig.2.37) In other words, the dew point is the temperature to 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 depends also on the moisture equilibrium in a given material.

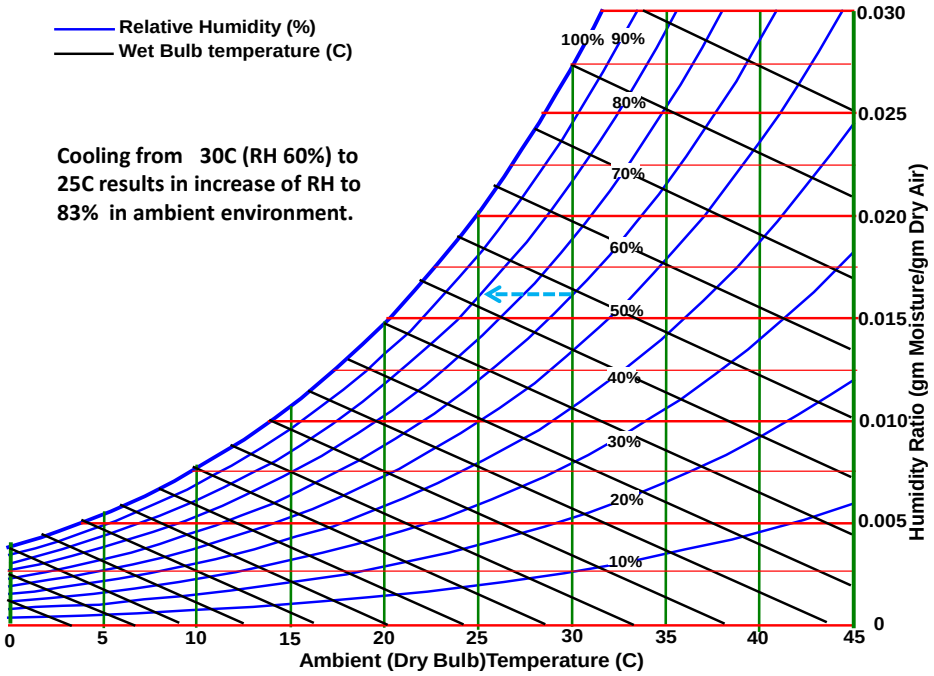


Fig. 2.37 Calculation of the dew point in a given environment. Environment in the room cooled down during night resulting in increase of RH in the ambient environment and condensation inside the Petri dish. (Chart courtesy of Dr. M. Mecklenburg).

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

Water condensed inside the Petri dish exposed to daylight in Light Experiment (see fig.2.38). It resulted most likely from fluctuations of temperature increased by daylight compared to temperature inside the Petri dish. As you see on the chart in fig. 2.37, that shift from 25 ^{o}C at night to 30^{o}C during the day was sufficient to result in water condensation; light energy fluctuated between 0 to over 9000 Lux. The condensation was one of the contributing factors to the abundant fungal growth in daylight colonies.

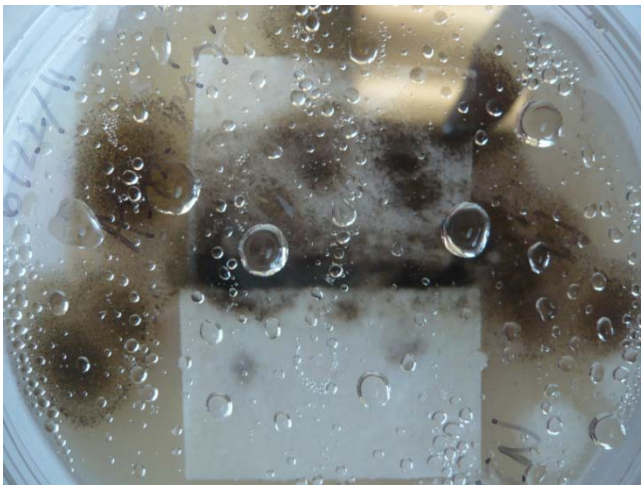


Fig. 2.38 Condensation of water inside the Petri dish during cultivation on a window sill, exposed to day-light was one of the contributing factors of abundant growth of fungi. 6/27/2011; *Aspergillus niger*, 6 day cultivation, daylight exposure.

Visible light: 0-9400 Lux
UV light: 0-500 mW/M²
RH in room: 30-42% (100% inside the Petri dish); T in the room: 25-30°C

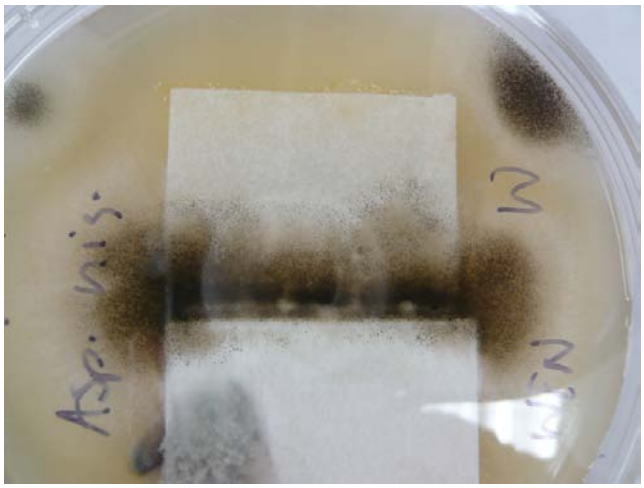


Fig. 2.39 Colony of the Aspergillus niger cultured for 6 days in dark. Water did not condense inside the Petri dish. That combined with low nearly dark conditions resulted in less abundant growth and pigmentation. 6/27/2011; *Aspergillus niger*, 6 day cultivation, dark conditions.

Visible light: 0; recorder on 4 occasions as 20Lux
UV light: 0 (initial spikes recoded prior to instrument setting). RH in the room: 48-52% T: 21°C

Based on the analysis of minimum and maximum value of the environmental conditions in both settings (daylight and dark, listed above) the pigmentation and growth pattern appear to be governed by light and water in addition to paper absorption ability.

Dew point is one of the concepts associated with fungal growth, **water activity** is another. Often elevated humidity is equated with initiation of fungal infestation, however that statement is only partially true, as long as it leads to the formation of water on surfaces; fungi will grow in environment with elevated humidity on surfaces that are wet. As pointed by W. J. Scott (1953) for the first time, it is water activity, not water content that is correlated with fungal growth. Water activity (a_w) concept 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 the 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 substances tend to support more microorganisms. Bacteria usually require at least a_w 0.91, and fungi at least a_w 0.7. Transversely, it has since been established that growth of fungi and bacteria are inhibited at specific water activity values. (Wkipedia, water activity, accessed: 9/30/2011)

Water activity of 0.60 is considered the lowest limit for microbial growth. Below a_w 0.55 all metabolic activity ceases and DNA is denatured. (www.ecometrex.com/moisture.html)

<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 for a given a_w and specific organisms that large differences in survival rate were function of the nutritive solutes used to culture the microorganisms, thus pointing out that other components play a role in microorganisms survival, in addition to a $_w$ (Mugnier 1985). The other factors include 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 history of fungi, and how fungi solve the problem of sporulation in water-limited conditions affects fungal dispersi0on. In a simplistic explanation of this complex processes, dormant conidia are formed containing all necessary mechanisms to begin a life cycle when conditions improve. The dormant conidia contain a complete mitochondrial respiratory system and electron-transport system that become active immediately upon spore activation (Bramble 2009). Enzymatic subunits precursors synthesized and stored in the cytoplasm during conidia dormancy are imported into mitochondria upon initiation of germination (Bramble 1981, 1985, 2009).

2.3.3 Temperature

Similarly to other environmental factors, temperature affects microbial life functions in a context and cannot be considered as a single factor without a synergistic effect of relative humidity and light. Temperature however was found to play a major role in spore germination, as reported by Schenck (Schenck at al. 1975). Therefore, temperature is utilized as a primary means of sterilization by deactivating vital functions in spores. Many microorganisms are classified according to their preference for specific temperature as:

- Psychrophiles: with optima less than 10°C
- Mesophiles: with optima in the room temperature range (18-22°C)
- Thermophiles: with optima at or above 37°C

(Burge 2006)

Optimal temperature for fungi which grow on paper is considered to be 20°C -25°C, an average room temperature placing them in the category of mesophiles. Temperature when used above tolerance level for specific species is damaging to their vital systems, thus hot air is considered a form of spores deactivation; proposing 1-3 hr of 170-140C respectively for spores kill, while 4-6 hours in 71C is suggested for treatment of fungi in an infested house (Burge 2006). The effects of heat on fungi are related to the chemical reactions within the fungal cells. For optimum 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 slowing the growth. Eventually, the temperature can reach a point where growth stops, and cell components begin to be damaged by the heat. Enzymes are proteins that change structurally when heated to their limit of tolerance (Burge 2006).

2.3.4 Light

Light is one of the most important environmental factors influencing orientation and growth of almost all organisms on Earth including fungi. The molecular machineries translating its electromagnetic energy (photons) into the chemical language of cells' transmit vital signals necessary for adaptation of living organisms to their habitat. Fungi react to illumination in various ways which are species dependant. (Corrochano 2011; Corrochano and Avalos 2010) For example, light stimulated the production of some metabolites in *Fusarium* (Avalos and Estrada 2010; Asthana and Tuveson 1992) however did not affect growth but increased fruiting production upon exposure in *Myrothecium verrucaria* and *Pestalotia gracilis* (Yusef and Allam 1997). Moreover, light was reported as prohibiting growth of the pathogenic fungus *Botrytis cinerea* and *Trichoderma harzianum* (Paul et al 2005). Knowing that variations in response to light affect the production of pigments light was selected as an environmental factor in the Light Experiment (II.8).

Response of fungi to light is initiated within minutes of exposure. The experimental work reported by Schwerdtfeger and Linden (2000), and Shrode et al. (2001), both referenced by Rodrigues-Romero et al. (2010), indicated that intensity of response to light diminishes within 30-240 minutes from exposure. During that short period of time fungi alter their behavior to cope with the (often) harmful effects of light and to 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-depended systems (Schmoll, 2011, Tish and Schmoll 2009; Rodriguez-Romero et al. 2010). However, despite the fact that large numbers of processes in fungi are light-dependent, even with the application of sensitive techniques of molecular biology, biochemistry, and cell biology, the knowledge of exact mechanisms of light perception and signal transduction in fungi is limited.

What is known is that many species utilize a number of different wavelength-specific photo-receptors. The core of all receptor types is a chromophore, a low-molecular-weight protein molecule. Most phytochrome responses in fungi are attributed to differential gene expression, explaining the mechanism of transferring the light signal into the nucleus where gene expression takes place. Microbial response to blue-light is one of the best-studied light responses; all other light-sensing mechanisms are less well studied or largely unknown. What has been discovered recently is that fungi appear to employ both plant and animal type photosensors (Rodriguez-Romero et al 2010).

The responses to light differ according to the night-day cycle following a fungal circadian clock. As in higher organisms, this clock is light and temperature regulated (Brunner et al. 2008, Dunlap and Loros 2006). Furthermore, fungi sense not only light intensity but also directionality resulting in phototropism (Dictionary of The Fungi 2009). The "chromatic" vision in fungi suggests complex interconnections between different photoreceptors to modulate the response as a function of changing light conditions. The overall analysis of light response in fungi suggests that the light signal is used to adapt to the harsh environment encountered by hyphae, when they leave a substrate and

grow on its surface. Filamentous fungi which are encountered on paper-base cultural heritage material may use light signals as an indicator for the exposure of hyphae to air.

Presence of light has been reported in some cases as halting sporulation, which cannot be confirmed by this study. However, different genetic strains of the same species react differently to light; therefore the conclusion cannot be applied to all strains of *Aspergillus niger*. Because of the limited number of publications on light effect on pigmentation in the context of cultural material this study is reporting observations of the behavioral responses solely of one fungus, *Aspergillus niger* and further, limited to growth on two specific papers. The observations that can be attributed to light effect only are summarized below and detailed in II.8, Light Experiment.

I. Light has impact on fungal growth and pigmentation. **Distinctly different patterns of growth and pigment intensity** were observed in cultures grown in daylight and in dark. (fig. 2.40-2.43) When grown in daylight fungi produced loosely arranged colonies with the production of intense and rapid pigmentation. When grown in darkness, colonies expanded at a slower rate but in a more even pattern. Pigmentation was increased slowly and was evenly distributed on paper.

II. **Presence of sizing did not support growth of fungi.** Whatman 4 paper which had no sizing supported fungal growth, while Winsor&Newton, which was sized with gelatin, did not. Growth intensity was directly related to paper's ability to absorb water.

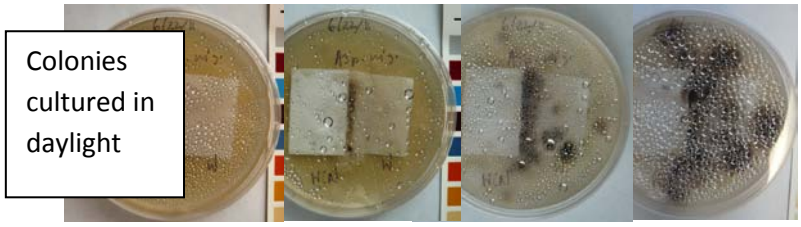
III. **Mycelial growth occurred mostly on the surfaces exposed to light**, and to a minimal extent on reverse of papers (side placed on nutrient plate). That pattern applied to both types of papers, regardless of intensity of growth. It may indicate fungi requirement for oxygen and possibly light in their development. Pigmentation however was more diverse on reverse than it was on recto of paper samples.

IV. **Rapid growth** and intensity of pigmentation of fungal colonies cultured in daylight corresponded to **greater mechanical weakening of paper.** It can be presumed that secondary metabolites, which are often acidic, disintegrated the paper fibers which led to their physical weakening. (fig.2.44-2.46)

V. **Chemical composition** of the surface of paper which contained calcium carbonate filler was **altered** differently in daylight than in dark cultures. (fig. 2.47-2.51.)

(Supportive documentation of the experiment, see Appendix V: Light Experiment)

Day Light exposure, days of observations: 1 through 5; 6/23/ through 6/27/2011



Day 1; 6/23/2011 Day 2; 6/24/2011 Day 3; 6/25/2011 Day 5; 2/27/2011

Minimal Light exposure, days of observations: 1 through 5; 6/23/ through 6/27/2011

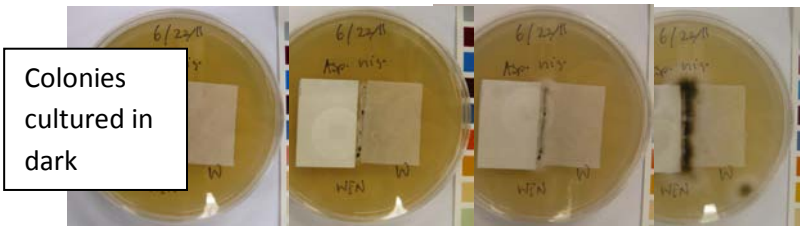
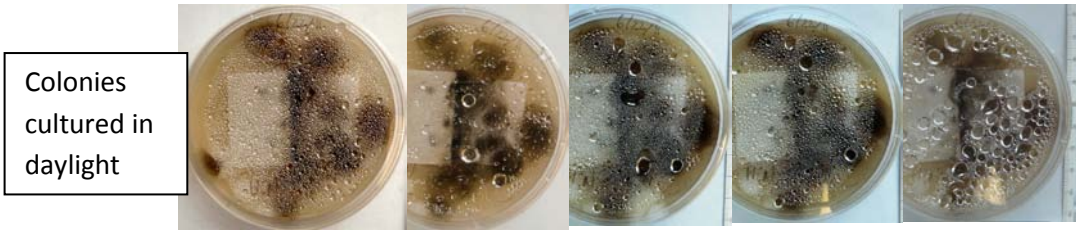


Fig. 2.40 Comparison of colonies expansion and pigment formation during the first 5 days of culturing in daylight and dark conditions. Rapid growth in daylight contrasted with slower growth and minimal pigmentation in dark conditions.

Day Light exposure, days of observations: 6 through 13; 6/28/ through 7/5/2011



Day 6; 6/28/2011 Day 7; 6/29/2011 Day 8; 6/30/2011 Day 9; 7/1/2011 Day 13; 7/5/2011

Minimal Light exposure, days of observations: 6 through 13; 6/28/ through 7/5/2011

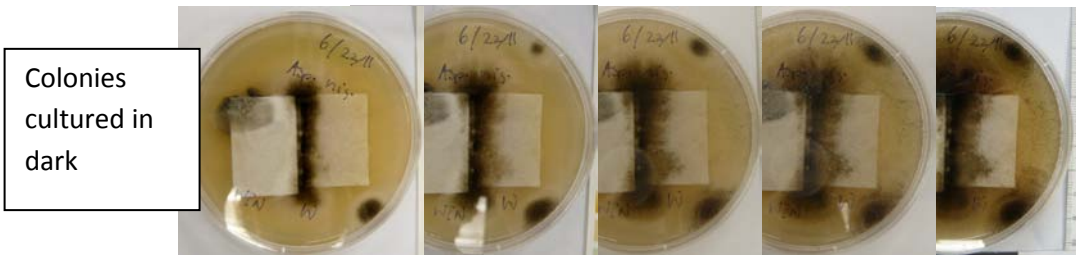


Fig. 2.41 Comparison of colonies' expansion and pigment formation in the remaining seven days of the experiment (7-13 days of cultivation). Intensity of pigmentation in daylight colonies reached plateau, while pigmentation continued to intensify in cultures grown in dark.

Fig. 2.42 and 2.43. Whatman4 paper pigmented by *Aspergillus niger* cultured in daylight (fig.6) and under dark conditions (fig.7). Pattern of growth and pigment deposition varied; colonies cultured in daylight were loosely arranged and airy, while the ones grown in dark were compact. Pattern of growth had a direct effect on modifying surface characteristics.

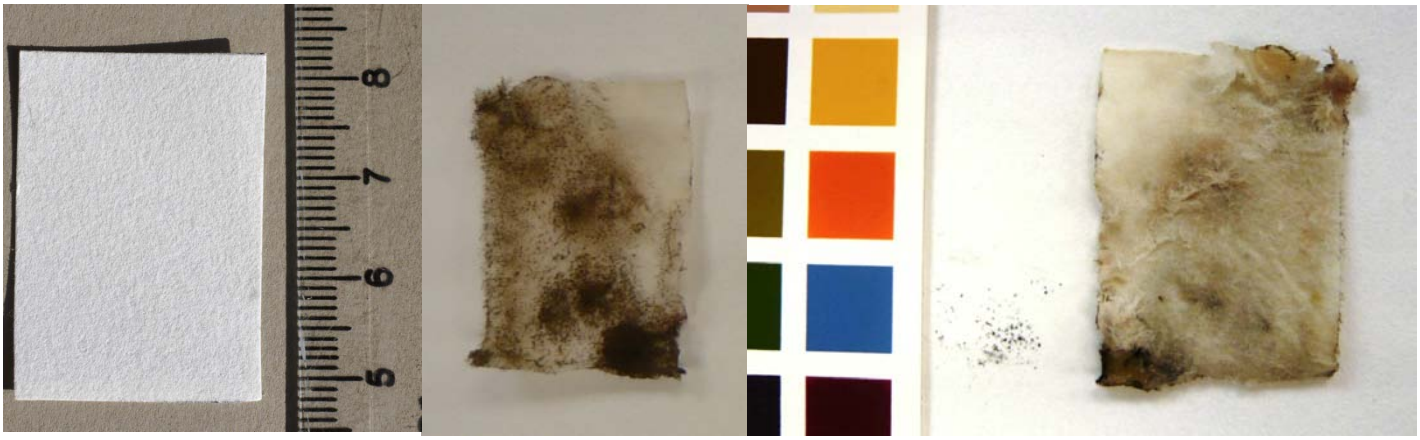


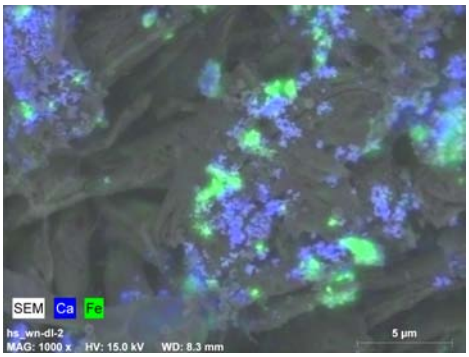
Fig. 2.44 Fig. 2.45 Fig. 2.46

Fig.2.44-2.46 Physico-chemical change of paper caused by fungal growth included pigmentation of paper, visible as staining, and structural weakening of the paper matrix. (2.44) Whatman4, control, prior to cultivation of fungus, (2.45) recto (side up towards light) of Whatman4 covered with colonies cultures in daylight (W4-DL); (2.46) reverse (side facing agar-agar in Petri dish) of the same sample showing severe deterioration of paper.

Physico-chemical deterioration of paper by fungi; Whatman4 paper

Fig. 2.47 and 2.48. Paper matrix of Whatman4 control (fig.2.47) before inoculation of fungus and after cultivation with *A.niger* , cultured in daylight. Fig.2.48 shows a physico-chemical deterioration of fibers. SEM-VP micrograph. Scale bar: 50µm

Chemical alteration of paper constituents: re-mineralization of filler; Winsor&Newton paper



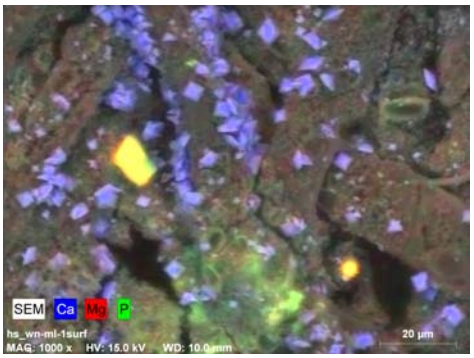


Fig. 2.49-51. Calcium carbonate filler in Winsor&Newton paper was modified by biogenic activity of *Aspergillus niger* to calcium oxalate. SEM-VP with EDS analysis confirmed the same chemical base composition with main component calcium, however of different crystalline configuration.

Differentiation of crystal formation was observed between daylight and dark-grown cultures. Fig. 2.49 shows randomly distributed calcium carbonate particles of the filler in W&N control. In fig. 2.50 calcium oxalate is clustered into congregates on daylight-grown culture; fig. 2.51 shows transition of random particles of calcium carbonate to well organized crystals of calcium oxalate in dark-grown culture.

2.3.5 Summary points have direct implications on predicting the behavior of paper-based collections when exposed to water in floods or in a rescue operation after fire events.

I. Environmental factors, characteristics of substrate, type of microorganism and time factor work in synergy towards biodeterioration, therefore their accumulative contribution needs to be considered when biodeterioration is evaluated.
II. Rapid growth and intense pigmentation can be attributed to fungal response to light and water absorption of paper. Whatman4 paper which absorbed water from substrate (agar-agar) showed rapid and intense growth in both light conditions, while W&N paper which did not absorb water showed minimal growth in both light conditions.
III. Fungi grew on the surface of paper exposed to light, showing almost no mycelial growth on the opposite side that was not exposed to light.
IV. Intense growth and pigmentation corresponded with the greatest level of paper deterioration.
V. Fungal growth cannot always be associated with the presence of sizing. W&N paper sized to repeal water did not support fungal growth.
VI. Chemical characteristics of paper were altered by fungi on paper which contained ad with filler. Because the pattern of change was different in daylight from that in dark, it can be attributed to light factor.
VII. Time factor plays a role in the biodeterioration process, although its role can be measured by different criteria; one is intensity of pigmentation, the other degree of deterioration. Intensity of pigmentation was greater in the initial stage of growth and light exposure, but physico-chemical deterioration can be speculated as increasing with time progression. That aspect however deserves separate studies.
VIII. The complex and multidisciplinary nature of investigating paper biodeterioration requires application of various instrumentations to gain a better understanding of the effect of the environment on fungi and their modifications of substrates. Various analytical techniques that were used in this investigation of light effect on pigmentation and paper biodeterioration contributed to a better understanding of fungi and substrate interactions and provided some insights into possible outcomes of biodeterioration.

3. Fungal stains on paper

3.1 Origins and definitions of stains

Although stain is a perceptual phenomenon an attempt is made to define and describe a stain in relation to a substrate on which it is found, namely paper, and to clarify the nature of particular stains caused by fungi. Interpretation of the stain's significance depends on the context in which a stain was formed, criteria of its evaluation and the aesthetic considerations of the one who describes the stain.

A stain is a mark created on a surface, which may penetrate into the substrate, below the surface. Many synonyms of the word 'stain' bare connotations indicating the origin of the mark, manner in which it occurred or was applied, and in some cases its characteristics and may suggest stain's footprint. As smudge for example, can be caused by a liquid or dry matter smeared over the surface and suggests an elongated mark, rather than a round one due to an action involved in its making. A splash suggests a liquid source of the stain and most likely a round footprint of the mark.

Stain synonyms compiled from various dictionaries include, but are not limited to, spot, blemish, dye, blot, smudge, smear, splotch, soil, discoloration, pigmentation, tarnish, taint, tincture.(fig. 3.1) One common denominator is pigmentation of a stain.

Fig. 3.1 Synonyms of the word 'stain' on paper, compiled from various sources: Webster's New World Dictionary of the American Language. D. B. Guralnik, Editor in Chief. Warners Books 1983. p. 582; Century Dictionary and Cyclopedia; WordNet 3.0; American Heritage® Dictionary of the English Language, Fourth Edition.

Stain formation, shape, appearance and behavior depend on interplay of many factors within three main areas:

1. Characteristics of substrate on which a stain is formed (in this case paper)
2. Characteristics of materials which make a stain (liquid or dry matter)
3. Forces at interface of stain material and substrate

Stain description depends on the material which produces the stain, liquid or dry in nature.
Liquid stain depends on liquid viscosity and material's porosity/wettability. Liquid stains produced by fungi result from secretions of enzymes into the substrate and most often than not penetrate the bulk of a substrate.
Dry stain depends on particles' size, compactness of particles, surface characteristics of particles, and their affinity to substrate. Fungi produce 'dry stains' by deposition of pigmented spores on surfaces and/or pigmented cells in the matrix.
Stain shape (footprint and profile) and **depth of penetration** reflect interplay of all the above elements. The possibilities seem countless, nearly infinite, if one considers rapid development of

new materials (substrates), novel liquids and methods of their application, and includes complexity of living biological systems that produce pigmentation, dry or in a liquid form.

Stain- substrate interface depends on the forces at play, gravity, capillary action, physical/chemical affinity of materials, environmental conditions such as relative humidity and temperature, although light energy in some cases needs to be accounted for and electron-charge of the surfaces coming in contact. Electron-charge characteristics of spores' surface are discussed in the following chapter, in the context of microbial adhesion to surfaces.

Considering the above factors, a stain can be defined only as an individual phenomenon within a particular set of circumstances and parameters. The openness of that approach is even more pertinent to fungi-induced stains, encompassing many variables resulting from continuous changes of living systems.

3.2 Fungi-induced stains; foxing

Bio-stains represent discoloration uniquely associated with activities of fungi, bacteria, yeast or algae. Pigmentation produced by microorganisms results from their complex bio-chemical processes occurring within their cell structures and found its applicable in numerous areas of industry. Fungal pigments are defined as secondary naphtoquinon metabolites (Medentsev 1996). Pigments production is a characteristic feature of certain species, but for others species occurs in response to stress or as a protective mechanism of their structures against external factors such as UV radiation. Colorations of fungal pigments vary among different fungal genera and /or species but also within the same species upon change of their growing conditions (Mapari 2006).

Stability of fungal pigments gained attention in the food industry as natural food colorants (Ali 2006 cited in Mapari) in medical field as antibacterial and antifungal agents and in agriculture as phytotoxins and insecticides.

Contrary to the importance of pigmented metabolites in industry, discoloration which they induce on cultural heritage material is undesirable. These stains alter not only the surface of artworks, sculptures, monuments, art on paper or documents on parchment, but are often associated with enzymatic digestion resulting in acid-related weakening of their structure (Gorbushina 1993). Pigmentation induced by microorganisms is resilient and resists attempts of their removal with physical forces or reduction of the colors' intensity by chemical means.

Cultural heritage collections affected by microbiological infestation are altered, often irreversibly, which diminishes their aesthetic and artistic quality, followed by lowering their market value. Surface aesthetics is of essential importance in artworks and is usually the first area affected by bio-deterioration. In spite of that none of the authors dealing with biodeterioration of cultural heritage have attempted to systematically analyze surface morphology of cultural heritage with respect to bioreceptivity of materials.

Studies of the aesthetic impact of bio-deterioration of cultural heritage proliferated during the last two decades has centered mostly in the areas to stone and wood deterioration (Gorbushina 1993, Kumar 1999, Santos 2009). Biodeterioration of paper has been limited to evaluation of so called foxing stains. Foxing stains, a rusty color, resembles fox fur for which the name originated have been associated with fungal growth. (Fig.3.2, 3.3) Relatively few papers deal with pigmentation secreted into paper and physical disintegration of paper caused by microorganisms (Szczepanowska, 1990, Zyska 1997, Michaelsen 2010, Principi 2011). Study of leather and parchment's biodeterioration,

although represented by a small number of authors, was presented in a monographic work by Strzelczyk and Karbowska (2000).

This work, therefore, constitutes the first attempt to formalize a systematic approach to correlate surface morphology of materials with their bioreceptivity in the context of cultural heritage (II.8 and II.9). A model approach, developed based on cellulose material can be applied to other types of cultural heritage collections.

Examples of foxing stains caused by fungi

Fig. 3.2 Powdery fungal residue of aerial mycelium on the surface of a gauche on paper, early 20th century.

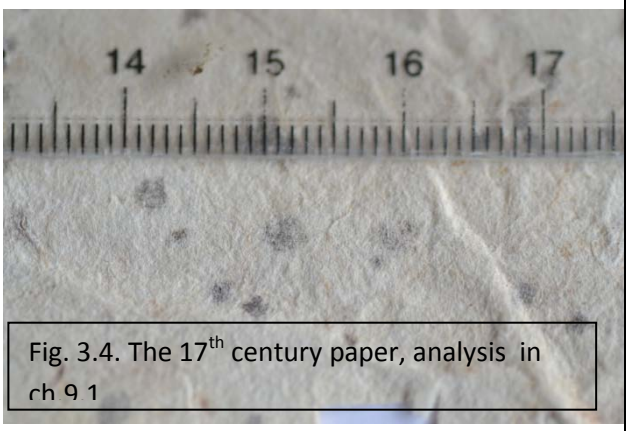
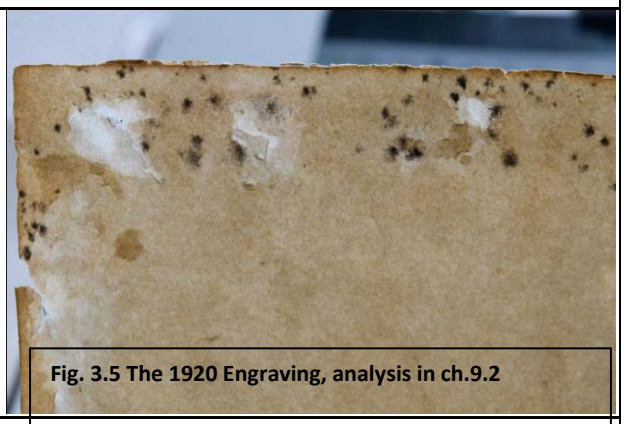
Fig. 3.3 Aerial mycelium of fungus growing on a mid 20th century print. Pigmentation was secreted into substrate under each colony.

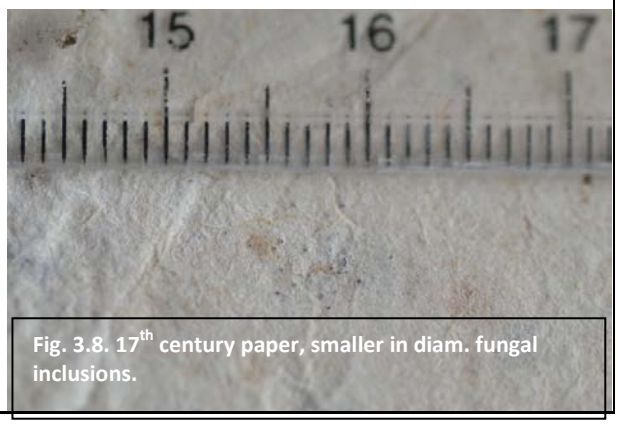
3.3 Black pigmented fungal stains

Melanin is a black pigment produced by fungi. Although in nature other organisms also produce melanin. The natural melanin pigments of vertebrates, insects, plants and microbial organisms serve vital roles in camouflage, in sexual behavior or as protection against solar radiation. Melanins, having such significant implications have been studied extensively across a broad spectrum of scientific disciplines. The mechanism by which melanin granules are assembled in pathogenic fungi and held in place is unknown (Eisenman 2005). In recent studies, utilizing solid-state nuclear magnetic resonance strategy it has been suggested that melanin in fungal walls is tightly bound by covalent forces and polysaccharide components may facilitate that attachment of the pigment to the cell walls (Zhong 2008).

The notion that pigment is tightly bound in fungal cells has been supported by this study. Black pigmentation was confined to the cell walls and the walls of fruiting structures. (II.9). Fungal forms found in the studied black stains on paper were evaluated with multivariate techniques including surface morphology and topography of surface with bio-deposits. (Chapters 8 and 9). Selected examples of black stains' shape, intensity and forms as observed on cultural material and are summarized in table I.

Table I: Variations in black fungal stains on paper

shape	Stains size	Pigment – visual observation	examples
Semi-round	1mm x1mm to 3mm x3mm	Gray, varied intensity	 Fig. 3.4. The 17th century paper, analysis in ch.9.1
Irregular edges	1mm x 3 mm	Black, varied intensity	 **Fig. 3.5 The 1920 Engraving, analysis in ch.9.2**
Defined spherical spots- single deposition **(note, those stains were not included in this study)**	**0.5mm x 0.6mm**	Brown-black	 **Fig.3.6 The 20th century silkscreen; black fungal inclusions**

Defined spherical spots- single dense deposition	**0.2-0.3 mm** **Each single inclusion**	black	 **Fig. 3.7 The 17th century paper, details in ch.9.1**
Defined spherical spots- single deposition **(note, those stains were not included in this study)**	**0.2-0.4mm each single inclusion**	gray-black	 **Fig. 3.8. 17th century paper, smaller in diam. fungal inclusions.**

3.4 Summary points

1. All black pigmented fungal structures, single cells, cells arranged in chains and clusters showed common characteristics that is thick dark pigmented walls.

2. Based on multivariate analysis black pigmentation was attributed to meristematic fungi and perithecia *of Chaetomium* sp which are discussed in chapter 2.1.

3. Secreted pigmented material appeared to be present on some paper fibers. That conclusion was drawn based on amorphous characteristics of the deposits in areas of stains and their much lighter pigmentation that that found in fungal forms. The function of extracellular material exuded by fungi is discussed further in the context of microbial adhesion to the host material, chapter 4.2.

4. Paper and fungal interfaces

4.1 Fungi : substrate interfaces; an overview

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 formation of biofilms are associated with pathogenesis of 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 effect in humans and plants. However, there is no reported research on interfaces of microbes and cultural heritage material. In fact, that deficiency has been pointed out by Orio Ciferri, Chairman of the Scientific Committee of the 1999 International meeting in Florence that was exclusively devoted to the relationships between microorganisms and cultural heritage. In the Preface of the conference proceedings (Of Microbes and Art) he stated: "...although enough data is 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". Further, in the same Preface, [,…] one could say that very often the causative agent has not been identified and very little is known concerning the microbe-host interactions." (2000)

The few available publications on biodeterioration of cultural heritage are focused on describing visual effects of bio-deterioration without attempting to explore phenomena occurring on interfaces or mechanisms of interaction with the substrate. Furthermore, most of the papers devoted to biodeterioration of cultural heritage deal bio-deterioration of stone, and only few with the bio-deterioration of paper and other materials. Bio-deterioration of paper remains largely unresolved.

This work is the first attempt to look at a multitude of phenomena occurring at interfaces of bio-deposits and paper-based material with the purpose of contributing to cultural heritage preservation. Paper is made of plant-derived material therefore some of the processes observed in plant pathology are used here as parallels to elucidate bio-deterioration of cellular, cellulose-based cultural material. It is worth noting that some of the fungi growing on cultural material are plant and human pathogens as well.

Fungi and substrate 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 system (such as solid : solid, solid : gas : liquid, solid : gas and gas : liquid) can apply (fig. 4.1).

However, complication lays in the fact that microorganisms are living and undergo continuous changes. Consequently the dynamic interactions between heterogeneous paper and microorganism involve multitude of forces. Furthermore, black pigmentation is produced by fungi in different parts of their body and/or secreted to a substrate forming stains. Thus it could be superficial or can form liquid like markings, as elaborated in chapter I.3 Fungal Stains.

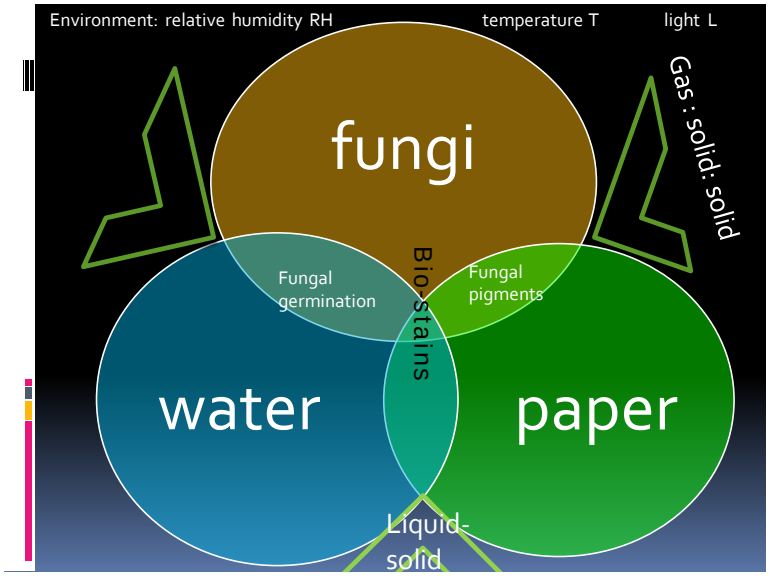


Fig.4 .1. Conceptualization of the three-systems' interfaces involved in bio-deterioration. (©H. Szczepanowska, 2011)

4.1.1 Fungi: air : substrate interfaces

Fungal interface with its environment is biochemically complex and multifunctional. Interface of fungi and air occurs during fungal spores' dispersion through wind and when fungal hyphae are protruding into the air, producing conidia bearing structures.

Interface of **fungi : substrate** (solid : solid) at the first contact of fungal spores with substrate is of great importance to the fungus because it ensures its attachment necessary for further growth; it involves interaction of physical forces and chemical interactions. That attachment is facilitated by adhesion of fungal spores to a substrate. Another type of fungi: substrate interface is formed by hyphae growing on the surface of a substrate, into its bulk, and chemically breaking down the substrate to utilize it as nutrient. The cycle of growth involves a variety of complex bio-mechanisms and forces therefore all of the interfacial phenomena are interconnected.

Fungi disperse their offspring (spores or conidia) via water or wind. Survival strategies of fungi facilitate that dispersion with specialized spore/conidia bearing structures and mechanisms (i.e. appendages) that ensure their successful propagation. Survival strategies developed among fungi which pertain to this study are concerned with interfaces of fungal elements with substrate, such as spores' adhesion to substrate and germination, cell surface and pigmentation, surface area of colony expansion and growth patterns. All these interactions are discussed in the context of interfacial dynamics of bio-mass and heterogeneous cellular substrate (fig.4.2). Furthermore, each of these interactions results in a different type of substrate alteration either due to enzymatic reaction or alteration of surfaces by deposition of pigments.

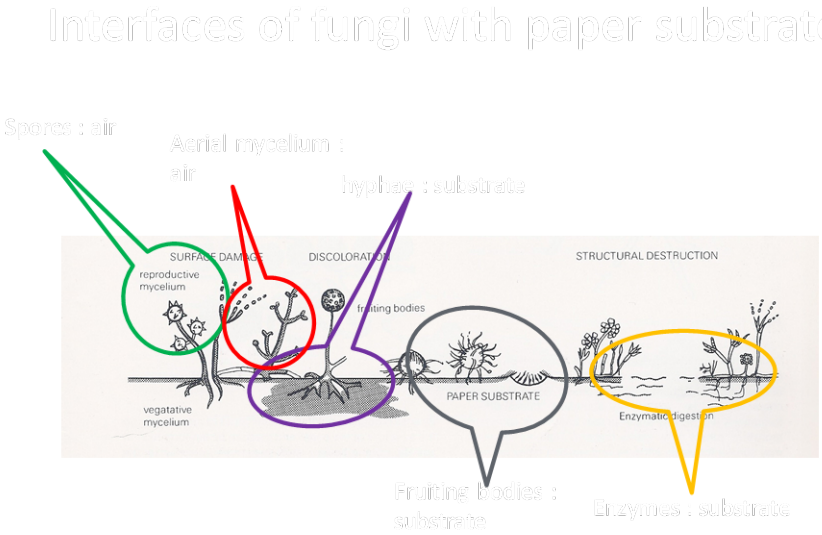
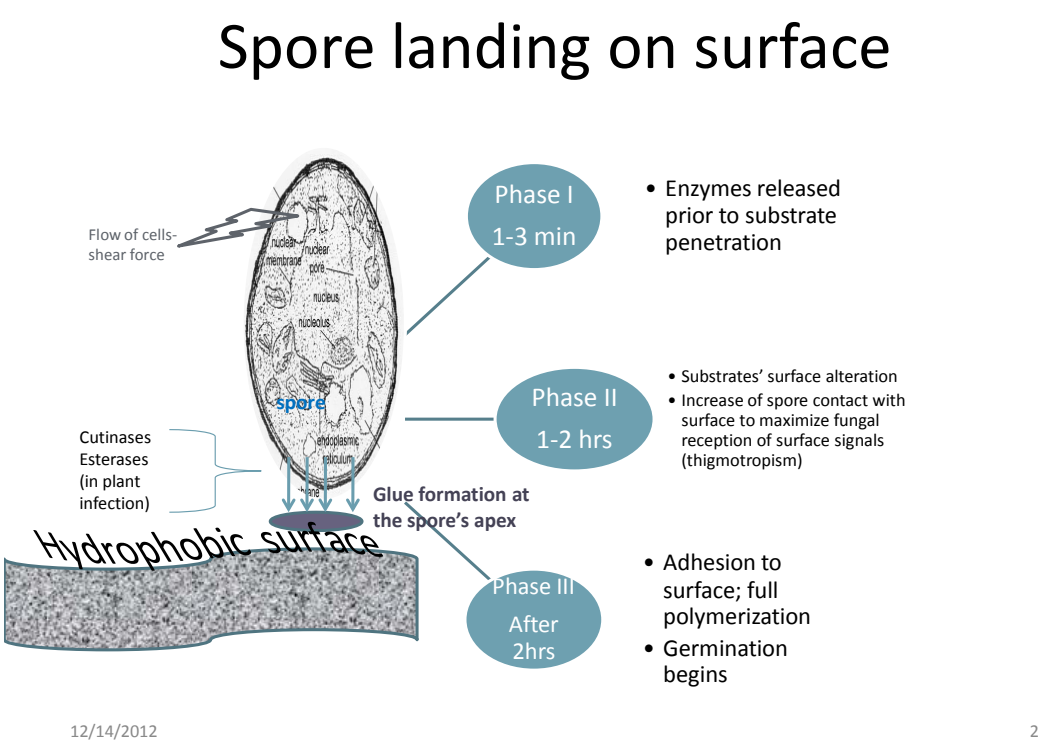


Fig.4. 2 Conceptualization of fungal interaction with paper-based cultural material. (based on a drawing fig.1, p. 31, Szczepanowska, IPC 1986). (not to scale)

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

1. spore/conidia landing on a suitable surface (surface must be hydrophobic with liquid water) (fig.4.3)
2. adhesion to surface and germination (fig.4.4, 4.5)
3. Growth of hyphae expanding into mycelium (fig.4.6)
4. Formation of fruiting structures with spores and conidia



4.1.2 Fungal spores : substrate interface

Fig. 4.3 Conceptualized sequence of events during the initial phase of a spore's contact with substrate's surface. Based on: Lynn Epstein and Ralph L. Nicholson, "Adhesion and Adhesives of Fungi and Oomycetes"; In: Biological Adhesives ed. A.M. Smith and J.A. Callow, Springer, 2006. (© H.Szczepanowska, 2011). (not to scale)

Fungal growth begins once spores or conidia find a suitable surface onto which they attach. Two essential elements are required, hydrophobicity of the surface and molecular water (Ward, 1980, Rutter, 1980, M. Rosenberg 1990, Smit, 1990, E. Rosenberg, 1990 Hazen, 1990, Wessels, 1997, Azeredo 2003, Schumacher 2008). It is surprising however that none of the authors dealing with hydrophobicity of surfaces pointed out that molecular water is available precisely because the surface is hydrophobic. Hydrophilic surface would absorb water, leaving no liquid film on its surface. Hydrophobicity has been discussed by researchers in a context of complex cells' communication (quorum sensing) and an electro-charge of spores' surface in aqueous environment. The views on the role of hydrophobicity in microbial adhesion have been debated for at least two decades, and recent developments are swaying towards contribution of other forces besides hydrophobic in the process of microbial adhesion. (See Appendix: Hydrophobicity review)

Once the spore lands on the surface and 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. The extracellular material which functions as adhesive undergoes polymerization and becomes insoluble (fig.4.4).

Adhesion of spore prior to germination

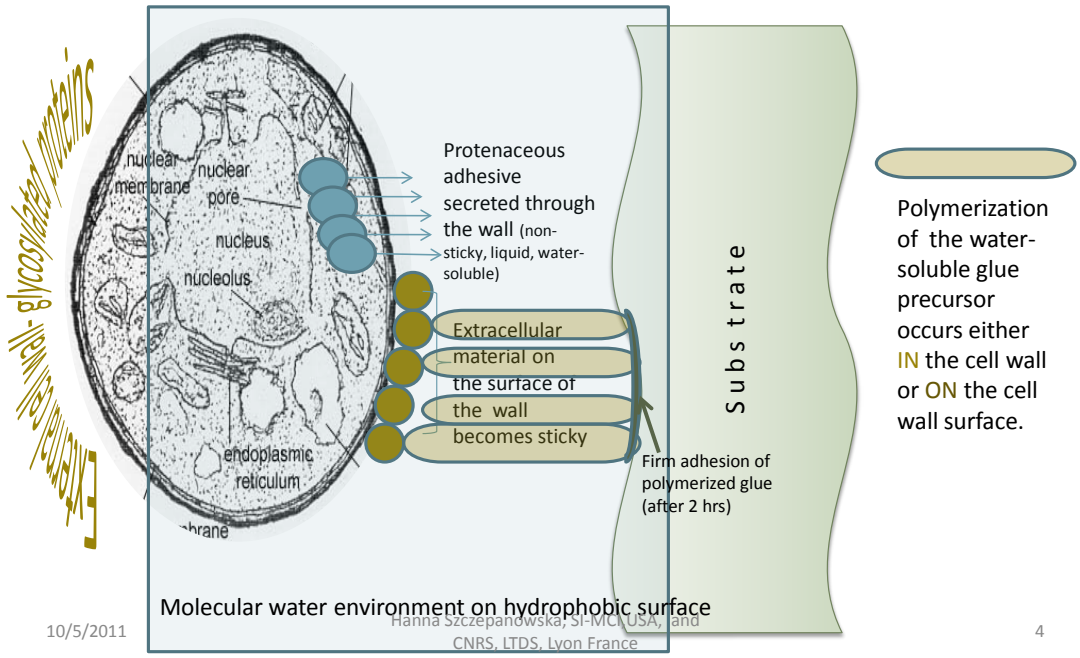


Fig. 4.4 A conceptual representation of extracellular material excretion and adhesion of a spore to surface. Based on: Lynn Epstein and Ralph L. Nicholson "Adhesion and Adhesives of Fungi and Oomycetes". In: Biological Adhesives ed. A.M. Smith and J.A. Callow, Springer, 2006. (© H.Szczepanowska, 2011).

A puzzling phenomenon of the secretion produced by a spore that changes its characteristics from water-like soluble substance to insoluble polymer once it passes through the walls still remains unresolved.

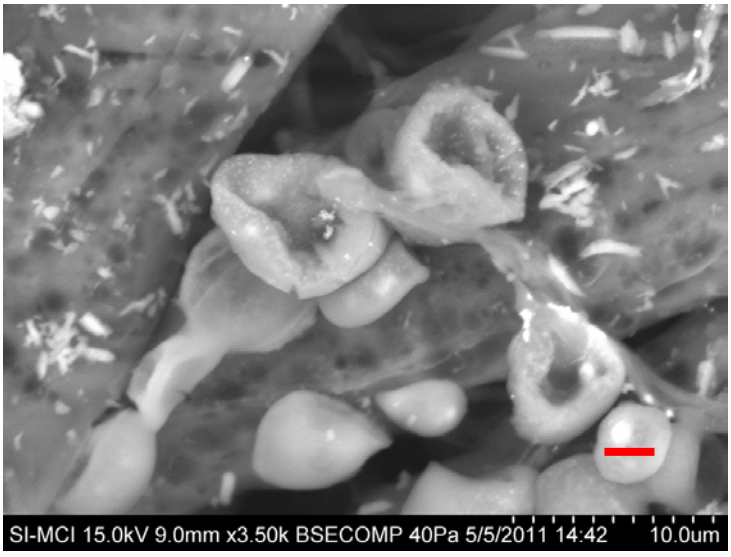


Fig. 4.5 Black pigmented fungal cells are attached to the surface of paper fibers. Extracellular secretions and most likely energetic forces hold the cells in place.

Stain type A, the 17th c Study Sheet, detail analysis in II.5.a. SEM-VP, sale bar: 20µm.

4.2 Microbial adhesion to surfaces

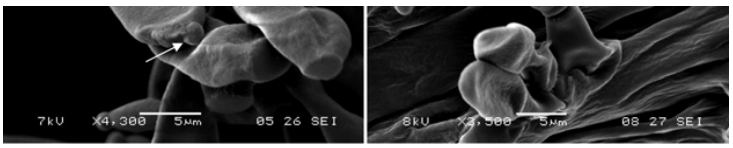
Adhesion of a fungal spore occurs upon its contact with a suitable surface, usually hours before germination. Although the fungal adhesion is known to exist and the reasons for its occurrence are understood the mechanisms of microbial adhesion are not entirely clear (Wang et al. 2010).

Interaction of a multitude of forces is involved in adhesion process which can be described as attraction forces between dissimilar molecular entities leading to close contact. These forces can be divided into mechanical and chemical in nature.

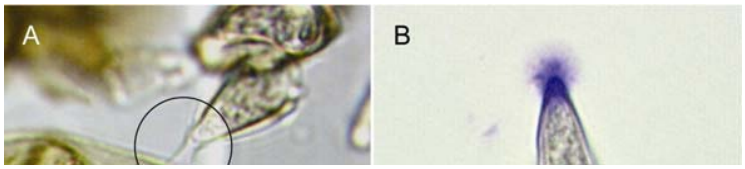
The adhesion of microbes to substrates has been studied since 1924 (Rosenberg et al. 1990). It has been discussed in terms of both chemical and physical interactions depending on who is investigating the issue. Spores' attachment and hyphal- adhered growth are aided by the production of an adhesive-like extracellular material (Gamarra et al. 2010).

Adhesion of spores or conidia to the surface is one of the key moments in the fungal life cycle. It is a process that occurs rapidly once the spore lands on a surface in favorable environment. Enzymes and protenaceous materials are involved in producing extracellular material that attaches a spore to the surface. Upon contact with such favorable surface, conidia release adhesive material localized in a droplet at the spore apex, which some authors name Spore Tip Glue (STG) (fig. 4.6). Histochemical investigations of STG indicated the presence of proteins and carbohydrates. With the aid of a 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 2008). Pigmented reside that was located on the paper fibers in black fungal stains in the No1 Study Sheet maybe a residue of extracellular secretion (fig.4.7). However at this time no tests for the presence of proteins were carried out to confirm its chemical composition.

Examples of conidia adhesion in nature



The apex of nonturgid conidia consisted of a globular structure (spore tip glue [STG], pointed with arrow, (left). Nonturgid conidia on conidiophore, (right). (adapted from Schumacher, page 763 fig. 1 a,b.)



Conidia of *Venturia inaqualis* treated with dyes after adhesion on polystyrene, after 15 min after application. A: Conidium adherent to another conidium with adhesive material at the conidial tip.. B: The adhesive pad at the conidial tip stained with Coomassie briliant blue. (adapted from Schumacher, page 766 fig. 5 a,b)

Source: Localized Adhesion of Nongerminated *Venturia inaequalis* Conidia to Leaves and Artificial Surfaces; C.F.A. Schumacher, U. Steiner, H.-W. Dehne, and E.-C. Oerke. DOI 10.1094/PHYTO-98-7-0760 2008 The American Phytopahtological Society; 760-768.

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Fig.4.6 Illustration of apical secretion of adhesive-like extracellular material. (adapted from Schumacher, 2008).

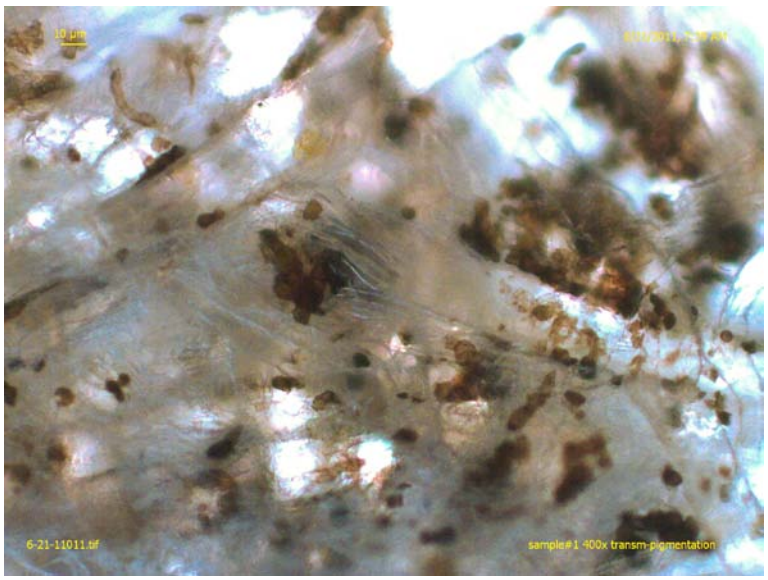


Fig. 4.7 Pigmented deposits in the areas of black stains are most likely extracellular secretions. The preliminary conclusion is based on the amorphous shape that does not indicate fungal cells.

Studies of bacterial and fungal adhesion indicated that a number of cell's surface physico-chemical factors contribute to the process of adhesion. In addition to extensively debated surface hydrophobicity (Bussher, at al, 1990, Oliveira at al, 2001), the presence of extarcellular polymers (Alison and Sutherland 1997, Azeredo and Oliveira 2000), and cell surface charge has been considered. The later determines the electrostatic interaction between the cell and the substrate (van Loosdrecht 1990).

In the most common situations, i.e. aqueous media with pH near neutrality, the microbial cells and solid substrate are negatively charged. This means that surface charge has a repulsive effect and acts contrary to adhesiveness. However, small patches of positively charged areas, according to the most recent discoveries citation) maybe 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 adhesive they also determine the structure of biofilms. Hydrophobicity is important in the first stage of contact and bio-films formation and the role of extracellular bio-polymers in the final stage of bio-film establishment. Attempts were made to measure the attachment of microbial spores and cells to surfaces using the contact angle methods is recognized as the most reliable way to determine cell's hydrophobicity (Doyle 2000). However, the results were not totally successful due to the small size of cells 3-9µm and the need to remove them from their natural environment which alters their natural responses.

4.3 Role of surface topography in fungal growth

The most intimate contact of fungi with surface has impact on the directionality of its expansion. Knowing that fungi grow by extension of mycelium (discussed in detail in chapter 2.1 Fungi) hyphal wall acts as an interface between the fungus and its environment. It responds to the environmental stimuli by directionality of its growth; the responses are species specific and the direction and type of the stimulus. Chemotropism, a reaction triggered by a chemical, in the case growth towards oxygen, was observed in relation to the paper pores and was discussed in chapter 2.2 Paper. Surface topography was reported as orienting directionality in plant pathogens which follow the surface groves towards stomata, a natural opening in pant leaf surfaces permitting them to enter the host (Dickson, 1969; Kwon & Hock; 1991, Allen et al 1991, Dictionary of the Fungi 2008). A visualization of this phenomenon is illustrated in fig. 4.8.

Surface features that influence fungal growth

Fungal interface with its environment is biochemically complex and multifunctional

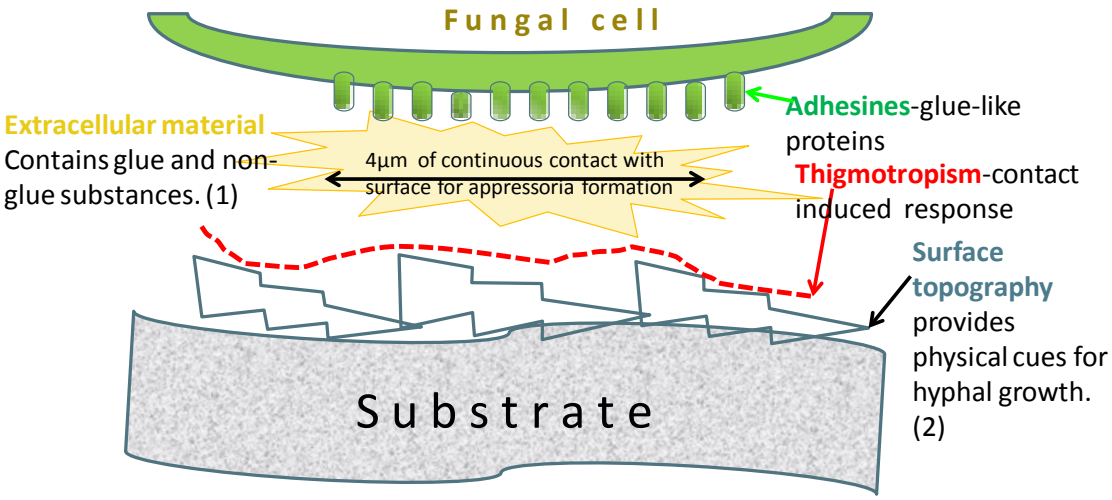


Fig. 4.8 Visualization of fungal interactions with substrate's surface, not to scale. An average size of fungal spores' ranges from 5-9µm. Spores flatten upon contact with surface to enlarge the area of attachment. 1.Nicholson, et al.1988;Pascholati et al.1993. 2. Hoch et al. 1987.

(Note : The visualization is based on article: R.J. Doyle, Contribution of the hydrophobic effect to microbial infection. Microbes and Infection, 2, 2000, 391-400. (© H.Szczepanowska, 2011)

Response to surface topography is termed thigmotropism and was observed in the fungal growth on the study papers. (fig. 4.9). Most likely the hyphae are not growing towards stomata, since the paper fibers are not a living material; however their rough surface seems to be utilized by fungi as anchorage permitting them to expand their growth. (fig. 4.9, 4.10)

Evidence of fungal tropism found in black stains on Study Papers, Group I, (details, see II.5)

Fig. 4.9 Dark pigmented cells arranged in chains follow the paper fibers, most likely utilizing their rough surface as anchorage.

Some cells appear to be attached to the surface of fibers; others are trailing off towards another surface, or more airy pores in paper. Evidence of thigmo- and chemotropism. The 17th c, Study Sheet, stain type A.

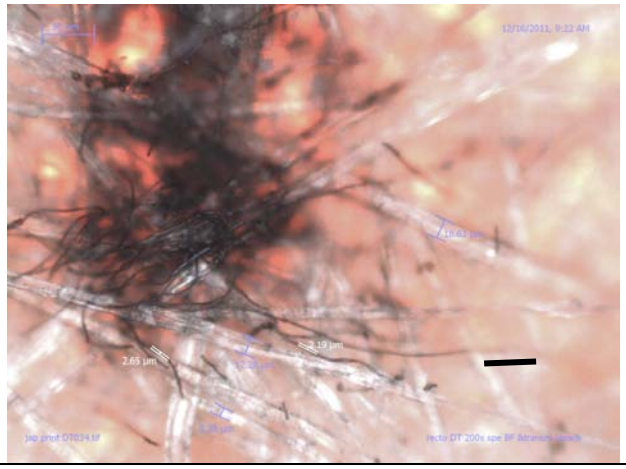


Fig. 4.10 Area stained by dark pigmented fungal filaments concentrated where the fruiting structure grew.

The filaments migrate through the substrate's bulk to the other side of paper. 1847 Japanese woodblock print. Scale bar, black , right bottom : 50 µm

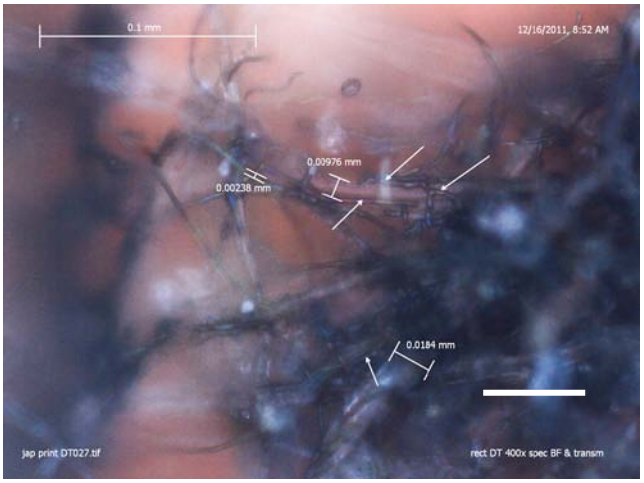


Fig. 4.11 Detail of the stain in fig. 4.10, showing dark filament interaction with paper fibers.

Similarly to other scenarios, the filaments follow the paper fibers and appeared to be attached to fibers' surface. Transmitted light micrograph, dark field.
Scale bar, white, bottom 50µm.

4.4 Summary points

1. Adhesion of microbes to surfaces involves a multitude of factors of physical and chemical nature. External stimuli trigger complex changes inside a spore from the moment it 'recognizes' the surface in a suitable environment, however the mechanism behind this initial forces are not clear.
2. Attachment to surfaces is one of the most important events in microbial life because it initiates a new life cycle.

3. Environmental conditions in concert with the characteristics of a substrate's surface play a decisive role in the initial stage of fungal development.

PART II: EXPERIMENTAL WORK

5. Investigation strategy

Problem. Fungi cause stains on paper. Current state of science does not explain how the stains are produced in fungal cells. In the context of cultural heritage it is unknown how the stains are formed by fungi growing on cultural heritage material and deposited on substrates. Furthermore, it is not clear how the environment affects stains' formation.

Aim of this investigation. Studying the dynamics of interfacial forces of fungi, living systems and substrate (here paper) will provide some answers regarding the fungal stains formation and deposition. The focus of our investigation was on deposition of pigmentation, if the deposit is on the surface of fibers or introduced by fungi into the fibers' interior. Answers to his question have direct Implications on how the pigmented deposits can be removed in ht future. One environmental factor, light, was selected to investigate its effect on pigment production.

5. 1 Examination approach

The examination approach, from global to local, began with the assessment of samples' context and macro-features of papers' surfaces and biodeterioration when present. The results of macro-evaluation were documented and analyzed before proceeding to more in depth, instrumental and chemical analysis of microstructures. Understanding the context of biodeterioration, such as visual characteristics of paper surfaces, bulk composition (fiber type) and presence of additives (sizing) were essential for the proper interpretation of analytical results. It is often difficult to conclude cause-and-effect relationship, as many factors are working in synergy in a complex biodeterioration phenomena. Methods of substrates manufacturing, especially in case of papers, surface finishing processes and additives, all contribute to the bio-mass development and characteristics. In this investigation an attempt was made to determine which of the surface characteristics contribute to fungal pigment deposition and how these features impact the interactions of paper with microorganisms.

Materials: Five papers were selected for this study. Two of those papers were subjected to fungi-induced pigmentation (group I), three of the papers were biodeteriorated artworks (group II). ***Group I included*** two types of paper, Whatman 4(W4) and Winsor& Newtown (WN). ***Group II*** consisted of three papers affected by fungal stains; a 17th century Study Sheet, 1920 Etching and 1847 Japanese woodblock print. All artworks were affected by biodeterioration. Details of fungal stains biogenesis and morphology are discussed in **II.9.**

Fungal species involved in this study, *Aspergillus niger* and *Chaetomium* sp. are briefly described, including their appearance, growth patterns, morphology, dimension of filaments, characteristics of fruiting structures and pigmentation they produce. **(I.2.1)**

Methods and Instruments: The focus of the experimental work was on investigation of interfaces of living systems and cellular substrate namely paper. Two groups of paper represented two different scenarios of biodeterioration. The underlying objective of the investigation of both groups of paper was the search for correlation between the types of papers and fungal pigmentation, patterns of staining and interfacial interactions of biomass and substrate in each scenario.

Group I- stains induced by known microorganism in controlled environment; **Group II -** stains occurred in the course of time in unknown conditions by unknown microorganisms. However, the course of investigation only slightly differed in those two scenarios. Evaluation of biodeterioration on artworks was conducted 'in situ' extrapolating as much information as possible without the need to procure samples of the original material; integrity of artworks cannot be jeopardized. Microscopic fragments underwent instrumental analysis where samples were available for extraction. **(II.9)**

Instruments: Impact of fungal staining on paper substrate was evaluated using both traditional optical microscopy and novel, surface tracelogy methods. Morphology of paper surface and matrix of controls (group I) served as a base line for comparative studies of surface and matrix affected by induced fungal stains. **(II.6 Materials and Methods)** Artworks in group II underwent examination using the same instrumentation, in situ and microscopic fragments underwent instrumental analysis where samples were available for extraction. **(II.9 Bio-genesis of Fungal Pigmentation)**

The following techniques were applied:

- STM Stereo-microscopy (surface characteristics of paper and biostains)
- TLM Transmitted Light Microscopy (morphology of microorganisms and paper fibers)
- CLSM Confocal Laser Scanning Microscopy (microorganism and paper surface interaction)
- CLA white light confocal laser profilometer (surface topography of papers with and without microorganisms)
- SEM-VP Scanning Electron Microscopy Variable Pressure (characterization of bio-deposits and paper surface)
- SEBT Synchrotron Electron Beam Tomography (microorganisms interaction with paper matrix)

Details of the examination protocols and principles of instruments' operation see **II.7. Instruments.**

Documentation: The analysis and investigations were documented in graphs, in a descriptive format and as digital images, using cameras appropriate for each type of the examination. The analysis of each case study constitutes a separate subchapter; **II. 8** Light Effect on Fungal Growth and Pigmentation (Light Experiment) and **II.9.** Biostains Genesis. Supportive documentation such as measurements and test analysis reports based on which the conclusions were drawn is included in **Appendices.**

5.2 Examination protocol

- Sample material: type, context and selection criteria
- Physical characteristics of study papers
    - Macro-evaluation of papers surface
    - Bulk, stiffness and wettability
    - Surface morphology and topography
    - Fibers composition of papers
- Chemical characteristics of sizing in paper
    - Starch, (test with iodine and potassium iodine)
    - Gelatin (test for protein BCA1)

- Microorganism observed on artworks and cultured on test papers; brief characteristics of their morphology and pigmentation
    - *Aspergillus niger* (inoculated to produce pigments)
    - *Chaetomiun globosum* (identified on artworks)
    - Meristematic fungi (identified on artworks)
- Biodeterioration of paper
    - paper surface (surface topography, confocal scanning laser microscopy)
    - fungi morphology (optical surface and transmitted light microscopy)
    - Interfaces of fungi and paper (optical microscopy, cross-sections of samples embedded in resin, synchrotron electron microtomography
- Documentation of individual case studies and experimental results
- Partial Conclusions: implications of findings on designing care of cultural heritage

5.3 Presentation of findings

Partial conclusions are drawn from the experiments and analysis of actual stains encountered on artworks summarizing each subchapter and listed as Summary Points. The final conclusions include the results of experimental work in light of the current theoretical research in pertinent fields. Additional supportive background information, data and measurements gathered in the course of experiments which served as bases for graphics, plots, or diagrams are included in Appendices.

6. Materials and Methods

The materials used in this study included five different papers, tree types of fungi and chemical reagents. The examination and analysis were carried out using an array of diagnostic instruments. Each material –paper, fungi, chemical reagents and methods of their application are described in pertinent subchapters.

6.1 Selection criteria of the study papers and fungal species

Paper: Five different types of paper were investigated in respect to biodeterioration caused by fungal stains. **Group I:** Whatman4 paper and Winsor&Newton watercolor paper. **Group II:** Three artworks: Study Sheet, ca 17th century, 1920 Engraving and Japanese woodblock print, dated 1847. **Selection criteria**: Three criteria, type of manufacture, fiber content and presence of sizing were used in the selection of papers for his study. Table I.

Fungi: One strain, *Aspergillus niger* was used in the experimental stain-production on papers in group I. **Selection criteria:** *Aspergillus niger* is one of the most studied fungus, known for its black pigment production. **Fungal stains:** Dark pigmentation produced by fungi on artworks was caused by several morphologically different fungi. One of them, *Chaetomium* sp., was unequivocally identified on the Japanese woodblock print; two others, *Taeniolella* sp. and *Ascotricha* sp.[2] were identified on 1920 Engraving and on the 17th century Study Sheet respectively.

Table II: Criteria used in selection of the study papers

Sample #	Sample set or location	paper	sizing	art technique	fungi
Group I papers, used in the experimental production of fungal pigments					
Whatman 4	W4; W4-DL, W4-ML	Machine made	None	Not applicable	*Aspergillus niger* - inoculated
Winsor & Newton	WN; WN-Dl, WN-ML	Machine made	present	Not applicable	*Aspergillus niger* – inoculated
Group II papers, artworks on paper					
No1: 17th century paper, samples	1.xx	Handmade, Western	present	Red pigment mark	3 types of fungal stains; *Taeniolella* sp. meristematic fungi

[2] Note that *Taeniollela* sp *and Ascotricha* sp were identified by an outside source. (appending , identification of species, correspondence). The identification was not confirmed by any additional study.

No2: Woodblock print 1847	2.xx	Handmade, Oriental	Residual	Color, woodblock	1 type identified: *Chaetomium* sp.
No3: Engraving 1920	3.xx, or location on artwork	Machine made, Western	residual	Engraving, printer's ink	1 type; *Ascotricha* sp. meristematic fungi

6.2 Evaluation of the study papers

6.2.1 Macro-evaluation

The first group consisting of two papers, Whatman 4 (filter paper) and Winsor & Newton (watercolor paper) was used in the experimental production of fungal pigments. *Aspergillus niger* was cultured on both papers in two different light conditions, daylight (DL) and dark (ML). (see II.4. Light and pigmentation). Morphology of papers' surfaces was recorded before the experiment begun and after pigments were produced and deposited on paper.

The second group of papers included two artworks and one, ca 17th century, Study Sheet, all diminished by black fungal stains. These three papers represented different types of paper manufacturing techniques, Western and Oriental, and were stained by four different fungal formations. However, not all fungi could be positively identified.

Physical characteristics of all five papers are summarized in table II.

Table III: Physical characteristics of the study papers

Group I	**Whatman 4 paper**	**Winsor& Newtown watercolor paper**
Two papers used in the experimental stain production by *Aspergillus niger.*		

Fig. 6.1 Recto and verso of Whatman 4 are identical. Paper is rough and fiberous.

Fig. 6.2. Imprint of Winsor&Newton paper. Surface characteristics of both sides: fibrous and rough. Different water sorption was noticed in relation to placement in the sheet.

Whatman4 paper is a filter paper produced without any additives for the use for qualitative laboratory analysis. It is made of pure cotton fibers. It is extremely fast filtering paper with excellent

retention of coarse particles. Used when high flow rates in air pollution monitoring is required and the collection of fine particles is not critical. Typical thickness: 205 µm. weight 96 g/m^2. (www.whatman.com) Circular discs 125 mm diameter were used in the study; cat. No 1004 125. Whatman1 was used for measurement of stiffness in Fold Test, because it is produced in sheets.

Winsor& Newtown paper is artists' paper produced as support for watercolor. The paper is an intrinsic part of the artwork. Traditional gelatin sizing is applied on the paper surface to make it hydrophobic in order for artists to alter the design using water without disturbing paper's fibrous texture. Gelatin gives a harder surface which also allows scraping and rubbing without damaging the paper itself. The rough sheet has been pressed between the 'felts' on the paper machine and the roughness of the felt (blanket) is embossed into the wet sheet. (www.winsornewton.com)

Group II, three historic and artistic study papers, overall characteristics; fig. II.2.b.3-8, H. Szczepanowska

No.1: Study Sheet, 17th c.	**No2: 1920 Engraving**	**No3: 1847 Japanese woodblock print**

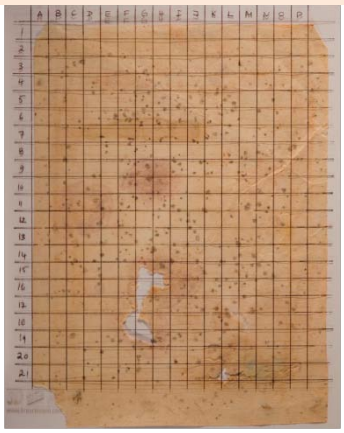
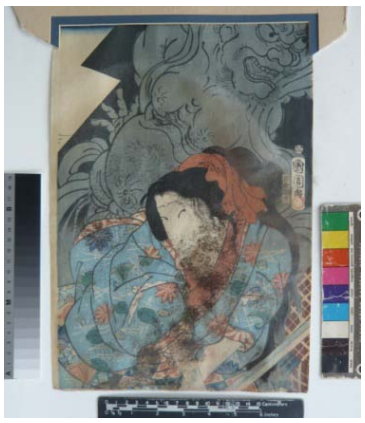

		

Fig. 6.3 Recto (front) of the sheet. Characteristics of recto and veso differed.	**Fig. 6.5 Recto of the sheet and vereso were identical in appearance, both were smooth. Paper was crisp; light buff color. H: 35.2cm W: 27.5cm.**	**Fig. 6.7 Recto of the sheet and verso are identical, both showing well defiend fibers. Paperwas soft and pliable; whitel color. H: 34.3 cm W: 24cm.**
Recto is fiberous verso is smooht. Adhesive spotches were present on verso. Paper wa soft and pliable; mottled color. H: 23.5cm W: 17cm. Fungal stains were scattered throughout the sheet on recto and less visible on verso.	Black fungal stains were locatated along the top on recto and verso.	Dark brown fungal fruiting strcutures were located on the surface, on recto and verso.

Fig. 6.4 Detail of recto; surface with scattered fungal stains. Two morphologically different fungi produced dark pigmentation.

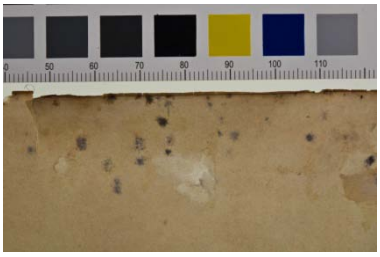


Fig.6.6 Detail of recto, top edge; surface with scattered black stains.

Fig. 6.8 Detail of recto, raking light (side view), surface with dark fungal deposits.

Biodeterioration of each type of staining has been analyzed and is reported in separate subchapters, 9.1-3.

6.2.2 Bulk, stiffness and wettability

Bulk of paper refers to the fiber composition and additives, both factors affecting paper's behavior and in turn fungal interaction with paper. For example, additives will effect stiffness and may affect wettablity, the latter having direct impact on fungal growth. To determine the stiffness of the study papers a fold test was performed. (figs. 6.9; 6.10; 6.11)

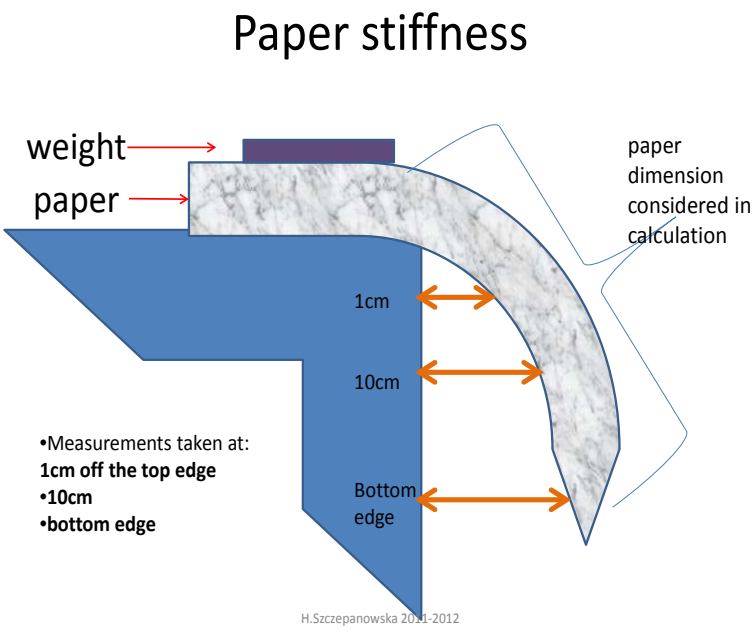


Fig. 6.9 Experimental measurement of papers' stiffness. ©H.Szczepanowska, 2012.

This practical evaluation was performed to gain insight into papers' characteristics. The principle of this test relies on measuring the ration of bend in each direction. Since paper is a very hygroscopic material, its stiffness properties are affected by the relative humidity and temperature of the

ambient air. It is also important to remember that it is the current moisture content of the sample which affects the stiffness. The moisture content of the material depends on the current climate but it also depends on earlier climatic conditions because of the so-called moisture hysteresis effect. Paper can reach different moisture contents and thus different stiffness even in the standardized climate of 50% RH and +23° C, depending on whether the paper has come to this climate from a dry or a humid climate. To ensure some uniformity of this test all papers were stabilized over 24 hrs period in the same room conditions.

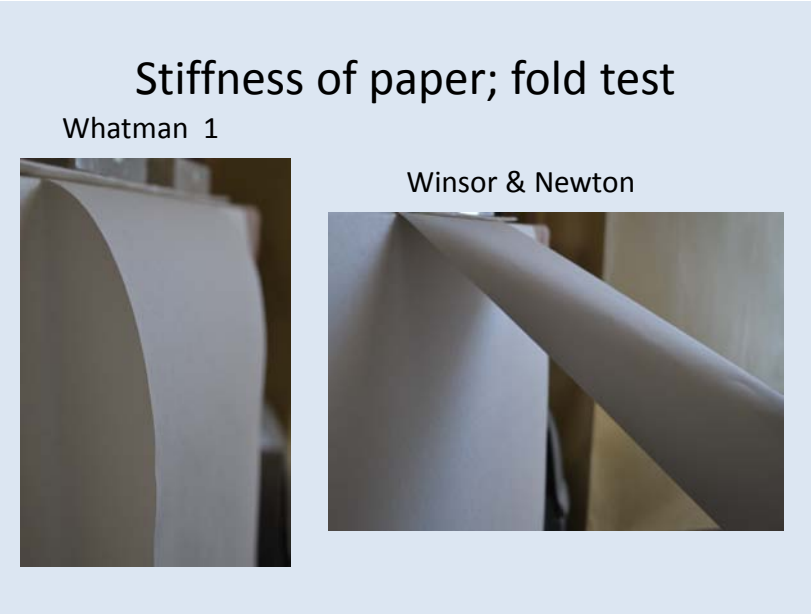


Fig. 6.10 Stiffness of Whatman1 and Winsor&Newton papers determined in the fold test. ©H.Szczepanowska, 2012.

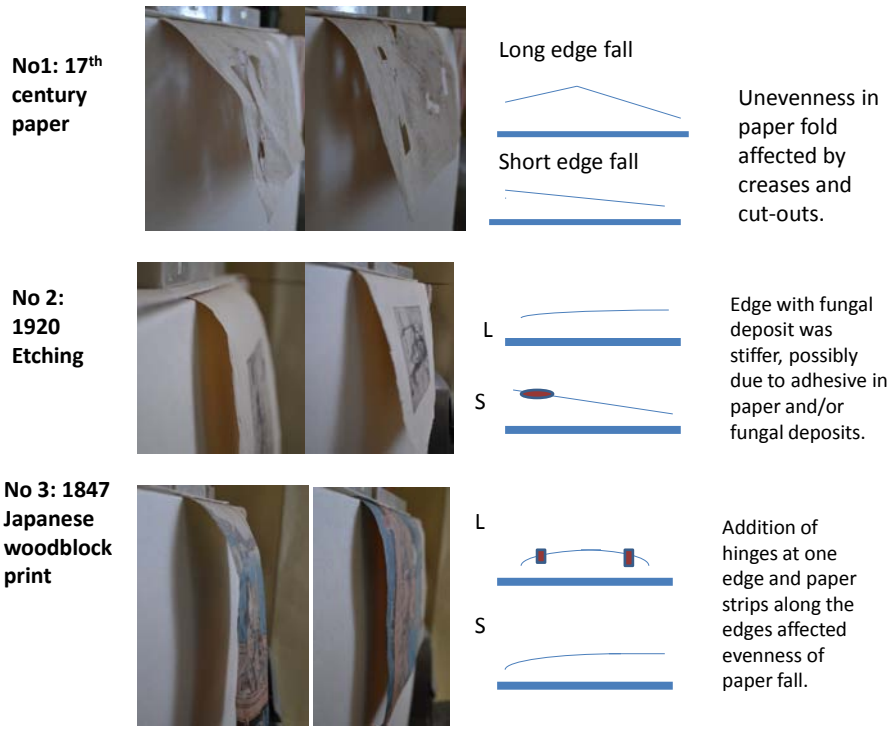


Fig. 6. 11 Stiffness of paper of three artworks, Group II Papers. ©H.Szczepanowska, 2012.

The stiffness of paper has been defined as the bending moment per unit width of the specimen and per unit curvature of the specimen at the torque axis. (Carson 1952). Many sophisticated and complex tests such as ultrasonic measurements (Cantwell et al. 2008) have been performed in the paper industry to determine 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. That 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. However the methods and instruments used in measuring stiffness are not uniform and according to some reports not completely standardized (Lorentzen & Wettre, 2011). A simplified fold test was performed to gain insight into the studied papers' characteristics.

Results. Assuming that stiffness of paper indicates additives, papers with greater amount of additives should support fungal growth. Presence of additives has always been associated with additional nutritional supplement for fungi in case paper becomes wet. However, that has not been entirely confirmed by the experimental work in this study. Only in once case, of 1920 engraving, fungal stains were located along the edge where starch adhesive was applied. Presumably, after exposure to water the adhesive provided additional nutrient. Gelatin sizing on Winsor& Newton paper and glue splotches on Study Sheet No I did not promote fungal growth. Fungal stains were sparsely produced on gelatin-sized Winsor&Newton. No stains occurred on the Sheet No 1 in the areas where glue was present.

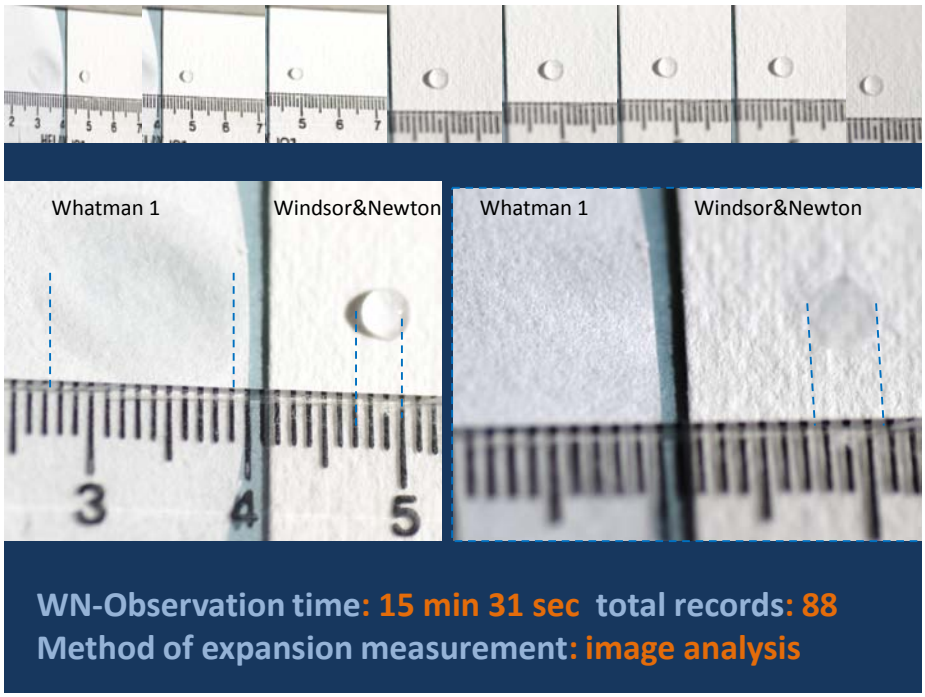


Fig. 6.12 Evaluation of water sorption by two papers used in the experimental fungal pigment production.

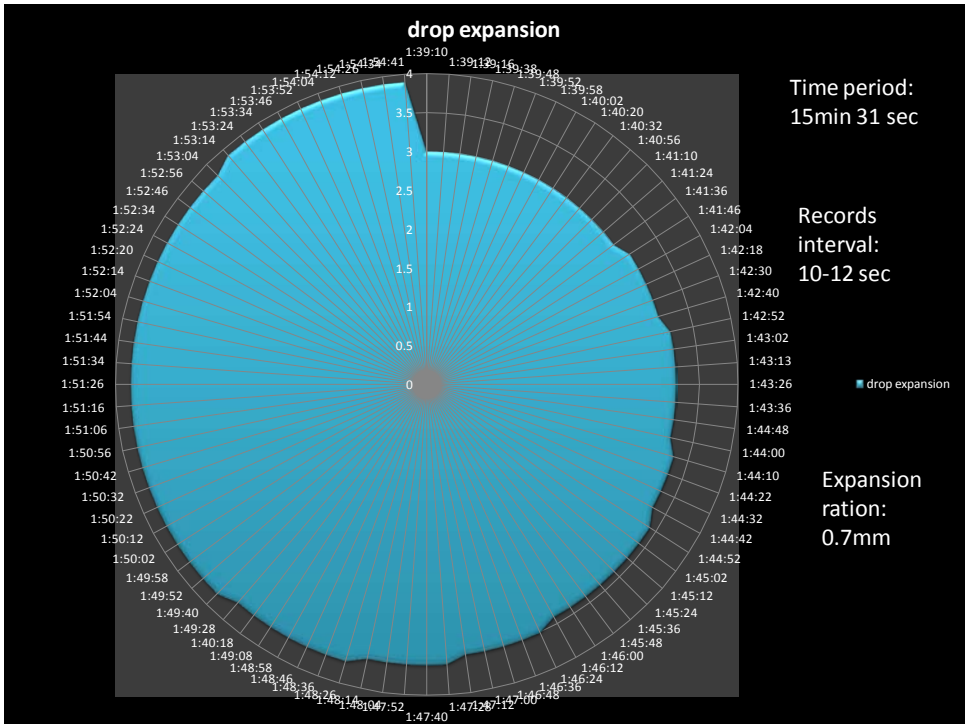


Fig. 6.13 Water drop sorption by Winsor&Newton paper was recorded optically in 10-12 second intervals. Collected records were plotted to illustrate and sorption of water on Winsor&Newton paper accompanied by expansion of the wet area. Whatman 4 absorbed water immediately after its application. Absorption of water has impact on the ratio of fungi grow, as can be seen on fig. 6.14.

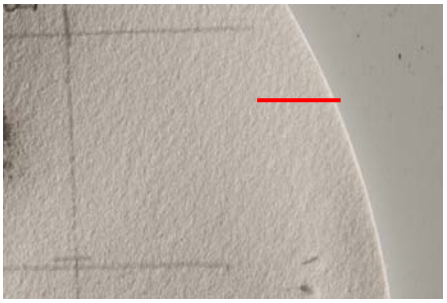
Fig. 6.14 Whatman4 which absorbed water rapidly showed greater growth and pigmentation of *Aspergillus niger* than Winsor & Newtown paper which did not absorb water.

6.2.3 Surface morphology and topography

Papers group I, used in the Light Experiment. Fig. 6.15-6.19, H.Szczepanowska 2012

Group I **Whatman 4: W4-C** (C stands for 'control')

Fig. 6.15 Macro-photograph, Whatman4 paper. scale bar: 5mm



Unaided-eye observation indicated rough, fibrous surface with no gloss on either side of the paper. Both sides of paper are identical considering their roughness. Rough surface provides natural environment for air-disseminated fungal particles as they land, protecting them from removal by air movement or other mechanical means, such as brushing off.

Stereo-microscopy		**SEM-VP**	**Surface morphology; CLSM**

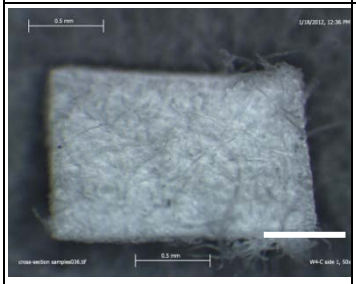
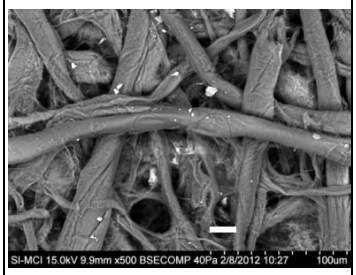
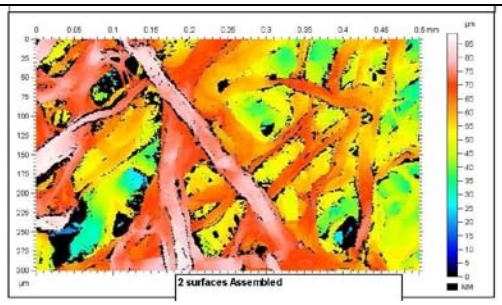

			

Fig. 6.16 Stereo-micrograph of W4 control paper sample; scale bar: 0.5mm		Fig. 6.17 Surface morphology, SEM-VP micrograph, Whatman4 control; SEM-VP ,scale bar: 20 µm	Fig. 6.18 3D topography; area measured: x500 µm; y300 µm; z 85 µm; white light confocal laser profiler. Surface morphology; CLSM

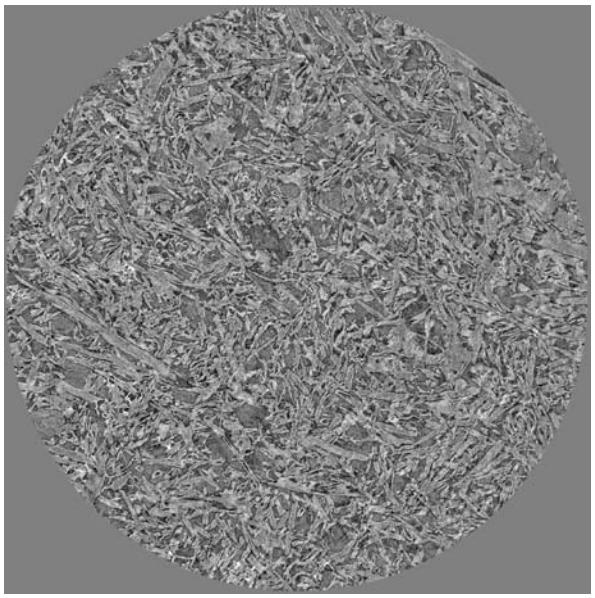


Fig.6.19 Cross-section of Whatman4 obtained in X-ray microtomography, ESRF Synchrotron Facility, Grenoble.

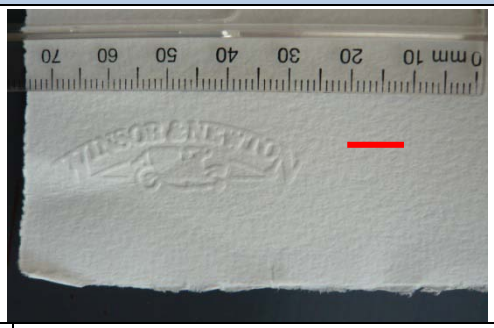
Paper characteristics: Well defined fibers, no additives present. Surface metrological tool (CLS-profiler) showed the surface topography. Height of the fibers reached nearly 90 µm confirming very fibrous and rough surface features. Surface characterization of control papers provided a base line that was used for the evaluation of surface alterations caused by fungal growth. Fiber analysis indicated cotton.

Papers Group I, surface morphology, cont; fig. 6.20-6.24, H.Szczepanowska, 2012.

Group I	**Winsor & Newton; WN-C** (C stands for control paper)

Fig. 6. 20 Macro-photograph, Winsor&Newton paper. scale bar: 10mm.

Note the characteristic, embossed mark of Winsor & Newton papers.



Stereo-microscopy	**SEM-VP**	**Surface morphology; CLA**

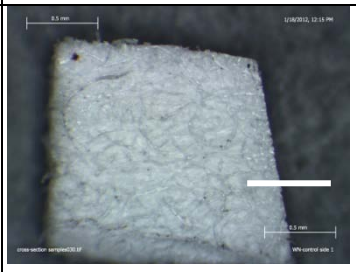


Fig. 6.21 Stereo-micrograph of WN control paper sample; scale bar: 0.5mm

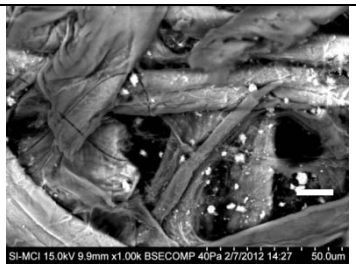


Fig.6.22 Surface morphology, SEM-VP micrograph. SEM-VP, scale bar: 20 µm

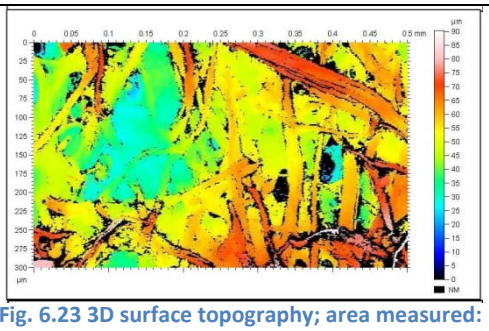


Fig. 6.23 3D surface topography; area measured: x 500 µm; y 300µm; h 85 µm. The highest detected level of fibers reached 70 µm. Surface morphology; CLA

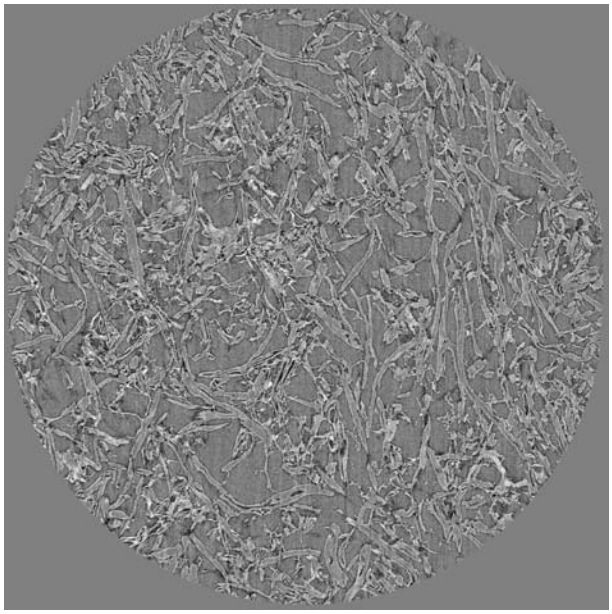


Fig. 6.24 Cross-section of Winsor&Newton paper obtained in X-ray microtomography, ESRF Synchrotron Facility, Grenoble.

Paper characteristics: Fibers are well defined, additives present. Further analysis indicated presence of calcium. Surface metrological tool (CLA) revealed lesser differentiation of surface topography than of W4. The paper is fibrous with well defined texture. Areas between fibers are smoother than in Whatman 4 which is indicated by 'green' plateau between fibers, indicating the additives. Fibers analysis indicated cotton.

Papers Group II, Historic Papers; *surface morphology,* cont. fig.6.25-6.29 H.Szczepanowska, 2011.

Group II	**No.1: Study Sheet**	**macro-characteristics**

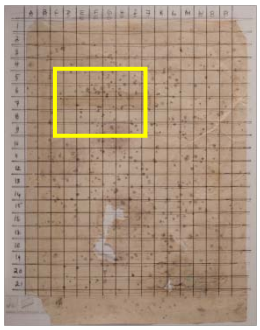


Fig. 6.25 Surface characteristics of recto of Sheet No 1 differed from verso. Close-up of macro-image marked in yellow.

Fig. 6.26 Recto of Sheet No 1: fibrous with defined texture. All types of fungal stains are pronounced. Scale bar: 10mm

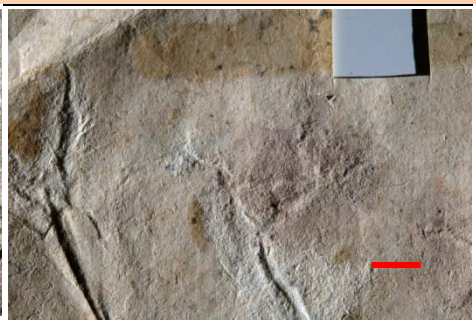


Fig.6.27 Verso of Sheet No 1: smooth with brown splotches of glue. Black fungal stains are less visible than on recto. Green/olive and purple stains are of the same intensity as on recto. Scale bar: 10mm

No 1 Study Sheet; Stereo-microscopy, 3D composite image

Fig. 6.28 Surface features and fungal deposits. White color of paper has been altered as a result of fungal stain. Scale bar: 0.1mm.

3D composite image of a black stain in area 2A; Sheet No1, recto.

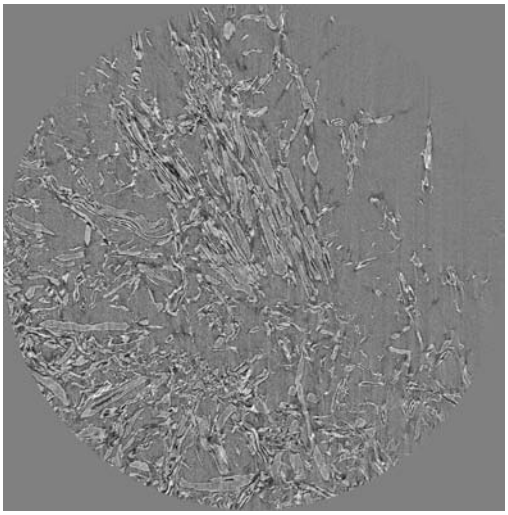


Fig. 6.29 Cross-section of No1 paper obtained in X-ray microtomography, ESRF Synchrotron Facility, Grenoble.

This particular area was severely damaged by microorganisms and shows loose fiber arrangement.

Papers Group II, Historic Papers, *surface morphology* cont. fig. 6.30-6.38 H.Szczepanowska 2012.

No 1: Study Sheet 17th c	**Optical surface analysis**	
Stereo-microscopy	**SEM-VP**	**Surface morphology, CLA**

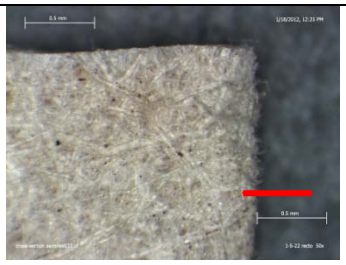
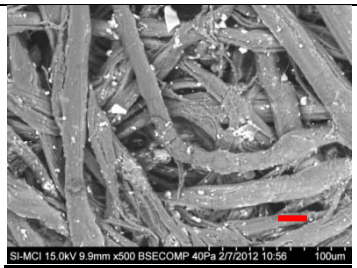
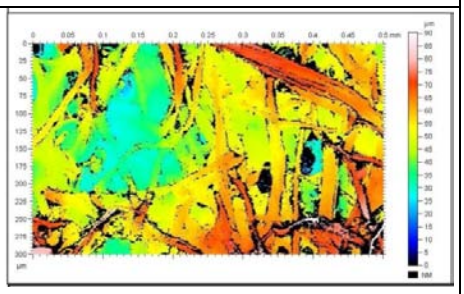
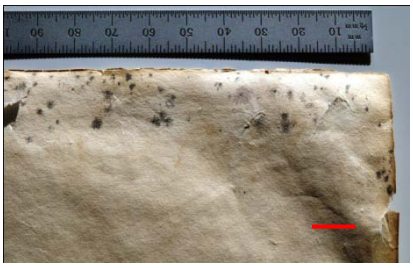
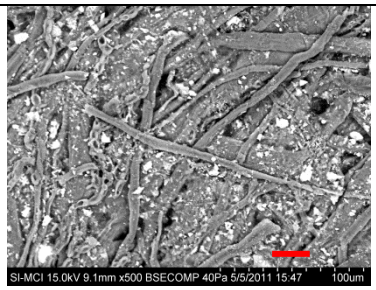
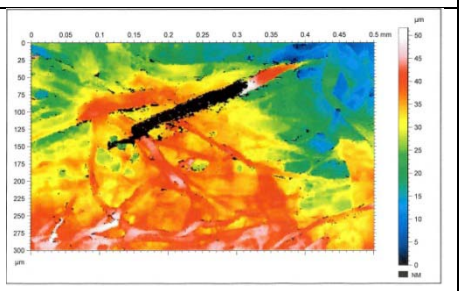

		

Fig. 6.30 Stereo-micrograph of paper No.1 surface. Shows well defined fibers and pronounced texture. Scale bar: 0.5mm.	**Fig. 6.31 Well defined fibers with impurities (most likely pigments) visible as 'crystalline' particles among fibers. Pigment analysis, App. III.Paper. SEM-VP Scale bar: 20µm**	**Fig. 6.32 Area measured: x 1mm; y 500µm; h 90 µm. The highest detected level of fibers reached 85 µm. Plateaus in the range 20-45µm most likely indicate areas of sizing where fibers were consolidated. Surface morphology, CLA**

Group II	No2: 1920 Engraving	Macro-characteristics
		
Fig. 6.33 Recto and verso of the 1920 Engraving were identical. Paper was smooth and crisp. Area of close-up is marked in yellow.	Fig. 6.34 Recto of the artwork, macro-image in raking light shows surface texture. Adhesive residue was present on both sides of paper. Scale bar: 10mm.	Fig. 6.35 Verso of the same area, raking light emphasizes texture of paper. Adhesive residue was present and is visible as darker brown splotches. Scale bar: 10mm.
Stereo-microscopy	**SEM-VP**	**Surface morphology, CLA**
		
Fig. 6.36 scale bar: 0.5 mm. Stereo-micrograph shows surface of No.2 paper smooth and compact.	Fig. 6.37 Fibers are embedded in compact matrix of sizing and fillers. SEM-VP, scale bar: 20 µm	Fig. 6.38 Measured area: x 0.5mm; y 0.3mm; h 52µm. Areas of plateaus corroborate optical observation showing smooth paper.CLA.

Papers Group II, Historic Papers, *iii surface morphology* cont. fig. 6.39-6.43 H.Szczepanowska 2012.

Group II	1847 Japanese woodblock print	Macro-characteristics

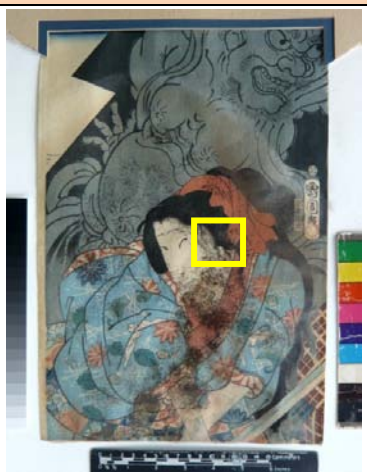
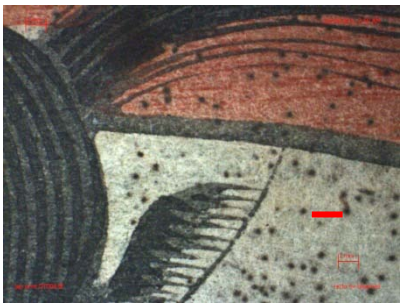
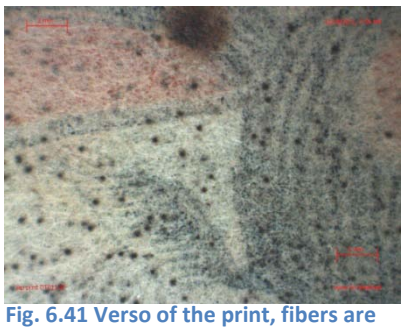

		

Fig. 6.39 Recto and verso of the Japanese woodblock print were covered with fungal deposits. Area of a	Fig. 6.40 Recto of the print, fibers are well defined and pronounced. scale bar: 1mm Microscopic examination of the printed areas, especially in black ink, is necessary for the differentiation between black fungal deposits and fibers with black ink.	Fig. 6.41 Verso of the print, fibers are equally well defined as on recto; scale bar: 1mm

close-up micrograph is indicated with a yellow square.		
Stereo-microscopy	**SEM-VP**	**Surface morphology-macro**

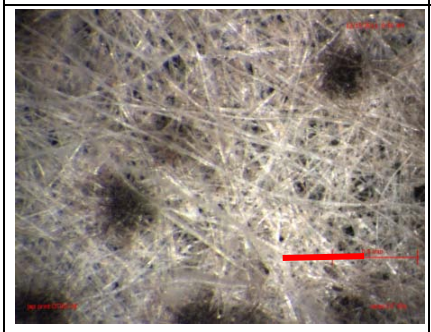
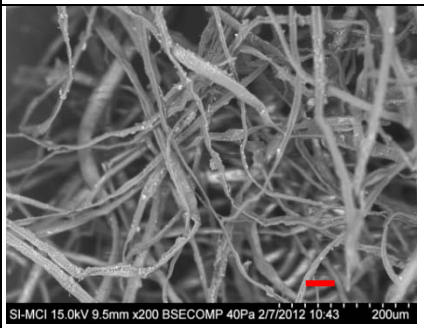
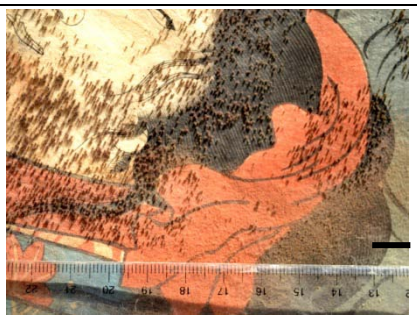

		

Fig. 6.42 Surface is fibrous, with well defined single fibers in a mesh-like web. Pigmented fungal deposits are on recto and verso of paper. Stereo-micrograph, scale bar: 0.5 mm	**Fig. 6.43 Loosely arranged fibers in area not contaminated by fungi. SEM-VP, scale bar: 40 µm**	**Fig. 6.44 Most of the paper surface was covered with large fungal formations, as seen here. No CLA surface morphology was carried out. scale bar: 10mm**

6.2.4 Fibers content: W4, W&N and the historic papers

Fibers content

Papers used for the experimental stain production and as support for the execution of artworks was examined with optical microscopy to determine its fibrous composition. In addition to the analysis of fibers and sizing, red pigment on the 17th century paper was analyzed, for two reasons. First, to learn more about the date of the paper and second, to use the results as reference in case the same residue is found in different locations on paper fibers. This red pigment was scattered randomly in different places on verso, and without the analysis its presence could be misinterpreted.

Instruments used: stereomicroscope (SM) transmitted light microscope (TLM), scanning electron microscope in variable pressure (SEM-VP), confocal laser scanning microscope (CLA) and electron beam X-tomography at synchrotron in Grenoble's ESRF facility. The principles of instruments operational systems are described in detail in chapter **7 Instruments and methods of characterization.**

Table IV: Oriental paper fibers;

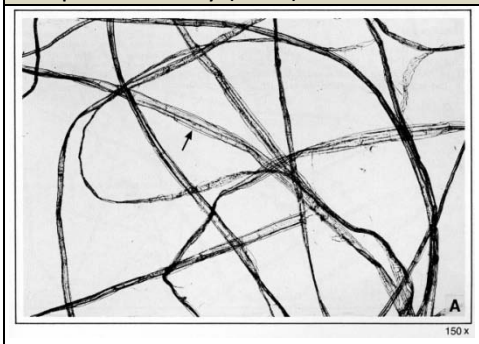
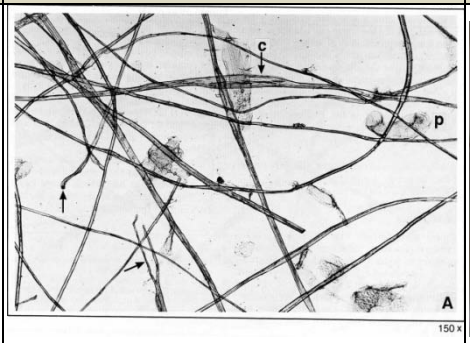
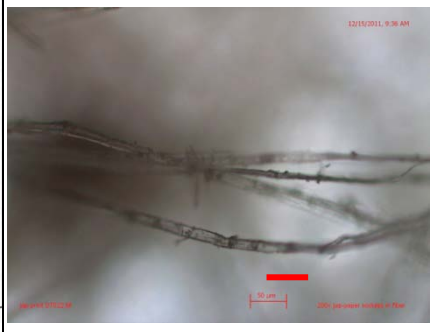
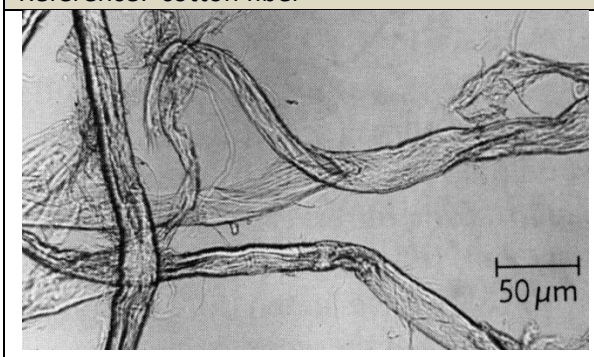
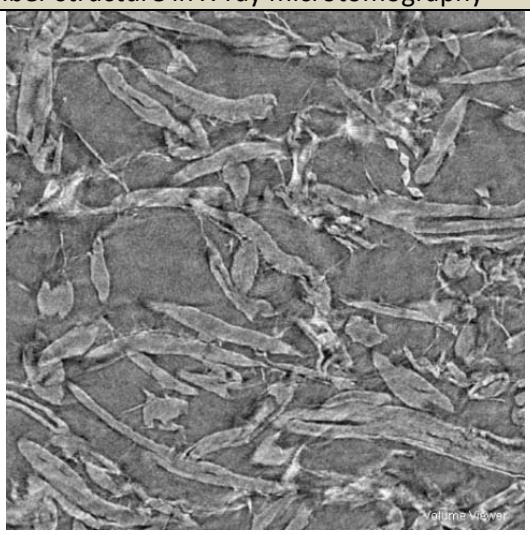
Paper-mulberry (kozo)	Mitsumata	Oriental study paper
 Fig. 6.45 Paper-mulberry (kozo) fiber length av. 10mm (6-20mm); fiber width av. 30µm (25-35 µm). **Image: Fiber Atlas, Ilvessalo-Pfaflf, p.349 fig.11.20.**	 Fig. 6.46 Mitsumata fiber length av. 3mm (2-4mm). fiber width av. 9µm (7-14 µm). **Image: Fiber Atlas, Ilvessalo-Pfaflf, p.351 fig.11.21A.**	 Fig. 6.47 Fiber from the Japanese print 1847, fiber av width: 13 µm. scale bar: 50 µm. Based on the size and morphological features, it is Mitsumata fiber.
Native to Japan, Korea and China	Native to Japan, China and Himalayans	1847 Japanese print paper

Table V: Western study papers: fibers content

Reference: cotton fiber	Fiber structure in X-ray microtomography
 Fig. 6.48 Cotton lint fibers: Fiber length: average 18 mm (10-40mm) Fiber width: average: 20µm (12-38 µm) (Ilvessalo 1995) (image source: Banik and Bruckle, fig. 4.24, p. 101, after D. Mayer).	 Fig. 6.49 Fiber twists along axis appearing like convoluted flattened tubes. Cross-section is showing lumen, semi-curled fibers. X-ray micro-tomography, ESRF Grenoble of Windsor& Newton paper.

17th century Sheet No.1-surface

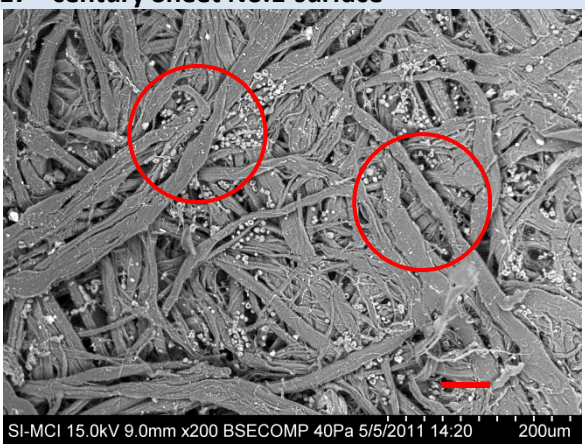


Fig. 6.50 No.1 paper; fiber width: 12-36µm; The characteristic cotton fiber twist is noticeable. scale bar: 50 µm.

17th century Sheet No.1-cross-section

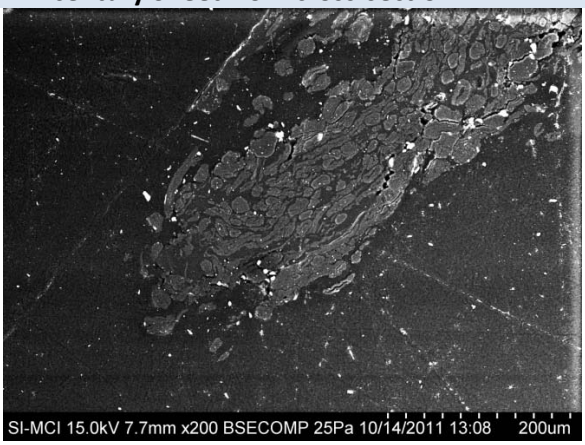


Fig. 6.51 Formation of cotton fibers with lumen in the center is visible, although lumen collapsed. scale bar: 50 µm

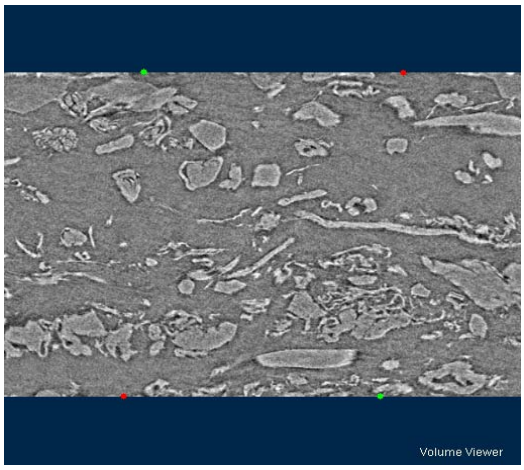


Fig. 6.52 No.1 paper, cross-section on area L20c obtained in X-ray microtomography shows cross-section of cotton fibers. H of cross-section,

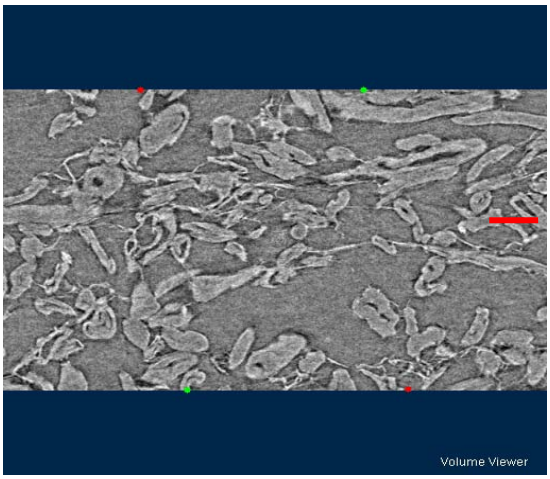


Fig. 6.53 Winsor&Newton paper, cross-section obtained in X-ray tomography, showed in cross-section cotton fibers. H of cross-section,28 µm.

1920 Engraving-surface

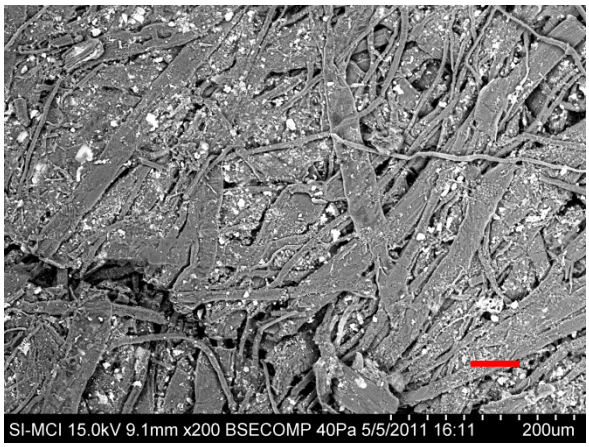


Fig. 6.54. Cotton fibers are visible, however the paper matrix is compact and fibers are less defined. SEM-VP micrograph, scale bar: 50 µm.

1920 Engraving-cross-section

Fig. 6.55 Inclusions of fungal structures were clearly identifiable in transmitted light microscopy in cross-section of paper embedded in resin. Scale bar: 50 µm.

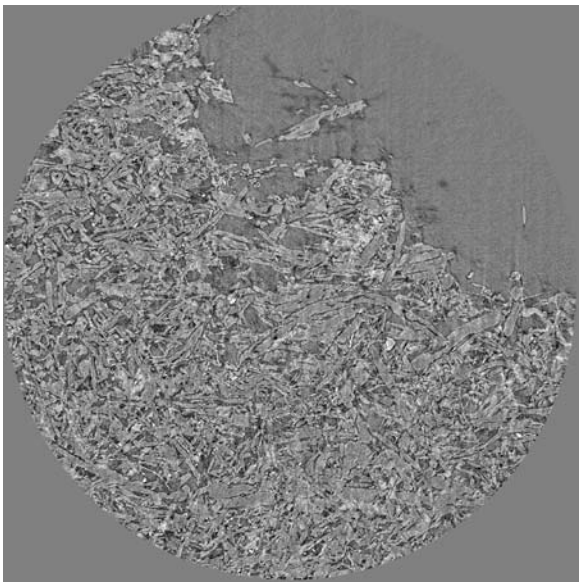


Fig. 6.56 Cross-section of the paper matrix obtained in X-ray microtomography shows compact paper.

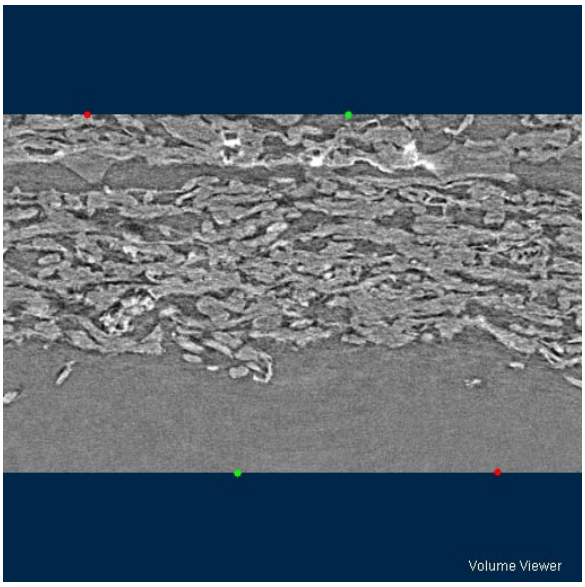


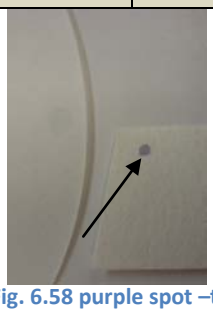
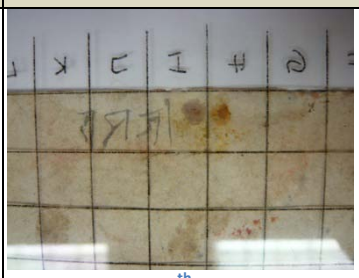
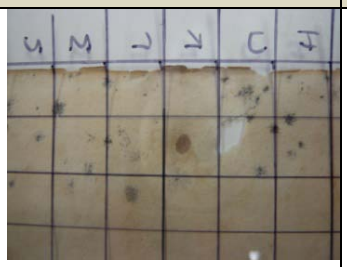
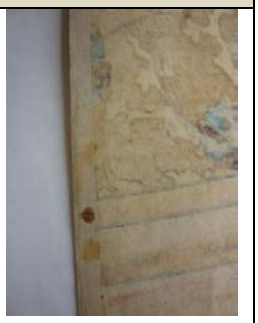
Fig. 6.57 Cross-section through the paper strata reveals cotton fibers and n densely arranged fibers. X-ray tomography.

6.2.5 Tests for sizing, gelatin and starch, and pH level

The tests were carried out to determine the presence of the most commonly used sizings on paper, gelatin and starch. In addition, acidity level was measured with flat electrode and pH meter. Summary of tests are included in Table VI. Supplemental information and imaging are in **Appendix: Paper Tests Results.**

Table VI: Results of test for acidity level, the presence of starch and protenacious gelatin.

fig. 6.58-6.6.61 H.Szczepanowska 2012.

test	W4-C	WN-C	No1:17th c paper	No 2: 1920 Engraving	No 3: 1847 Jap print
	 Fig. 6.58 purple spot –test for protein (gelatin). W4 and WN tests.		 Fig. 6.59 No1:17th c paper tests.	 Fig. 6.60 No 2: 1920 Engraving tests.	 Fig.6.61 No 3: 1847 Jap print tests.
pH	5.7	6.9	6.25	6.8	5.55
starch	Not tested	Not tested	present	present	not determined
gelatin	none	present	present	present	present

Note: The listed number of acidity level, (pH) is an average of 3-6 readings on each paper.

6.3 Summary points

Out of many possible features of paper, only those were considered for discussion which potentially might have had impact on bio-mass formation or growth. In particular characteristics of the paper surface, presence of sizings, and water absorption were evaluated. Each of these features, especially water absorption, influenced fungal development. Surface characteristics modified by sizing, which in turns effects water absorption, are discussed further in chapter dealing with fungal production of pigmentation.

7. Instruments and methods of characterization

7.1 Examination techniques, an overview

The diagnosis of biodeterioration included examination of the substrate (paper) and microorganisms (fungi); therefore the selected techniques had to be suitable for both types of materials. Traditionally both paper fibers and biological specimen are examined with transmitted light microscopy and scanning electron microscopy. In his study fungi and paper morphology was investigated using confocal laser scanning microscopy (CLSM) and white light confocal profilometer. Experimental use of X-ray microtomography was applied in the investigation of fungal and paper interfaces.

Although the same protocol of examination was applied to papers inoculated with *Aspergillus niger* and to the examination of artworks, sampling of artworks was restricted. Considering the limitations of examining the original artworks and strict rules applied to sampling the material, the examination protocol relied on the choices that are permitted in the real life situation. The artworks were examined intact, with minimal sampling allowance, obtained with permission of their owners.

To follow the examination protocol, as outlined in II.1, the instruments and techniques utilized in this study are categorized as optical instruments and analytical laboratory instruments.

The first group of optical instruments included:

- stereo-microscope, SEM-VP
- confocal laser scanning microscope (CLSM)
- white light confocal scanning laser profilometer (CLA)
- X-ray microtomography, ID19 designated beamline

The second group of laboratory instruments included the following:

- environmental monitoring units, Elsec 764 (described in II 8.1.1)
- chemical assays for testing sizing (described in II.6.2.5)
- pH meter (described in II.6.2.5)

Documentation of the tests' results and microscopy was captured with digital cameras:

- macro-imaging, digital cameras: Leica Delux-2 and Nikon D3100 with exchangeable macro-lens Tamron SP Di, 90mm1:2.8 macro 1:1 with UV filter
- digital micrographs were captured with a camera outfitted for microscopes, and used exchangeably between stereo- and transmitted light microscopes

All the samples underwent multivariate analysis in multi-scale, starting from 'global to detail' similarity to approaches in other fields of surface analysis (Mathia et al 2011).

7.2 Binocular microscopes fig. 7.1, 7.3-7.5 H.Szczepanowska

7.2.1 Stereomicroscope, reflected light

Instrument	Specifications and principles of operation	application
	**Fig. 7.1 Stereomicroscope, binocular zoom with 6x-50x magnification range; type: Wild M8 Heerburgg shown here with attached digital camera.** The power source used was Intralux 6000-1, Volpi, Swiss made.	Surface characterization, macro-imaging. Surface characteristics of paper in each set of samples were examined in respect to their finish, unique features, sizing, roughness, absorption and discoloration. The examination with this stereomicroscope provided an overview of the surface characteristics, features such as the size of colonies and distribution of the black pigmented matter and guided the course of further analysis.

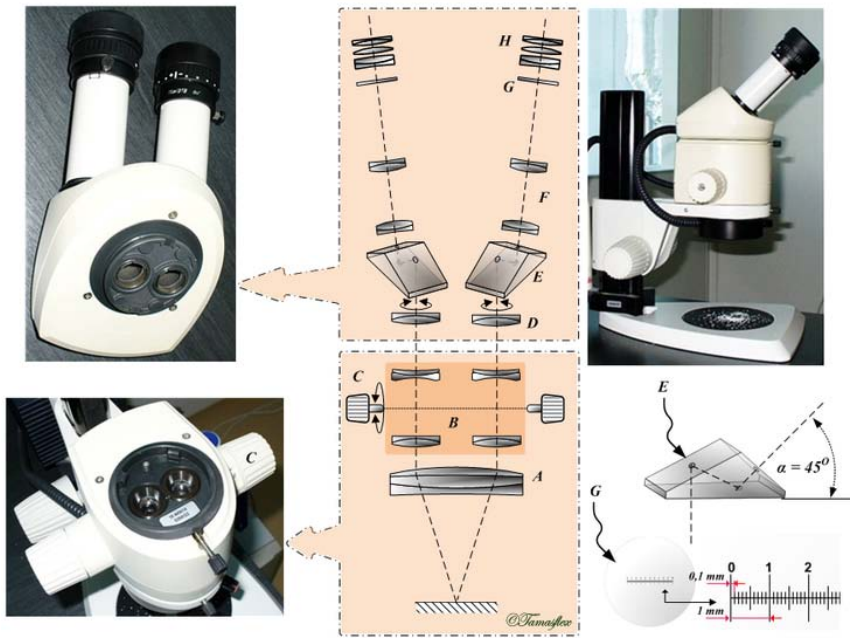


Fig.7. 2 The principle of light path in a stereomicroscope system.

Light is reflected from the surface of an object. It uses two separate optical paths with two objectives and two eyepieces to provide slightly different viewing angles to the left and right eyes. In this way it produces a three-dimensional visualization of the sample being examined. Great working distance and depth of field here are important qualities for this type of microscope.

7.2.2 Transmitted light microscope

Instrument	Specifications and principles of operation	application
 	**Fig. 7.3 Leica DMLM, transmitted light microscope, with 3-gear focus drive, individual torque adjustment and stage height stop.** **Light is transmitted through an object.** Specifications: Leica DM LM, magnification ranges: 50 x, 100x, 200 x and 400x. Focus can be adjusted with the precision of 1um to 4um. Power source: Leica AC volts 0-15. Images were captured with Leica digital camera, attached to the viewing tube; type: Leica EC 3, Microsoft Systems, Heerbrugg. **Fig. 7. 4 Head of Leica DMLM, with quadruple objective nosepiece for bright/dark field examination.**	Morphology of paper fibers and fungal elements was examined. Furthermore, it revealed the structure of biological specimens growing on paper and their configuration in the paper matrix, characteristics of cell walls and distribution of pigments in the bio-mass and the substrate Three different types of transmitted light: bright field, dark filed and phase contrast illumination was utilized. Colonies were measured and their dimensions were recorded on the procured images using millimeters and microns scale bar. Date of the examination, sample's description and magnification power were included on each obtained image.

Fig. 7.5 The Japanese woodblock print was examined on supports that maintained its flatness.

Application of transmitted light microscopy posed a challenged for the examination of artworks which could not be sampled. They required custom-constructed support to carry out that examination.

Transmitted light microscopy, principles of operation

Observations were carried out using three illumination techniques: phase contrast illumination, dark filed and bright light illumination.

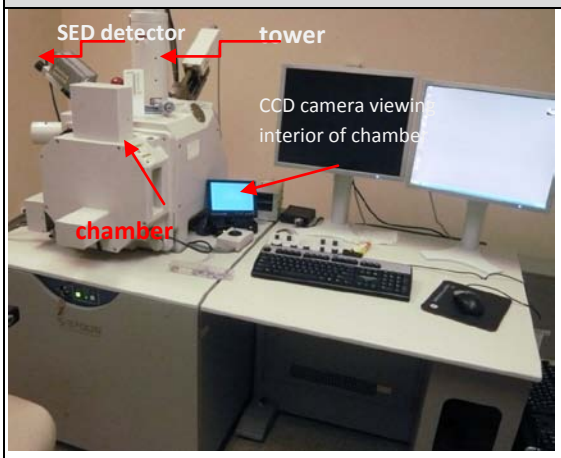
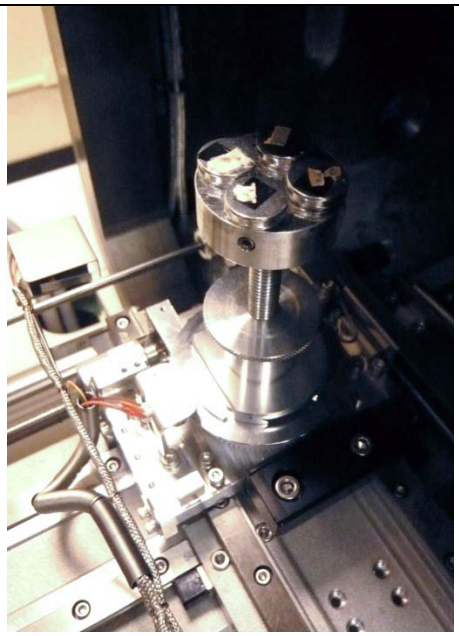
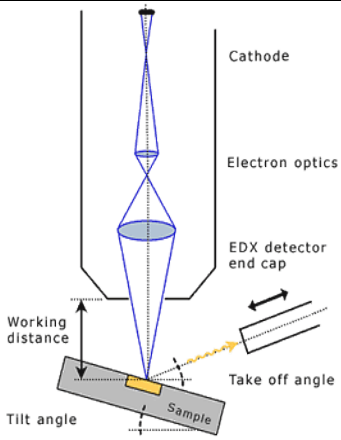
- Phase contrast illumination, sample contrast comes from interference of different path lengths of light through the sample
- Dark field illumination, sample contrast comes from light scattered by the sample
- Bright field illumination, sample contrast comes from absorbance of light in the sample

Phase Contrast Microscopy is specifically designed for examination of living cells and microorganisms. It is a contrast-enhancing optical technique that can be utilized to produce high-contrast images of transparent specimens. The phase contrast technique employs an optical mechanism to translate minute variations in phase into corresponding changes in amplitude, which can be visualized as differences in image contrast. One of the major advantages of phase contrast microscopy is that living cells can be examined in their natural state without being killed, fixed, and stained. Source: http://micro.magnet.fsu.edu/primer/techniques/phasecontrast/phaseindex.html

7.3 Scanning Electron Microscopy (SEM-VP) and SEM with EDS;

fig. 7.6-7.8 H.Szczepanowska.

The electrons interact with the atoms that make up the sample producing signals that contain information about the sample's surface topography, composition and other properties. The micrographs produced in SEM show depth of field accentuating surface characteristics. This SEM was used as SEM—VP meaning in variable pressure (or environmental SEM) in BSE mode. VP permits to use an uncoated sample which shortens the time of examination. Low pressure used in VP does not distort the specimens. In BSE signals are produced by secondary electrons and back-scattered. Backscattered electrons (BSE) consist of high-energy electrons originating in the electron beam that are reflected (or back-scattered) when interacting with the specimens atoms. The electron beam used in this examination had an energy ranging from 10 keV to15 keV. The beam was focused by one condenser lenses and able to measure a spot about 0.4 nm to 5 nm in diameter; it scanned the sample in a raster fashion.

Instrument	Specifications and principles of operation	application
	**Fig. 7.6 SEM-VP Hitachi S 3700N.** SEM-VP was used for imaging of the surface and SEM-EDS for elemental chemical analysis.	SEM-VP permits to examine specimens without coating. Energy dispersive spectrometry is available at this instrument as well. SEM-EDS was utilized for the examination of chemical changes on papers on which A. *niger* was cultured.
	**Fig. 7.7 Scanning electron microscope used in variable pressure (SEM-VP).** Scanning electron microscopy (SEM) uses electron beams, instead of light as in an optical microscope, to scan the surface features of a specimen. The range of magnifications is from 100x to 60,000x.	Sizable interior of the chamber permits inspection of large artifacts. Usually four samples were inserted on the stand for one examination session.
 **Fig. 7.8 a. SEM-VP Hitachi S 3700N, chamber into which the samples were placed for examination.**	Examination conditions: 12 kV to15 kV energy range of the beam; 40Pa pressure BSE mode (back scattered electron).	 **Fig. 7.8. b General relations inside SEM. Source: www.bruker. com**

SEMs typically measure surface topography on a much smaller spatial wavelength scale to stylus and optical instrument, but can be used over relatively large ranges. The main drawback with 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).

7.4 Surface metrology

7.4.1 Confocal Laser Scanning Microscope (CLSM)

Two instruments used in this investigation relied upon confocal optical principle. That was confocal scanning laser microscope, Keyence VK-9700 CLSM and white light confocal profilometer AltiSurf 500. Both were used in the evaluation of surface topography of papers which were stained with fungal deposits. The purpose of that investigation was to determine if the CLSM can be effectively used in the evaluation of biodeterioration.

The principle of confocal microscope relies on using point illumination and a pinhole in an optically conjugate plane in front of the detector to eliminate out-of-focus signal. As only light produced by fluorescence very close to the focal plane can be detected, the image's optical resolution, particularly in the sample depth direction, is much better than that of wide-field microscopes. However, as much of the light from sample fluorescence is blocked at the pinhole, this increased resolution is at the cost of decreased signal intensity, requiring long exposures.

Principle of operation

Confocal Light Microscope	Confocal Laser Scanning Microscope

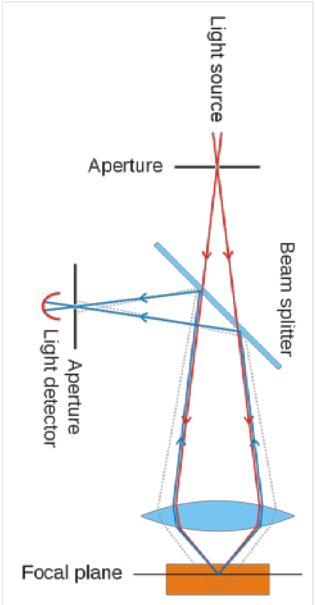
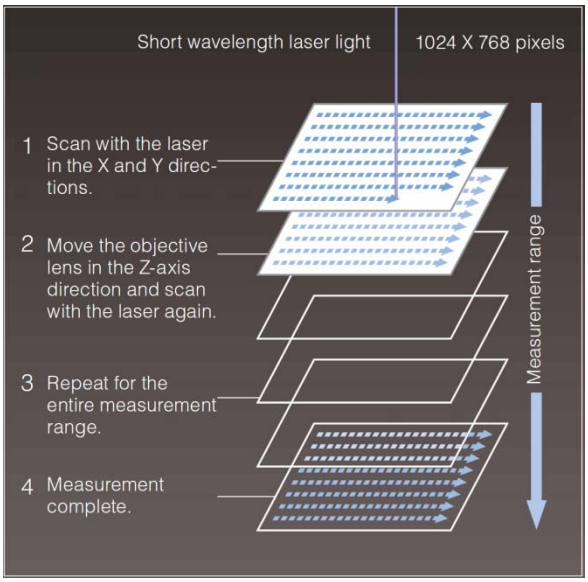

	

Fig. 7.9 Principle of confocal light microscope operation.	Fig. 7.10 Principle of CLSM VK-9700 operation.

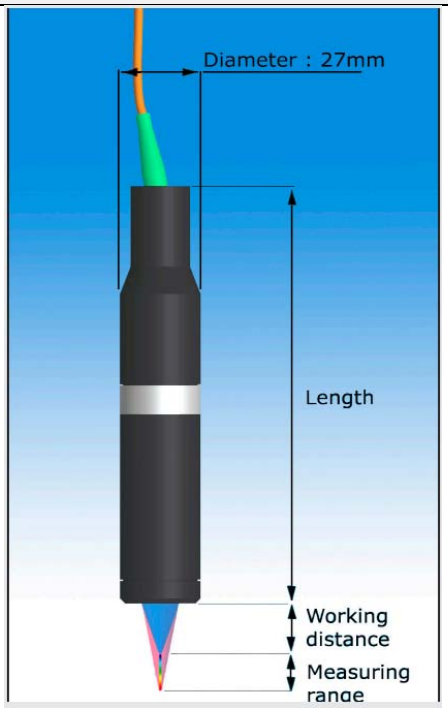
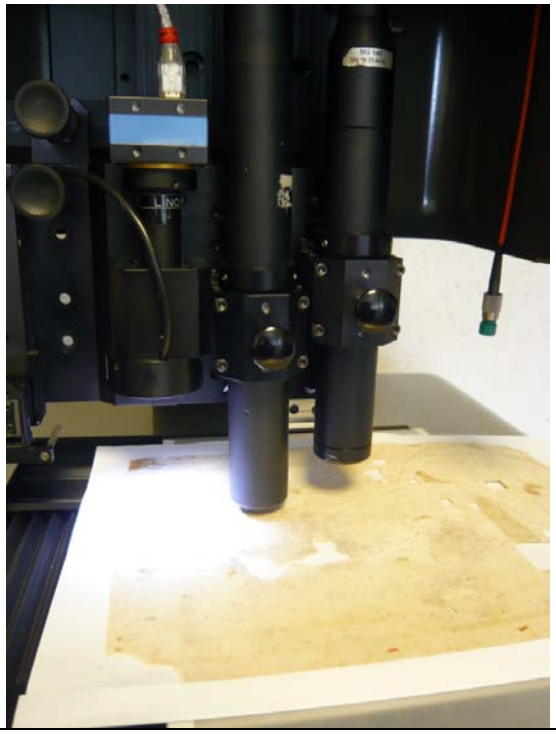
Chromatic confocal probes are single point optical sensors built around a confocal coaxial setting that use chromatic dispersion and decoding to obtain the surface distance. Such sensors are usually installed on scanning stages of surface texture measuring instruments, roundness instruments or coordinate measuring machines. Chromatic confocal probes can measure on, and through, transparent material, detect several interfaces between materials and, therefore, calculate thickness. The metrological characteristics of chromatic confocal probes are close to those of stylus probes and they are often used as a non-contact substitute on stylus profilometers (Blateyron in Leach 2010).	The VK-9700 scans across a target in the X and Y directions using a laser with a 408 nm wavelength, and a light receiving element gathers data from the reflected light on a pixel per pixel basis. After completing the scan in the XY-plane, the lens is driven in the Z-direction and the scan repeats for the remainder of the measurement range. From this information, the VK-9700 is able to construct a high-resolution, fully-focused image with height information associated with each pixel.

7.4.2 Instruments for measuring surface topography

Instrument	Specifications and principles of operation	application
 **Fig. 7. 11 Keyence color 3D Laser Scanning Microscope, VK-9700 Model.**	3 types of laser are available: red, He-Na and violet; only one can be installed in an instrument however. Red laser was used in this investigation. Magnification 200x-18000x; 1000x was used. Area: x-y 2048 x1536 pixels. The VK-Analyzer software can measure the height, width, cross-section, angle, or radius of curvature or curved cross-section profile. Using short-wave lasers and pinhole optics, a lateral resolution of 0.13 µm 0.01 Mil can be obtained.	3D surface topography of paper was measured to evaluate its roughness and generate profile. Both sides of each paper were measured, with and without fungal deposits where it was possible.

 Fig. 7.12 Confocal white light profilometer (CLA), AltiSurf 500.	The surface topography is measured in x, y and z direction based on confocal chromatic imaging principle.	The surface is measured with a light sensor eliminating physical contact with examined material, making it particularly appealing for applications to cultural heritage objects, as seen on Fig.7. 12.

7.4.3 White light confocal laser profilometer, (CLA) AltiSurf 500.

Instrument	Application
 Fig. 7. 13 Diagram of a stylus used in the White Light Confocal Profilometer AltiSurf500 (CLA). Source: www.altimet.fr	 Fig. 7.14 The 17the c Study Sheet, measurement of black fungal spots with AltiSurf500.
Spot size 2µm, area measured in most samples: 3mmx3mm, spacing between scans: 1µm.	The advantage of this instrument is its ability to measure the intact object, without the need to procure 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) in the specimen. The achievable thickness of the focal plane is defined mostly by the wavelength of the used light divided by the numerical

	aperture of the objective lens, but also by the optical properties of the specimen. The thin optical sectioning possible makes these types of microscopes particularly good at 3D imaging and surface profiling of samples.

7.5 X-ray microtomography, ESRF Grenoble, France

X-ray microtomography was carried out on electron beam synchrotron, designated beam ID 19, at the European Synchrotron Facility ESRF in Grenoble, France.

Fig. 7.15 ESRF facility in Grenoble, France. The examination was carried out on a designated beam ID 19.

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, and diffraction imaging (topography, analyser-based imaging) experiments. The beamline can work in the energy range 6 to 100 keV, but most of the experiments are performed in the 10-35 keV range. The monochromatic beam can either originate from a double Si 111 crystals monochromator ($\Delta E/E\ 10^{-4}$) or a state of the art multilayer ($\Delta E/E\ 10^{-2}$).(www.esrf.eu)

X-ray absorption tomography has been used during the past several years to characterize the architecture of cellular materials of different nature. However its application to the characterization of biodeteriorated cultural material is a novel approach. Ideally the best way of analyzing the architecture in the modeling of cellular materials is through the use of the three-dimensional information provided by the new tomographic techniques to produce a finite element model of the actual architecture. The general principle of the tomography technique relies on an X-ray source, employs a rotation stage and a radioscopic detector. A complete analysis is made by acquiring large number (in his case ca 1500) X-ray absorption radiographs of the same sample under different viewing angles. A final computed reconstruction step is required to produce a three dimensional map of the local absorption coefficients in the material which gives, indirectly, a picture of the structure. (Fig. 7.16, 7.17)

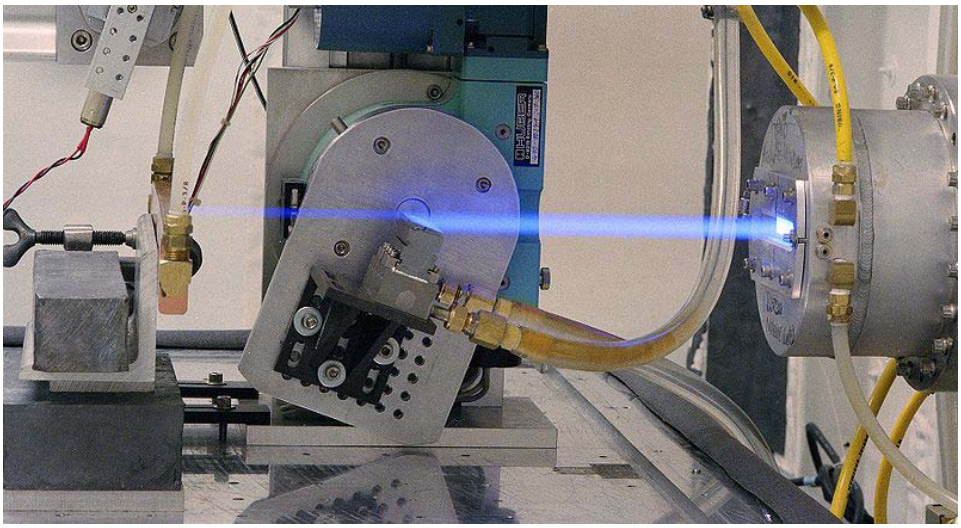


Fig. 7.16 Beam directed to the sample from the right and captured (left side of this image) by a radioscopic detector CCD FReLeon camera.

Fig. 7.17 Set up on the examination stage in ID19 where the samples were analyzed.

Parameters of the measurements on November 7, 2011 were as follows:
The 6 MeV electrons produced hard X rays, next monochromated at 30 keV with a double Si single crystal. The absorption images were collected using a fast read out 2048*2048 CCD camera. Each pixel represents 0.7 µm. The images included in this report were generated from 300 slices, which is an equivalent of 21µm.

Samples preparation (see details in Appendix: ESRF Samples Preparation Protocol):

1. Sample size: 1mm x 1mm
2. Sample is attached onto a sticky side of paper-sticky
3. Sticky paper is attached to a glass capillary
4. Capillary with a sample is mounted into the stand shown in fig. 7.17.
5. Stabilization of samples in a monitored RH environment, at T 21^{o}C prior to analysis in synchrotron.

Table VII Sample preparation for X-ray microtomography

Fig. 7.18 Cutting out 1mmx1mm paper samples.

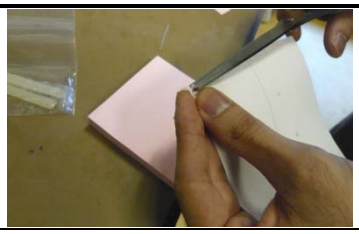


Fig. 7.19 Mounting the sample to a self-adhesive on sticky -note.

Fig. 7.20 Sample attached to a glass capillary with wax.

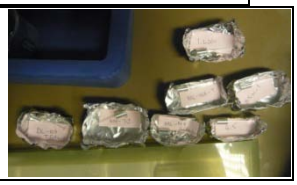


Fig. 7.21 Samples ready for RH conditioning.

7.6 Summary points

1. The selection of instruments for the examination was dictated by the limitation posed by cultural heritage material from which sample material often cannot be extracted. Non-destructive techniques, which allow examination in-situ, can be applied in a real life situation. Techniques such as SEM and X-ray tomography require very small amount of materials for diagnosis providing wealth of information from a very small amount of material.
2. Examination from macro-to micro is essential for proper interpretation of micrographs and radiographs. It is especially important for the interpretation of monochromatic mages which do not differentiate particles in color.
3. Multitude of analytical techniques used in this investigation revealed information that complemented each other.

8. Light effect on fungal growth and pigmentation (Light Experiment)

8.1 Fungal stains production

Objective of this experiment was to observe impact of light on pigments production and resulting modification of the paper surface. Pigmented black stains, similar to the ones occurring naturally, as powdery, colored conidia and liquid-like secretions to substrate were produced on paper by culturing one know microorganism (*Aspergillus niger)* on two different types of papers (Whatman 4; W4 and Winsor&Newtown; NW) and in two light conditions, daylight (DL) and dark (ML). Interfacial relationship between bio-mass and substrate, surface and bulk of paper with induced biodeterioration were investigated with various analytical methods. (fig.8.1)

The experiment was run four times, in two and three parallels. (Table VIII)However, only the first trial was successful in that the expected black pigment was produced undisturbed with minimal contamination. The other three trials were contaminated with Actinomycetes, a microorganism producing antibiotic which halted development of *A. niger.* (Appendix IV: Light Experimental).

8.1.1 Experiment protocol

Development of a systematic methodology was the first part of this experiment; it followed the outline:

I. Selection of materials; suitable fungal strains and two papers of different composition
II. Experiment design:
   a. Inoculation of paper samples
   b. Environmental monitoring (visible, ultraviolet light and temperature)
   c. Daily data collection during a 10-days culturing period (optical and microscopic imaging)
III. Analytical evaluation of biodeterioration (various analytical instruments)
IV. Data processing, analysis and conclusions

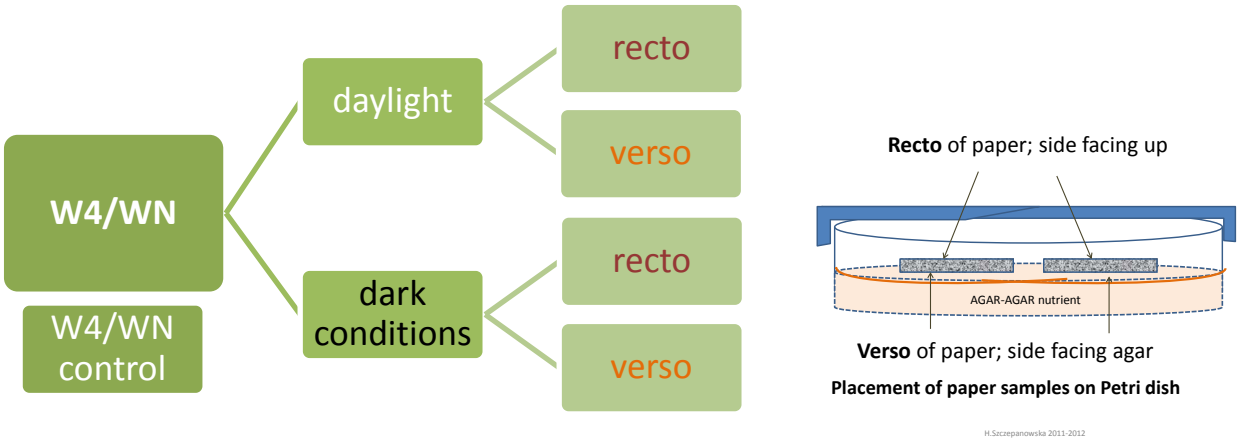


Fig. 8.1 A schematic layout of the Light Experiment. Surface modifications resulting from fungal growth and pigmentation were evaluated within each paper set and between paper sets (W4 and W&N). Diagram on the right illustrates concept of 'recto' and 'verso' of paper samples.

8.1.2 Materials and methods

1. Strain selected :
   Asperigillus niger was obtained from Carolina Biological Supplier, grown on Agar-Dextrose culture. (Fig. 8.2 and 8.3)
2. Paper Substrates
   a. Whatman No 4
   b. Watercolor paper, Winsor & Newton
3. Procedure: (fig.8.4)
   a. Sterilization of paper prior to inoculation
   b. Paper samples were transferred onto agar-agar media with sterilized tweezers
   c. *Aspergillus niger* was inoculated on each pale, between two paper samples, (fig.8.5).
4. Light was monitored with Elsec 764 environmental units in both locations in 10 min intervals. (fig.8.6)
5. Photographic records of growth, macro and microscopic images were collected in 24 hrs intervals. (Appendix, Light Experiment, contains full set of images).
6. Observations on progress of growth and pigmentation were noted on prepared note-sheets with date of the observation and light conditions. (Appendix: Light Experiment, Notes)
7. Interactions between bio-mass and paper fibers were analyzed with an array of instrumental methods.

Note: ***Aspergillus niger was*** selected because it is known to produce very definite, black pigmentation (Jorgensen 2011, Liakh S.P., Lysenko S.V.1978, Carley, 1967).

Fig. 8.2 and 8.3 Aspergillus *niger Source*: Caroline Biological Supplier of Pure Culture; grown on Potato Dextrose Agar, 25˚C. 8.2: Top view of the culture. 8.3: View of the culture plate in transmitted light illustrating intensity of the black pigmentation, characteristic for *Aspergillus niger.* ©H.Szczepanowska 2011.

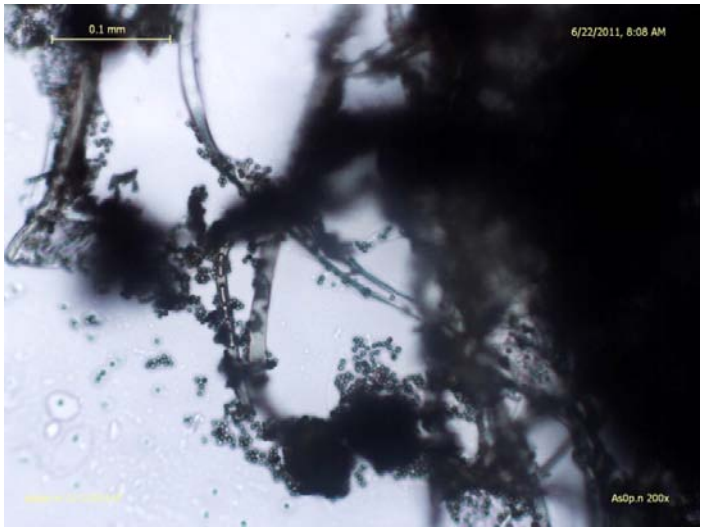


Fig. 8.4 Aspergillus *niger*, black pigmented spores. Most of the black spores are attached to a sporangium others are scattered. Transmitted light, 200x, sample procured from the culture plate. Scale bar: 0.1mm

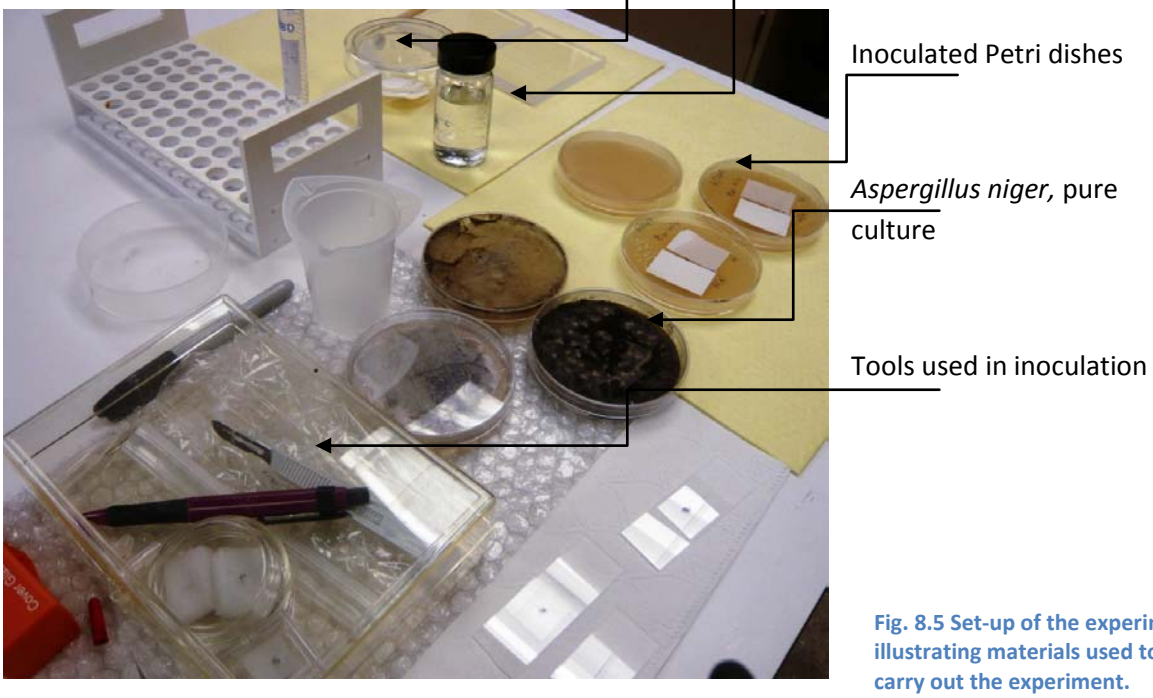


Fig. 8.5 Set-up of the experiment illustrating materials used to carry out the experiment. ©H.Szczepanowska

Fig. 8.6 Petri dishes with paper samples were inoculated with *Aspergillus niger,* along one side, in the center, between the paper samples, marked here with red oval.

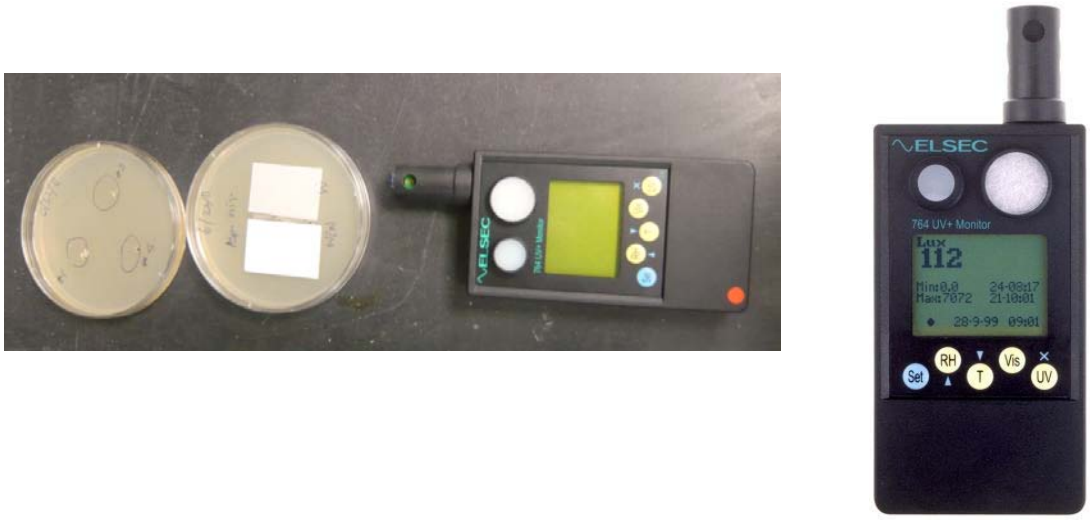


Fig. 8.7 Plates were located in two different light conditions - one on a window seal, exposed to daylight and the other one in dark room. Environmental parameters, temperature, light and humidity were monitored with Elsec 764 units, on 10min intervals.

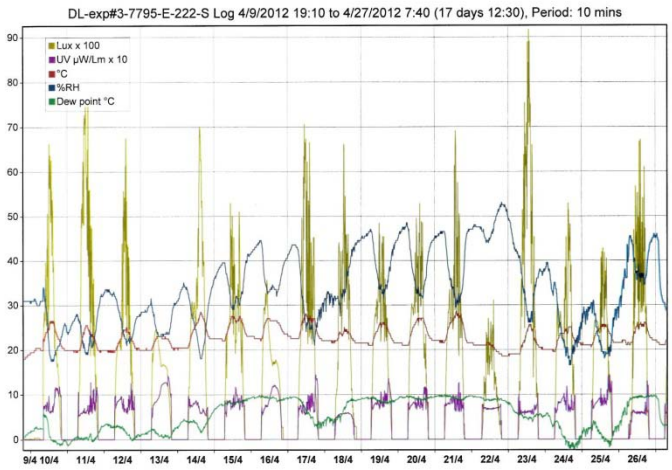


Fig. 8.8 The environment was monitored through the duration of all four experiments. This plot indicates external temperature in daylight conditions in April 2012.

Av.25 T ^{o}C (red), relative humidity (av.44 %RH-blue), visible light (0-900 Lux-green) and UV light (20-28 µW/L-purple)

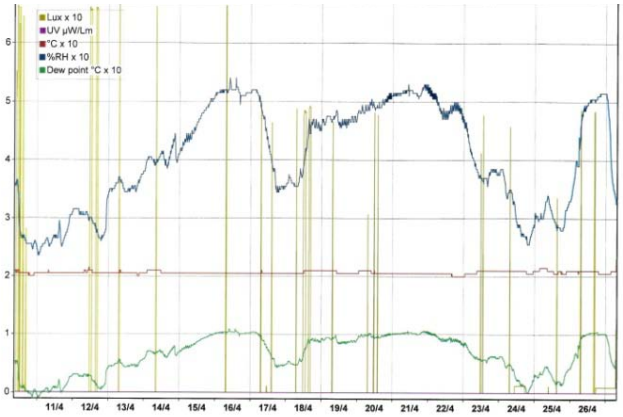


Fig. 8.9 This plot shows condition of the environment monitored for cultures in dark.

External temperature was stable at 20° C (red), RH 25%-55% (blue), no visible or UV light, besides momentary spikes when images were taken. Monitoring period: April 2012. (Records collected during each experiment are included in Appendix: IV Light Experiment)

Table VIII Summary of experimental repeats and fungal pigments production in cultures in four trials

Exp.No/date	1: June 22-July 5, 2011	2:April 10-20, 2012	3:April 17-28, 2012	4:April 30-May10, 2012
Period of observations	13 days	10 days	11 days	10 days
Parallel repeats	DL-1 plate ML-1 plate	DL-3 plates ML-3 plates	DL-2 plates ML-2 plates	DL-2 plates ML-2 plates
Pigmentation	*A.niger* black pigment : DL and ML	DL1:Actinomycete, yellow pigment DL 3: Actinomycete, white pigment	ML4: Actinomycete, yellow pigment	DL 7 : Actinomycete, yellow pigment

Note: Contamination by Actinomycetes in experiment No 2 through No 4 became apparent after 2-3 days of cultivation. Actinomycetes produce antibiotics therefore it halted growth and production of *Aspergillus niger*. Pigmentation of paper therefore was studied on set of samples obtained from experiment No 1 only. (Details, see Appendix IV. Light Experiment)

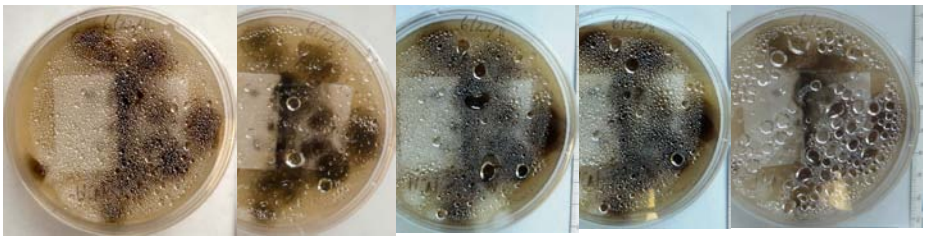
8.1.3 Pigmentation produced under daylight and in dark conditions

Growth of colonies and ratio of pigmentation were more rapid in the first 5 days on plates exposed to daylight than on plates cultured in dark. Between the 5th and 8th day growth was moderate and after that colonies reached a plateau in daylight colonies; pigmentation continued to intensify on pates in dark condition. Comparison of the last phase of growth in both sets, see fig. 8.7 and 8.8. Intensity of pigmentation produced in cultures in dark reached the same level as those cultured in daylight after 8 days from inoculation; see results in graph, fig.8.12.

Time factor versus growth in two different light conditions affected qualities of aerial mycelium as follows:

- **Daylight:** colonies grew rapidly, reached peak in 5-6 days, produced loose, airy aerial growth.
- **Dark:** colonies grew slowly at a lower rate, produced more compact aerial mycelium and more dense pigmentation. Growth and pigmentation continued through the duration of the experiment, 13 days.
- Pigment secretion into substrate was more intense in daylight cultures than in dark- grown.
- Whatman4 paper supported abundant and rapid growth and pigmentation in daylight and dark. Winsor&Newton paper supported minimal growth in both light conditions.

Day Light exposure, days of observations: 6 through 13;
6/28/ through 7/5/2011

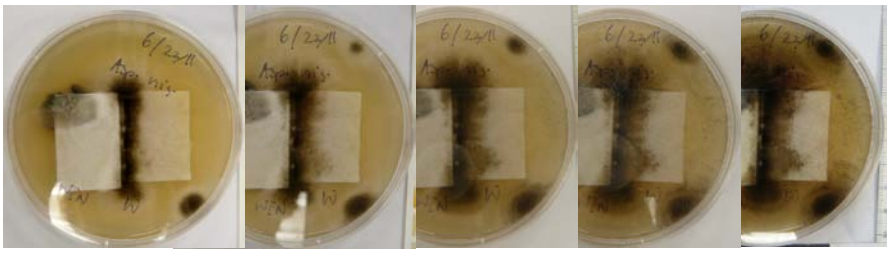


Day 6; 6/28/2011 Day 7; 6/29/2011 Day 8; 6/30/2011 Day 9; 7/1/2011 Day 13; 7/5/2011

Extensive growth of colony appeared to have stabilized after day 5 and not much expansion was observed through the remaining 5 days. Accumulation of water condensation inside the plate increased and changed the pattern; droplets were larger than during earlier observations.

Fig. 8.10 Growth and pigmentation on W4 and WN in daylight. W4 is on the right side in Petri dish, WN on the left side. Presence of water inside the Petri dishes plates cultured in daylight most likely contributed to growth of fungus.

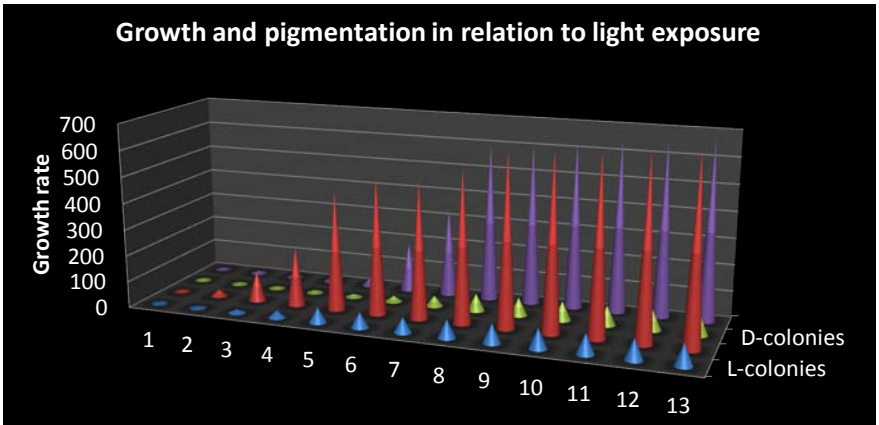
Minimal Light exposure, days of observations: 6 through 13;
6/28/ through 7/5/2011



Day 6; 6/28/2011 Day 7; 6/29/2011 Day 8; 6/30/2011 Day 9; 7/1/2011 Day 13; 7/5/2011

A progression of growth was observed, in addition to pigment formation. There was no water condensation inside the plate.

Fig. 8.11 Growth of *A.niger* in dark condition in the last phase of culturing (5 days). Note the intensity of pigmentation that was increasing until the last day of observations.



	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

Fig. 8.12 Graph illustrating colonies expansion and pigment production in cultures grown in daylight and in dark. Red plot indicates pigmentation produced in daylight, purple, pigmentation produced in dark; green and blue, expansion of colonies in light and dark respectively. ©H.Szczepanowska

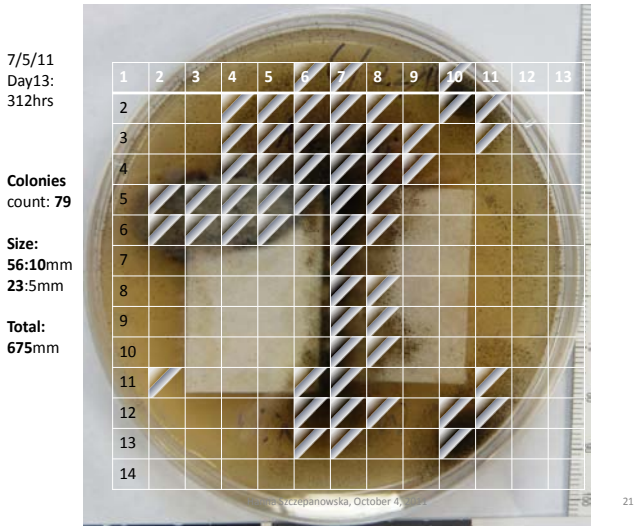


Fig. 8.13 An illustration of the method of calculating colonies expansion and pigment production . Grid was overlaid on each plate, each square representing 10mm.

Those squares which were filled completely counted as one, 10mm-size colony, those which were partially filled, counted as 5mm colonies. This calculation is an approximation only. Numerical data was plotted in Excel program. The resulting plot is showed in fig. 8.12. (Appendix: Light Experiment) ©H.Szczepanowska

8.2 Surface modifications of paper by fungi cultured under light and in dark conditions

Fig. 8.14-8.18 H.Szczepanowska 2011

Whatman 4 (W4)	Daylight, recto (W4-DL))	Daylight, verso

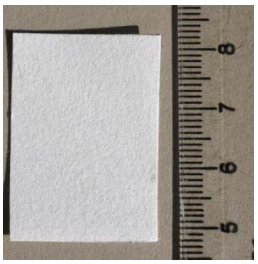
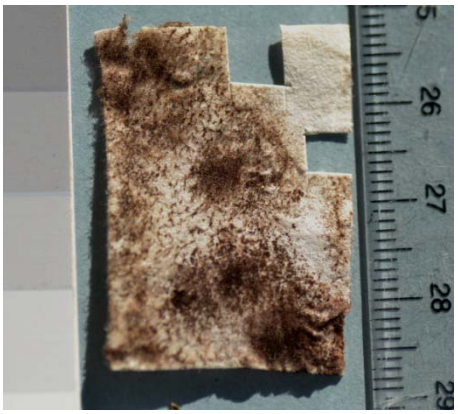

	

Fig. 8.14 Control sample of W4 before culturing of fungus.	Fig. 8.15 Colonies cultured in daylight are loosely arranged on the surface that was exposed up (to light). Pigmentation is intense and scattered over the entire surface.	Fig. 8.16 Light pinkish pigmentations migrated to verso. Intensity of stains corresponds to location of colonies on the other side.
	Dark conditions, recto (W4-ML)	**Dark conditions, verso**
		
	Fig. 8.17 Colonies grown in dark are compact and pigmentation densely deposited on the surface of paper. Pigmentation is on the side exposed to light.	Fig. 8.18 Light pinkish pigmentations migrated to verso. No correlation between intensity of stain to location of colonies on the other side. Minor growth observed on verso.

Fig. 8. 19-23 H.Szczepanowska

Winsor&Newton	**Daylight recto (WN-DL)**	**Daylight verso (WN-ML)**

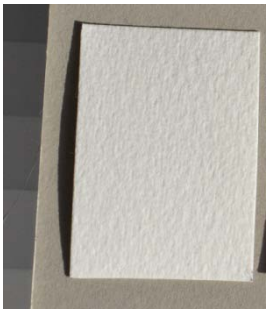


Fig. 8.19 Control sample of WN paper before exposure to fungal biodeterioration.

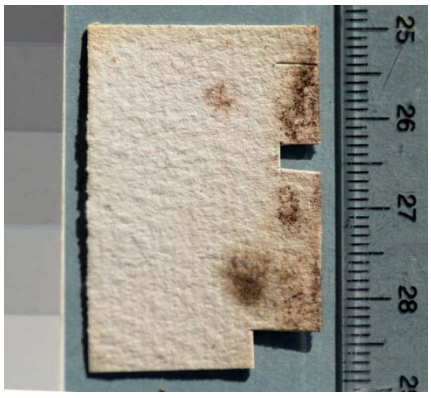


Fig. 8.20 Colonies grown on recto, the side exposed to 'dark' were sparse and grew slowly.

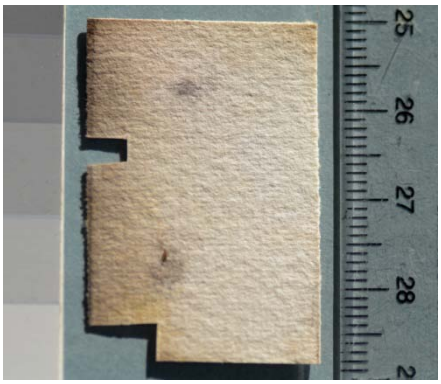


Fig. 8.21 Verso of paper, side facing agar gel. Pigmentation was secreted to paper in areas where colonies grew on recto. Two dark stains corresponded to the growth of colonies on rect.

Dark conditions, recto

Fig. 8.22 Mycelia growth of colonies cultured in dark was smaller than on paper exposed to light. Large black area at the top was caused by contamination.

Dark conditions, verso

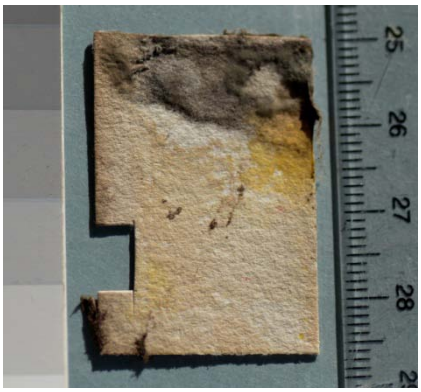


Fig. 8.23 Pigmentation by inoculants *A. niger* was minor. Yellow staining was produced by Actinomycetes.

Paper was affected structurally by fungal growth. That became evident once the samples were removed from culturing plates. That observation has far reaching implications in practical situations, for example during rescue operation of paper-based collections after flood or suppression of fire, which also involves wetting the materials.(fig.8.24; 8.25)

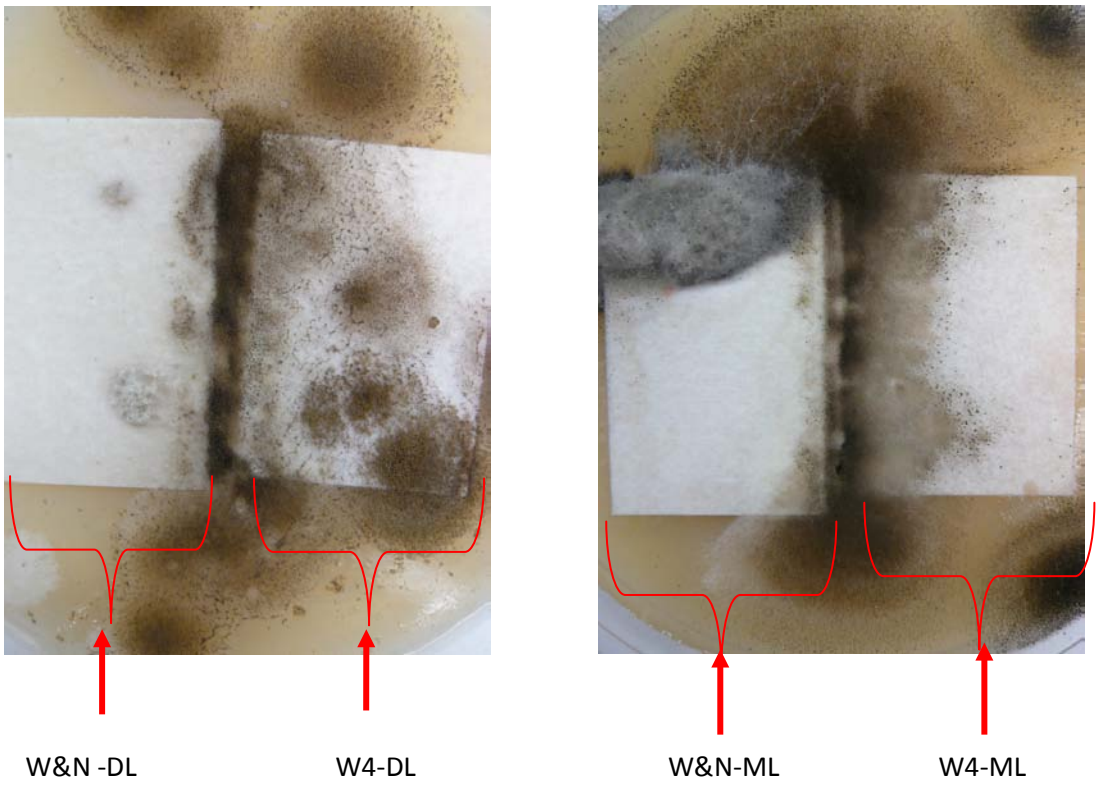


Fig. 8.24 and 8.25 View of paper samples immediately after termination of the experiment and opening of the cover lid on Petri dishes.

Figure 8.24 shows pigmentation produced under daylight condition; fig. 4.23, , pigmentation produced in dark conditions. The W4 paper with intense growth and pigmentation was very weak when removed from Petri dish at the termination of the experiment. Fibers of paper separate from bulk and remained attached to agar- gel used in Petri dishes.

Optical microscopy of pigmented surfaces revealed small number of contaminants on WN paper that grew in addition to the inoculant *A. niger.* That is why small colored stains, yellow and red, were detected on paper. Further investigation of colored deposits revealed morphological features of Actinomycetes. (Appendix: Light Experiment, Microorganisms Morphology)

Transmitted light microscopy examination was carried out on all samples. The most significant changes of surface and damage to fibers were observed in SEM-VP therefore only those examples are included here. A complete set, see Appendix: Light Experiment, Optical Microscopy. Interface of fungal deposits was captured with non-contact white light profilometer and X-ray micro-tomography carried out on synchrotron, at the European Synchrotron Radiation Center, Grenoble, France.

8.2.1 Surface morphology of papers with bio-deposits; Fig. 8.26-8.30. H.Szczepanowska 2011

W4 daylight (DL), recto

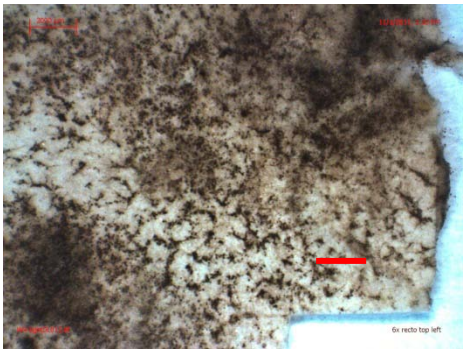


Fig. 8. 26 Pigmentation and pattern of particles deposition on papers with daylight cultures is more intense in comparison with colonies cultured in dark condition. scale bar : 2mm

W4 dark conditions (ML), recto

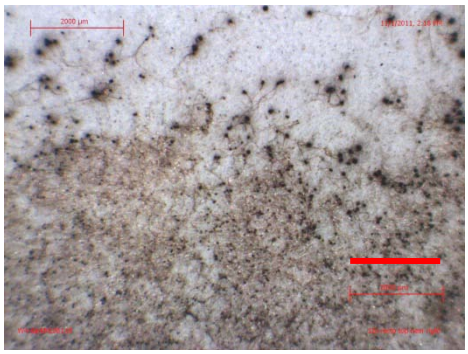


Fig. 8.27 Intensity of pigment produced in colonies cultured in dark was much lesser that that produced in light. Distribution of pigmentation was more even on the surface. scale bar : 2mm

W4-C (control)

Fig.8.28 Fibers are well defined and arranged on a control sample of W4. No additives are present. SEM-VP micrograph scale bar: 40 µm.

W4-DL recto; SEM-VP

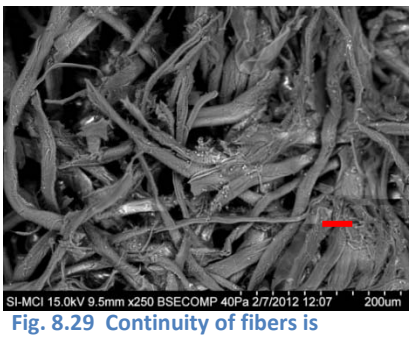


Fig. 8.29 Continuity of fibers is interrupted by chemical damage caused most by fungal growth that led to mechanical damage of paper fibers. scale bar: 40 µm

W4-ML recto

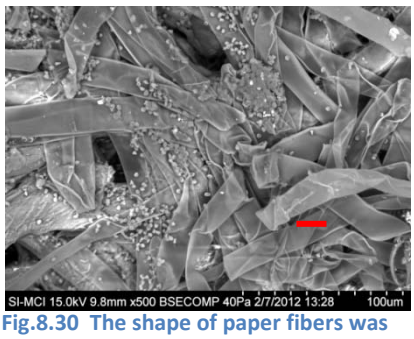


Fig.8.30 The shape of paper fibers was altered as a result of fungal activities. They appeared to be flat and collapsed. Fungal propagulates (spores) are deposited among fibers. scale bar: 20 µm

8.2.2 Surface topography was measured with optical, non-contact, white light confocal laser profiler; AltiSurf500; Fig.8.31-8.33. © H.Szczepanowska

W4-C

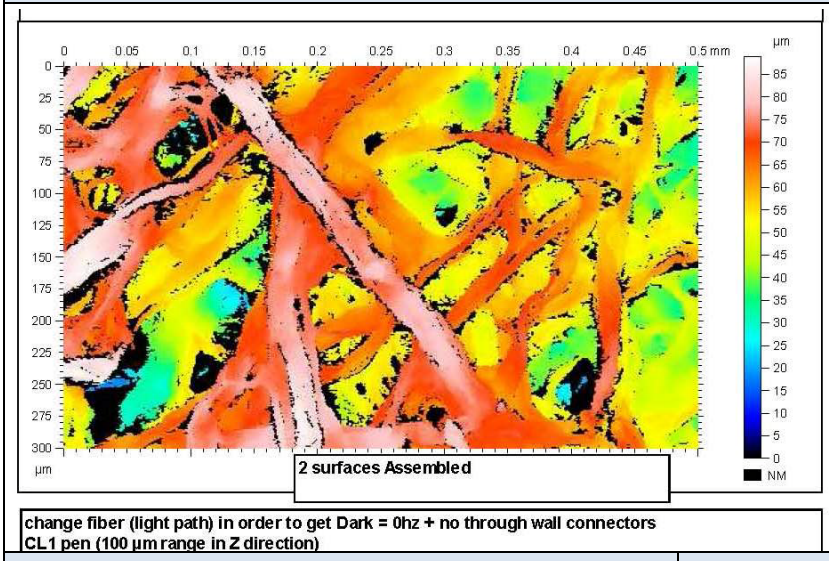


Fig.8.31 On the control sample of W4, fibers are well defined with no sign of any residue. Area measured: Y:300µm X: 0.5mm Z: 85 µm

W4-DL

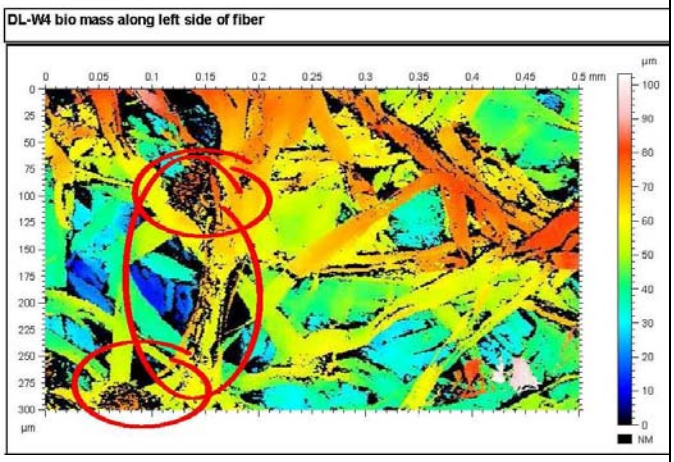


Fig. 8.32 Fungal residue is visible as dark areas, 'voids' of data. Location corresponds in both cases (DL and ML) with location and shape of fungi on target-image.

W4-ML

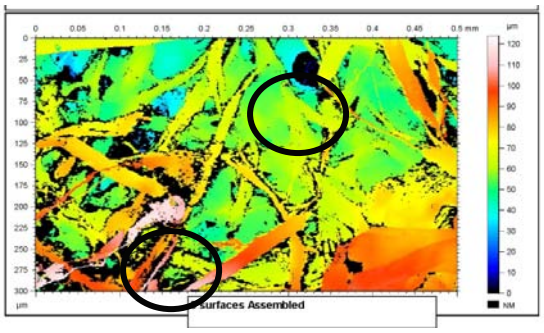
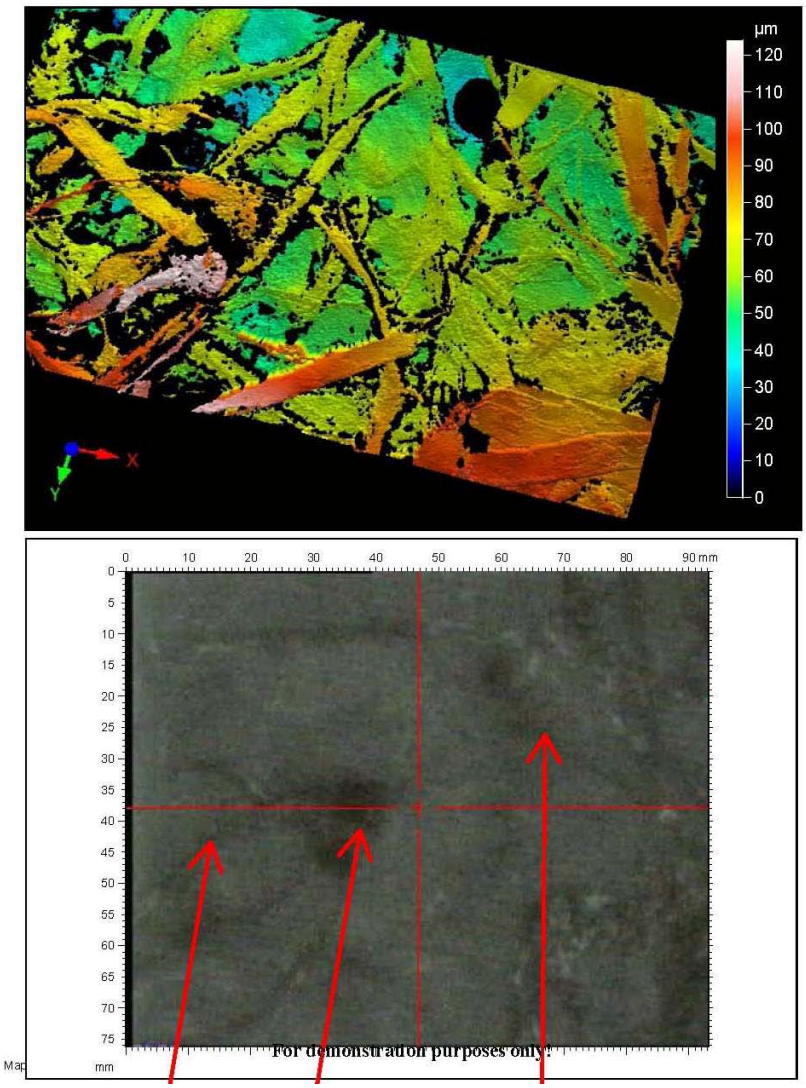


Fig. 8.33 Shape of 'voids' areas corresponds with the shape of fungi visible on the paper surface, as seen on fig. 8. 32.

Measurement of upper fungal formation: X:0.115-0.06mm; Y: 97-96 µm; Z: 76.21 µm

Corresponding image and 3D map see fig. 4.32

Fig. 8.34. W4-ML, 3D topography and corresponding location and shape of fungal residue captured with target camera. © H.Szczepanowska



A meaningful interpretation of the results requires familiarity with the sample characteristics and repetitious measurements to ensure that the target topography is properly measured and visualized.

The same protocol of investigation was applied to Winsor& Newton papers with fungal deposits. Even though the fungal colonies were much smaller and in much lesser numbers that those which grew on Whatman 4 paper their composition was more diverse, indicating that most likely contaminants grew side-by side with the fungus of interest, *Aspergillus niger.*

WN daylight (DL) recto	WN dark condition (ML) recto

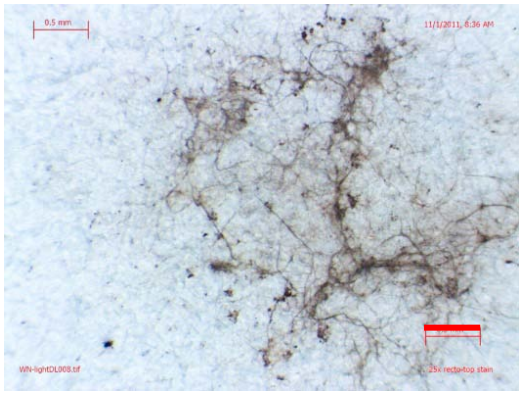
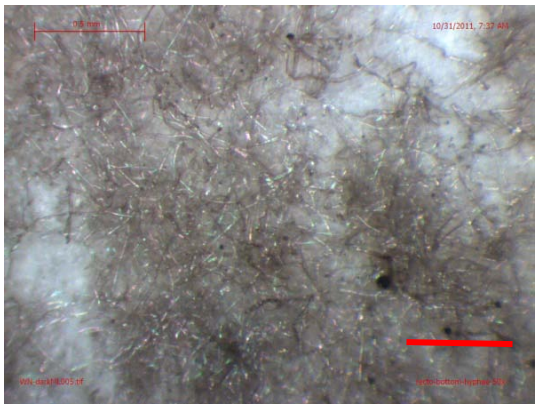

	

Fig. 8.35 Sparse fungal aerial growth in top stain on the sample, one of two present on recto of Winsor&Newton paper with colonies grown in daylight. Scale bar (red, bottom): 0.5mm	Fig. 8.37 Evenly distributed aerial mycelium without fruiting structures was observed on WN with colonies grown in dark; bottom part of the paper sample. scale bar (red, bottom): 0.5mm.

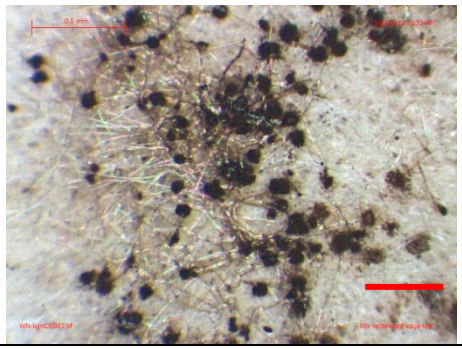



Fig. 8.36 Another type of aerial growth, bearing fruiting structure, in a lower part of the sample. Scale bar (red, bottom) : 0.5mm.	

WN-C (control)	WN-DL recto	WN-ML recto

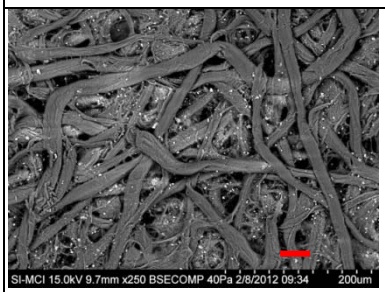
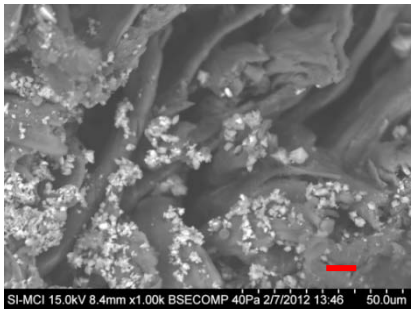
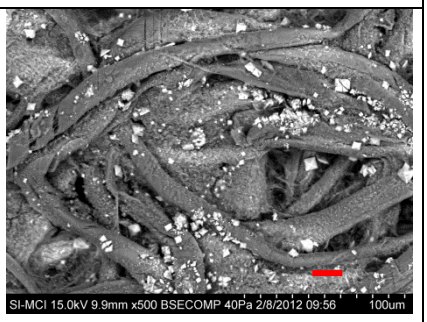

		

fig. 8.38 Fibers are well defined on Wn control. Additives, sizing and calcium-based fillers are visible as scattered particles. SEM-VP micrograph. Scale bar: 40 µm	Fig.8.39 Additives were altered by fungi into clumps of crystal-like deposist. Chemical analysis , see Appendix: SEM-EDS Analysis Report. Scale bar: 10 µm.	Fig.8.40 Additives were altered by fungi into defined crystal-like formations. Actinomycetes spores were present in the area of yellow stains. Scale bar: 20 µm

Chemical composition of WN papers was altered by fungi growing under light and in dark conditions (WN-DL and WN-ML). SEM-VP indicated presence of well defined crystal of calcium in samples of WN on which fungi grew. Further investigation using SEM-EDS, (SEM- equipped with electron dispersion spectroscopy EDS) confirmed presence of calcium and iron in DL and primarily calcium in ML. Fig. 8.39-8.44. Complete report of the analysis, see Appendix V: SEM-EDS Analysis Report. (Fig. 8.41-8.44 © H.Szczepanowska.)

WN-C

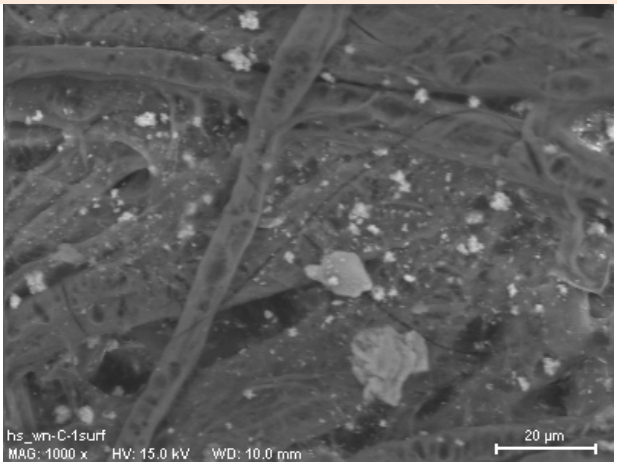


Fig. 8.41 WN-C before paper was subjected to fungal growth. Filler is calcium carbonate according to the WN manufacturer. scale bar: 20 µm.

SEM-EDS

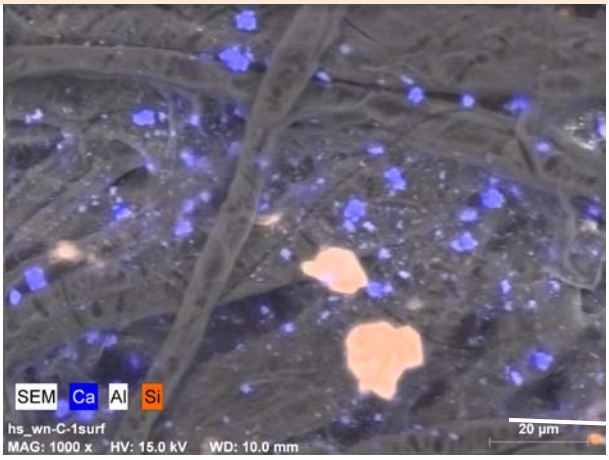


Fig.8. 42 SEM-XRD elemental analysis confirmed the chemical composition of calcium compound. scale bar: 20 µm

WN-DL; SEM-EDS

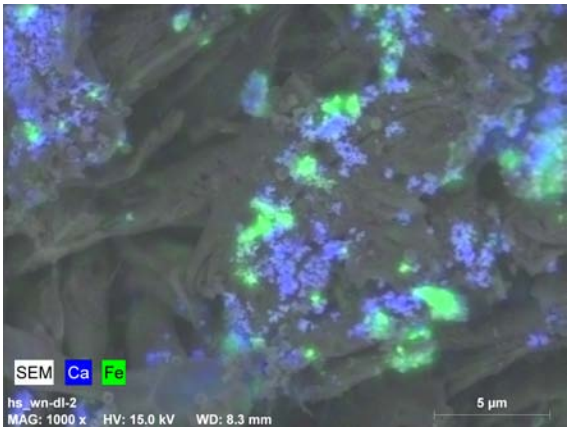


Fig. 8.43 Scale bar: 5µm. Calcium oxalate crystals congregated into clusters of different formations that those produced in dark.

WN-ML; SEM-EDS

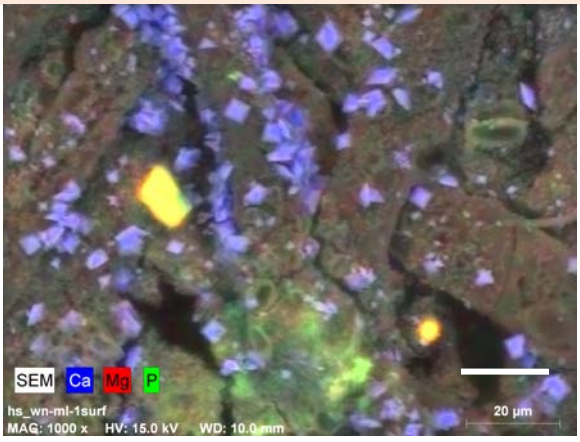


Fig. 8.44 Scale bar: 20 µm Well defined crystals of calcium visible on surface. Their presence was observed in X-ray tomography.

8.2.3 Interface of microorganisms and substrate with X-ray microtomography

Further investigation of surface modification by fungi utilized X-ray electron beam synchrotron tomography in ESRF, a designated beam line ID19 was used.

Investigation of biodeteriorated paper, overgrown with fungi using X-ray tomography has not been done before. Control samples of W4 and WN were imaged as well to use as base line for comparative purpose. Complete set of measurements is included in Appendix: X-ray tomography imaging. One representative set has been selected using the above technique. Fig. 8.45-8.47 ©H.Szczepanowska.

W4-DL; surface microscopy	W4-DL, X-ray micro-tomography

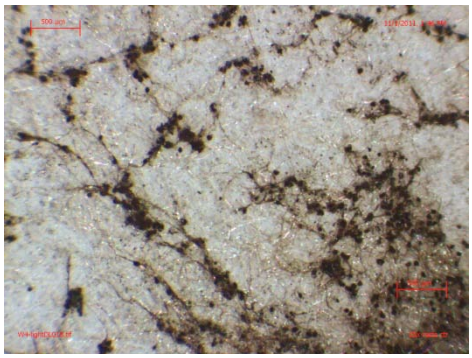
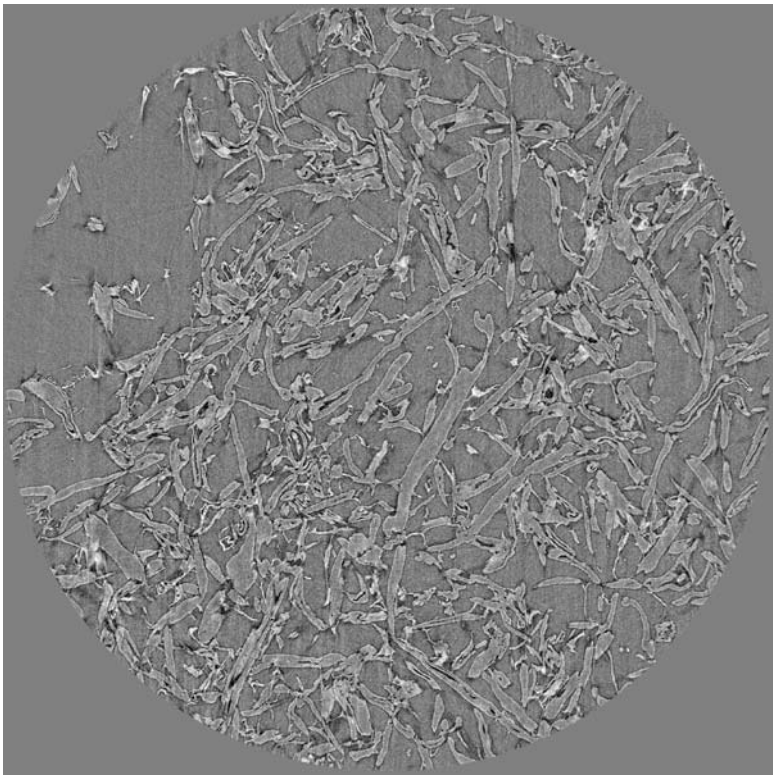

	

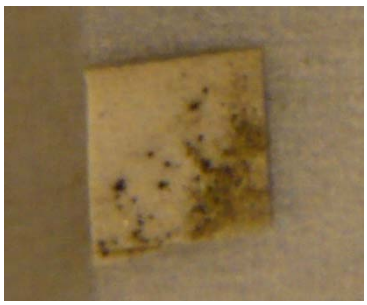
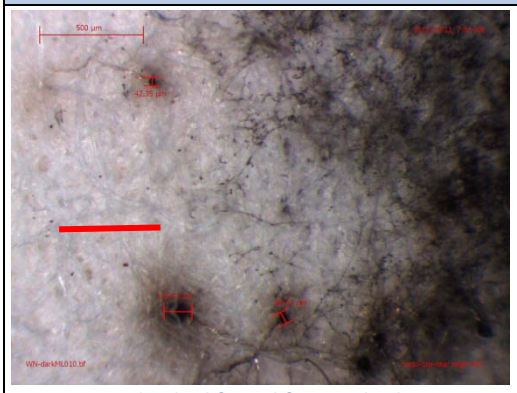
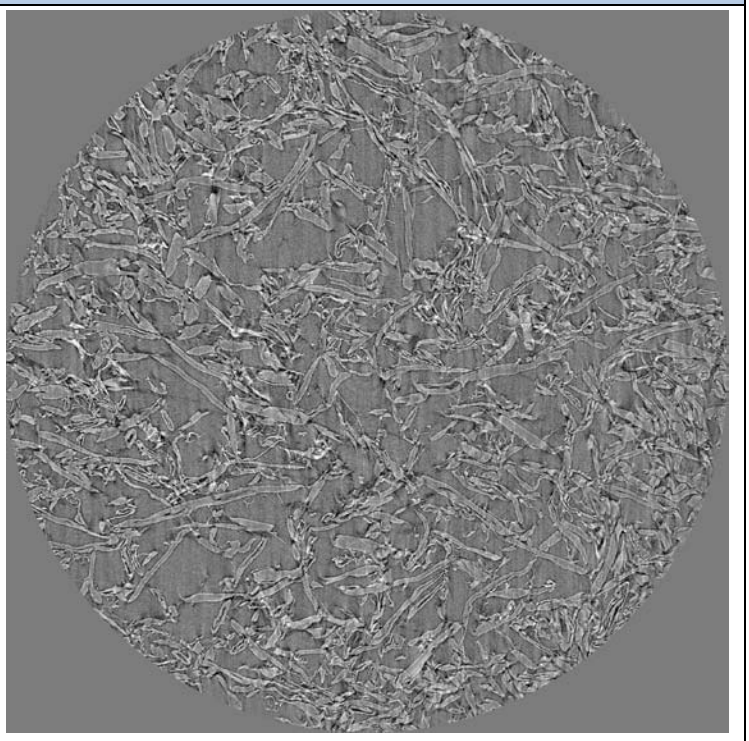
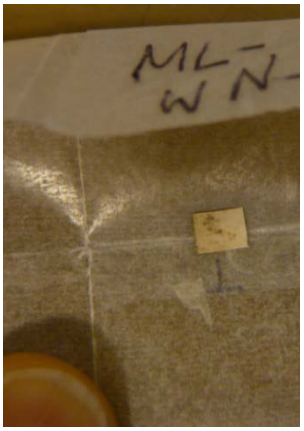
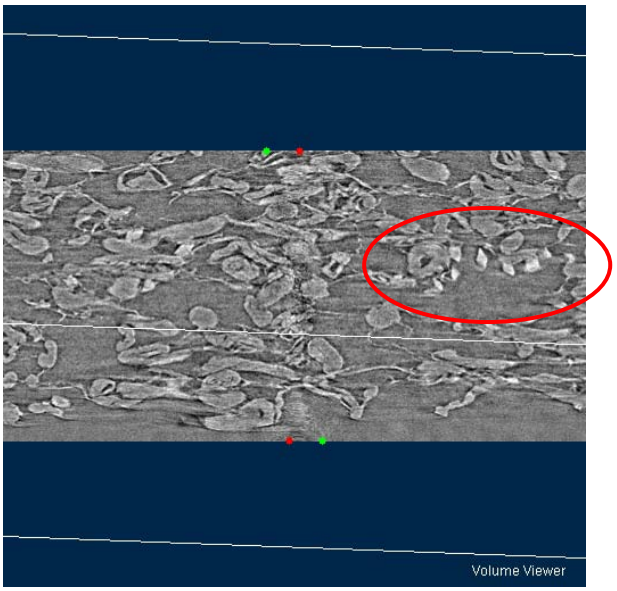
Fig. 8.45 1mmx1mm sample (below 8.46) was cut out for analysis in X-ray microtomography. W4-daylight, stereo-microscopy image; scale bar: 0.5 mm	**Fig. 8.47 A slice-image (tomograph) obtained from X-ray tomography of W4-DL sample, one of ca 1500 procured during the analysis.**
	Fungal residue are visible among paper fibers, however, close and detailed examination of the image is necessary to distinguish their shape. X-ray tomography provided additional information about paper characteristics, such as matrix compactness and fibers distribution.
Fig 8.46 Enlarged view of the sample which underwent examination in X-ray tomography.	

Fig. 8.48-8.50 © H.Szczepanowska.

WN-ML, surface microscopy	WN-X-ray micro- tomography
 **Fig. 8.48 Individual fungal fruiting bodies, size: 42-135 µm. Scale bar: 0.5mm.**	 **Fig. 8.50 One of the ca 1500 slices (tomographs) of the sample, produced during X-ray microtomography.**
Fig. 8.49 1mm x 1mm sample (below) was cut out for further analysis in X-ray micro-tomography. 	Fungal formations are visible among the paper fibers. They can be easily distinguished based on fungal morphology differentiating them from paper fibers morphology.
	**Fig.8. 51 X-ray tomography imaging confirmed the presence of calcium oxalate crystals in the paper matrix. Here the crystals area marked with red oval.** Winsor&Newton paper, with cultures grown in dark (WN-ML).

8.3 Summary points

1. Without a doubt light has impact on fungal growth and pigmentation. Distinctly different patterns of growth and pigment intensity were observed in cultures grown in daylight and in dark.
2. Rapid growth and intensity of pigmentation of fungal colonies cultured in daylight corresponded to mechanical weakening of paper. It can be presumed that secondary metabolites, which are often acidic, disintegrated the paper fibers which led to their physical weakening.
3. Rapid growth and intense pigmentation was not only associated with light condition, but also with water absorption. W4 paper which absorbed water from substrate showed rapid and intense growth in both light conditions. WN paper sized to repeal water did not support fungal growth.
4. Paper fibers were affected the most on W4-DL (daylight) sample in comparison with all papers used in this experiment (W4-ML, WN-DL and WN-ML).
5. Presence of sizing did not support growth of fungi. W4 which had no sizing at all supported fungal growth, while WN which was sized with gelatin did not.
6. Various analytical techniques that were used in this investigation contributed to a better understanding of fungi and substrate interfaces, by providing information that supplemented each other. The complex and multidisciplinary nature of the investigation calls for application of various instrumentations to gain a better understanding of interactions of fungi and substrate.

8.4 Discussion and implications of finding for cultural heritage

Considering multivariate nature of this experiment and dramatically different outcome of fungal pigmentation, each component that contributes to the pigments production ought to be tested separately, to eliminate synergistic contribution of all the factors.

The obtained results however indicated that light without a doubt alters fungal growth and pigment formation and deposition. However, it appears that the governing element is presence of water, playing a major role in response of fungi, rapidity of growth and intensity of pigmentation.

Water absorbed by substrate without any nutrients supported abundant growth (W4) while substrate which did not absorb water even though was sized with gelatin did not support fungal growth (WN). That is an important observation that diminishes a common believe perpetuated in most publications that presence of nutrient in paper substrate will support fungal infestation. It is true only if the substrate absorbs water. It is discussed in details in chapters 2.3 The Environment, and 6. Methods and Materials.

This findings permit to predict the course of deterioration ratio of cultural material. More specifically how paper-based collections will be affected by exposure to flooding or water in fire- rescue operation. Papers in the collection which are more absorbent will be affected by fungi more before those papers which are sized, such as watercolor papers. Intensity of pigmentation will be initially greater on the absorbent papers. However, longer the wet paper remains wet, greater the pigmentation intensity will occur. Time factor therefore is critical in designing rescue operations of flooded collections and those wetted during fire suppression.

9. Bio-stains genesis and morphology of three case studies of fungal pigmentation of artworks on paper

Group II of Study Papers consisted of two artworks on paper and one sheet of historic paper from the collection of the Knights of Malta. All three papers were affected by fungal stains; the 17th century Study Sheet, 1920 Engraving and 1847 Japanese woodblock print. The fungal stains occurred in the course of time in unknown conditions by unknown microorganisms. One microorganism was positively identified, as *Chaetomium* sp. on the 1847 Japanese print. Black pigmented cells on the other papers were attributed to meristemtic fungi. **(I.2.1.5)** Although the principle of investigation followed the designed course, there were some modifications, dictated by limitations posed by the original artworks. Minimal size samples amounting to few fibers and one fruiting body were collected from the Japanese print. Three semi-detached fragments of paper of the 1920 Etching were utilized as samples. The 17th c (No1. Paper) supplied more substantial sample material, because it served from the onset as the study sheet. A brief provenance and description of the original papers were included to provide background information for each study case.

9.1. The 17th century Study Sheet, Maltese Collection (No. 1 Study Sheet)

Paper's provenance: Archives, Cathedral Museum in Mdina, Malta. **Date**: ca 17-18th based on presence of minium and vermilion pigment. (Appendix; Study Sheet, 17th c. SEM-EDS Report.
Paper size: Height: 233mm Width: 170mm

9.1.1 Context of bio-deterioration.

Biodeterioration was evident as fungal stains and structural deterioration of paper. Predominant black stains were scattered throughout. Green, pink and brownish, liquid-like stains were located in the central part and some were associated with structural deterioration of paper matrix. (fig.1) Structural deterioration evident as paper losses was in the center of pink and brown stains, indicated strong enzymatic (cellulase) activity of fungi. In the area of brown stain presence of fruiting structures of fungi characteristic for cellulolithic fungi confirmed that enzymatic deterioration must have occurred. There was no presence of bio-residue in pink or green stains areas. Black stains were prominent on one side of the paper, which will be referred to as 'recto'. The opposite side is referred to as 'verso' in the subsequent text. On verso, small patches of pin-like black spots were located at the bottom, near the most severely deteriorated paper in brown stain.

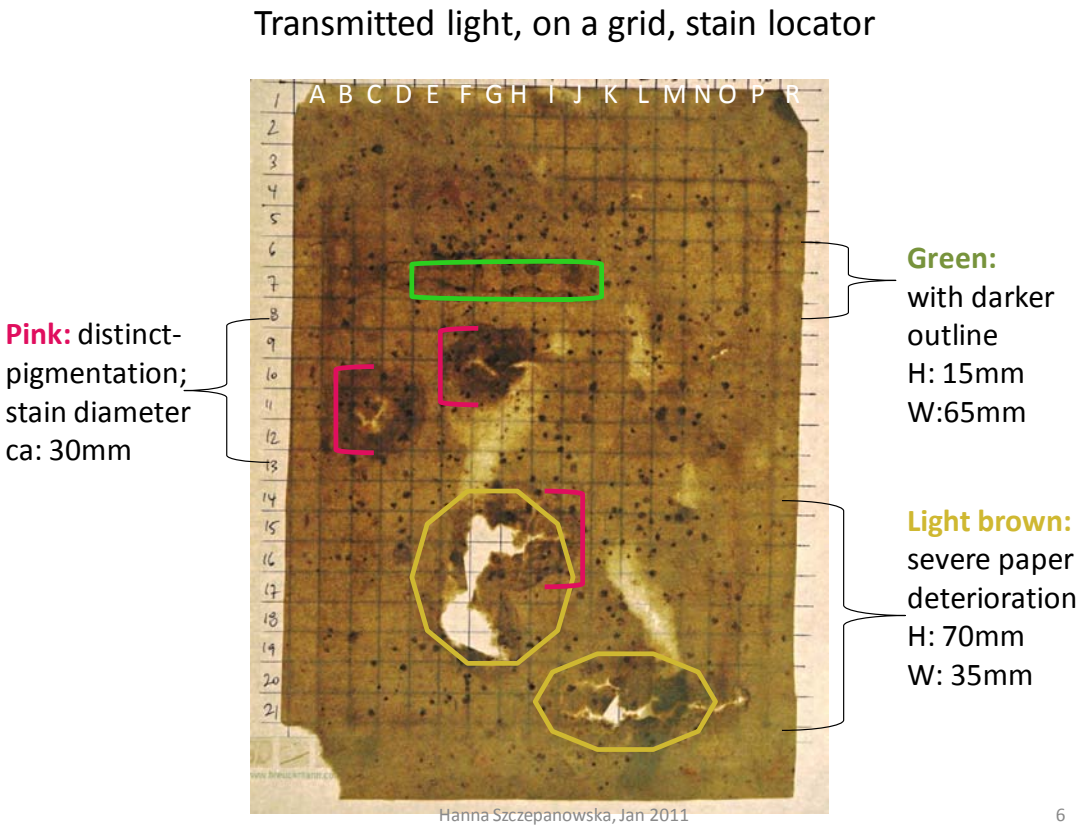


Fig. 9.1 Examination of No1. Study Sheet (17th c paper) on a transmitted light table indicated three different types of bio-deterioration induced by fungi.

The following stains were most likely produced by fungi: 1. Small black stains, scattered (Type A); 2. Liquid-like larger stains, pink, green and brownish; 3: localized, pinhole-like black inclusion in areas near paper losses (Type B). Grid locator dividing the sheet into 10mmx10mm squares was used as a reference to indicate the placement of the examined areas.

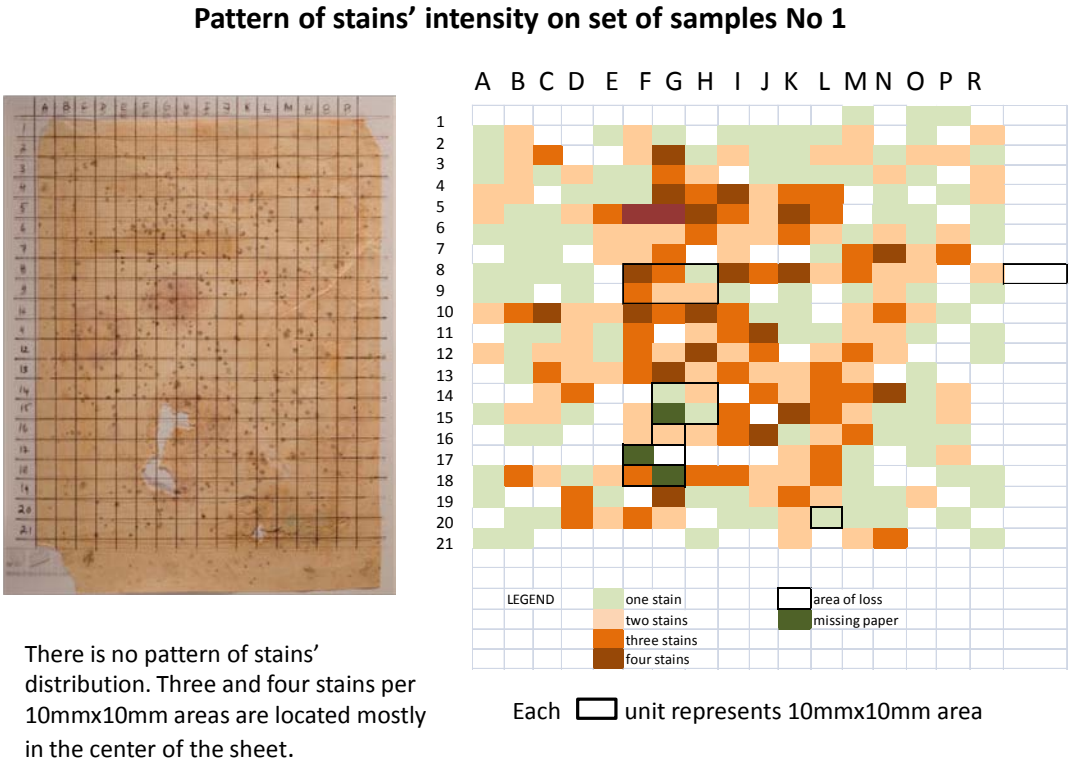


Fig. 9.2 Distribution of black stains on the No1. Study Sheet.

9.1.2 Examination protocol

1. Physical characteristics of paper, fibers and sizing analysis results are summarized in chapter II.2 Materials and Methods.

2. Analysis of red pigment residue was unique for this paper. The objective was to determine characteristics of the red pigment, which could provide information about dating of this paper. (Appendix: Sheet No 1, SEM-EDS Report, red pigment analysis)

3. Investigation of biodeposists followed the examination protocol designed for all samples in II. 5. Investigation Strategy.

- Biodeteriorated paper, surface characteristics of bio-stains,
- Transmitted light microscopy (fibers and fungi identification)
- SEM-VP surface and cross-section examination
- Surface morphology of biodeteriorated paper

9.1.3 Surface characteristics and morphology of bio-stains; No.1 Study Sheet

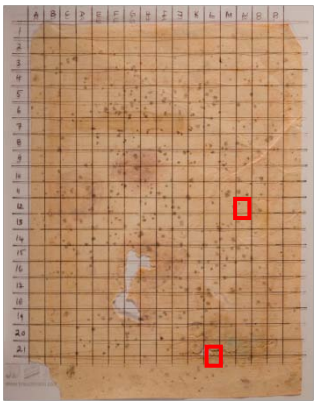


Fig. 9.3 Stains Type A, represent the most prevalent black stains scattered throughout the sheet; location 1- N11. Type B stains represent small inclusions of dark pigmented fruiting bodies; location 1-L20.

Fig. 9.4-9.9 H.Szczepanowska

Stain A: characteristics, recto

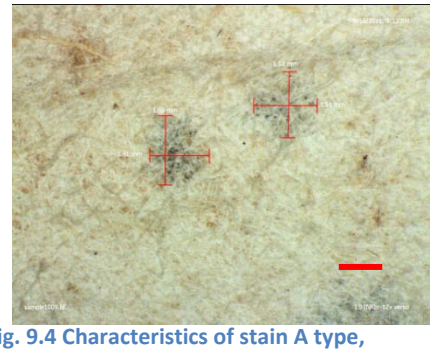


Fig. 9.4 Characteristics of stain A type, recto; location: 1-N11, recto. Scale bar 2mm ;

Stains' dimensions: **1.24mm x 2.14mm ; 1.92mm x 2.13mm**

Stain A: characteristics, verso

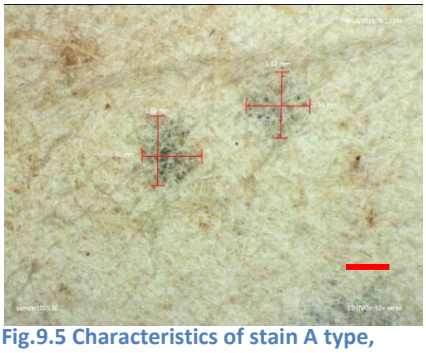


Fig.9.5 Characteristics of stain A type, verso; location: 1-N11, verso. scale bar 0.5mm. Location : N11 verso.

Stains' dimensions: **1.41mm x 1.60mm ; 1.51mm x1.53mm**

A: Bio-deposit in paper matrix

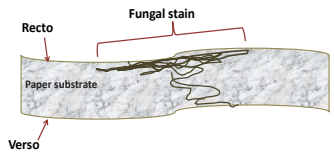


Fig. 9.6 Fungal filaments penetrate through paper matrix to verso, resulting in stains of lesser intensity. (Schematic drawing not to scale).

Stain B: characteteristics-recto

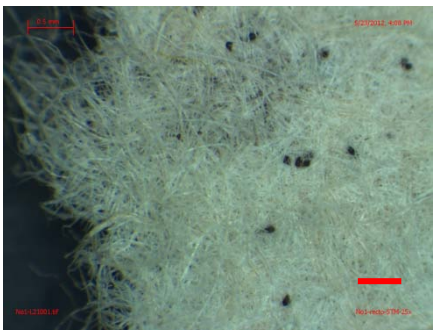


Fig. 9.7 Characteristics of stain B type, verso. Area on recto of paper shows black fungal inclusions less prominent than on verso. Location: L20. Scale bar: 0.5mm

Stain B: characteteristics-verso

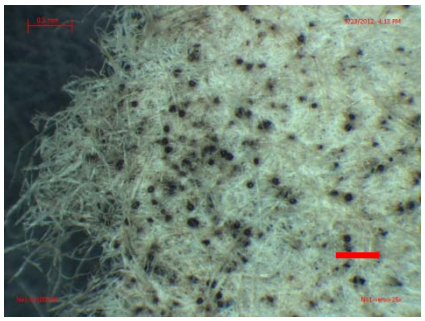


Fig. 9.8 Verso of the same area L20 with black fungal inclusions. Scale bar: 0.5mm

B: Bio-deposits in paper matrix

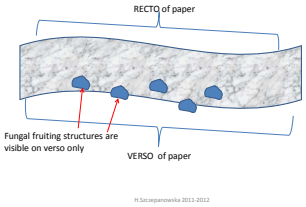


Fig. 9.9 Fungal fruiting structures were located near the surface on verso. (Schematic drawing not to scale.)

9.1.3 Surface characteristics and morphology of bio-stains; No.1 Study Sheet, cont.

Fig. 9.10-9.14 H.Szczepanowska

Stain A: Examination / area	**Optical surface analysis ; stereo-surface microscopy ; 6x-50x ; Wild Microscope**	**Transmitted light microscopy : 50x-400x, Leica DMLM**

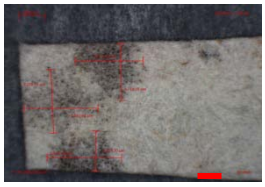


Fig.9.10 Stereo-micrograph of stains type A. Location : 1-5L. Scale bar: 2mm ;

Stains size:

4.71mm x 5.17mm
5.25mm x 5.59mm
3.32mm x 5.64mm

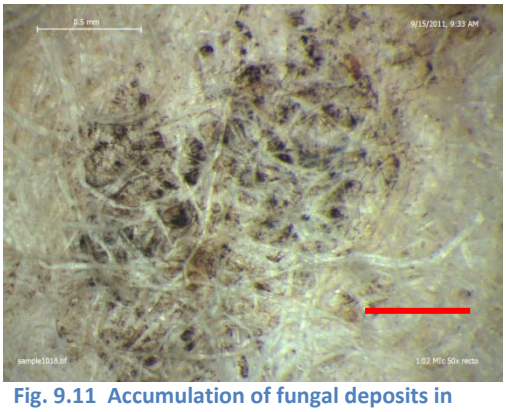


Fig. 9.11 Accumulation of fungal deposits in niches created by paper fibers; on the surface and just below the surface.

Paper fibers are being utilized by fungi as physical anchorage and protection against sweeping them off the surface. Scale bar: 0.5mm ; 50x

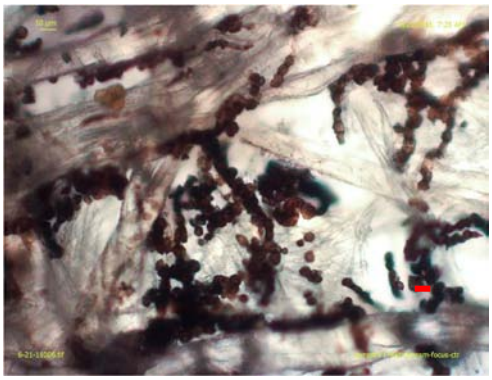


Fig. 9.12 Cells show pigmented walls, melanized, appear to be connected and trail on the surface of papers fibers.

Chains of cells branch out from paper fibers to connect with another fiber. That pattern of fungal growth and interaction with paper fibers was evident in all examined stains. Cells size: **5.7 µm - 6.5 µm.** Scale bar: 10 µm ; 400x

Transmitted and polarized light

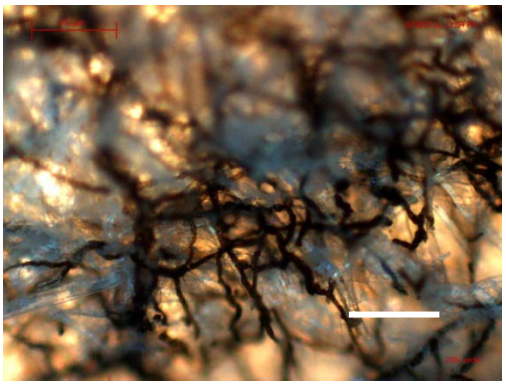


Fig. 9.13 Pigmented chains of fungal cells are intertwined with paper fibers.

There was no evidence that cell grew into the fibers. The tendency seems to be towards aggregation in air pockets and pores in paper. Scale bar: 100 µm

Transmitted white light microscopy

Fig.9.14 Characteristic pattern of cells chains partially attached to fibers surface and partially branching out towards air pockets. Scale bar: 10 µm

Fig.9.15-9.19 H.Szczepanowska

Stain B: Examination method/ area

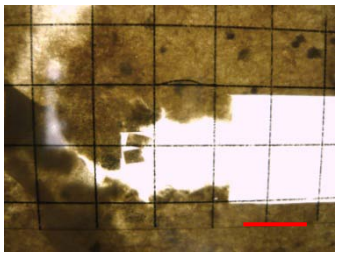


Fig. 9.15 Biodeteriorated paper, stain type B. Each sample, 2 mm x 2mm, underwent further analysis. Location: 1-L20. Scale bar: 10 mm

Optical surface analysis ; stereo-surface microscopy

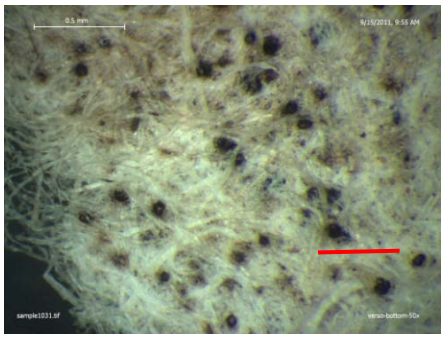


Fig. 9.16 Sample area with inclusions of fungal fruiting structures, 1-L20. Scale bar 0.5mm

Transmitted light microscopy

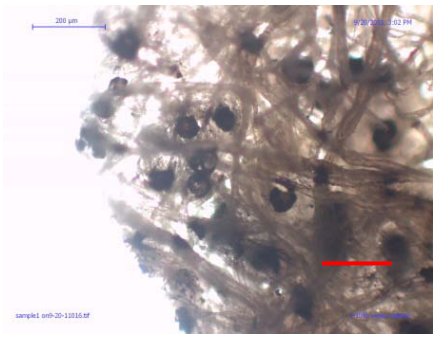


Fig. 9.17 Fungal structures are partially embeded into paper matrix, as seen on verso of paper. scale bar: 200 µm

Transmitted light microscopy

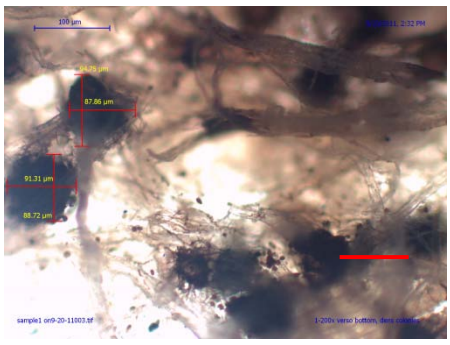


Fig. 9.18 Black stains caused by inclusions of dark pigmented individual fruiting structures; 1-L20.

94.75 µm x 87.85 µm
91.31 µm x 88.72 µm
scale bar: 100 µm

Transmitted light microscopy

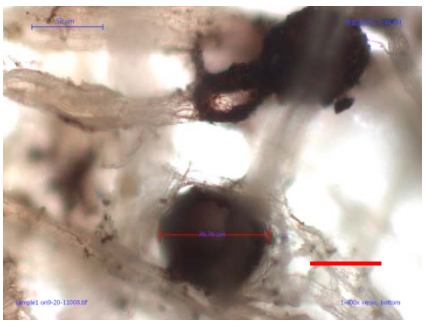


Fig. 9.19 Attachment of fruiting structures to paper fibers. Average size of each inclusion: 76.76 µm. scale bar: 50 µm

9.1.4 Morphology of microorganisms fig. 9.20-9.22 H.Szczepanowska

Stain A: Morphology of microorganisms, SEM-VP

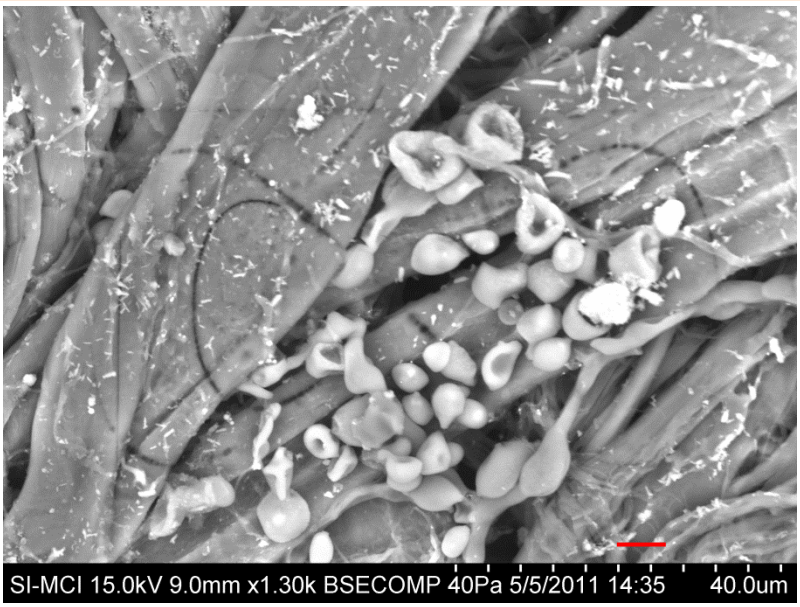


Fig. 9.20 Analysis of biodeposits on fibers indicated congregation of single cells. SEM-VP 1300x. Scale bar: 5 µm

Heavy-walled cells a appeared to be spores, based on their shape and dimensions. Indentations most likely are caused by desiccation of cells.

Fig. 9.21 Detailed view of the fungal cells in black stains deposits. Cells appear to be attached with extracellular material to the surface of fibers. SEM-VP micrograph, 3500x. Scale bar: 2µm

Fig. 9.22, 9.23 H.Szczepanowska

Stain B: Morphology of microorganisms

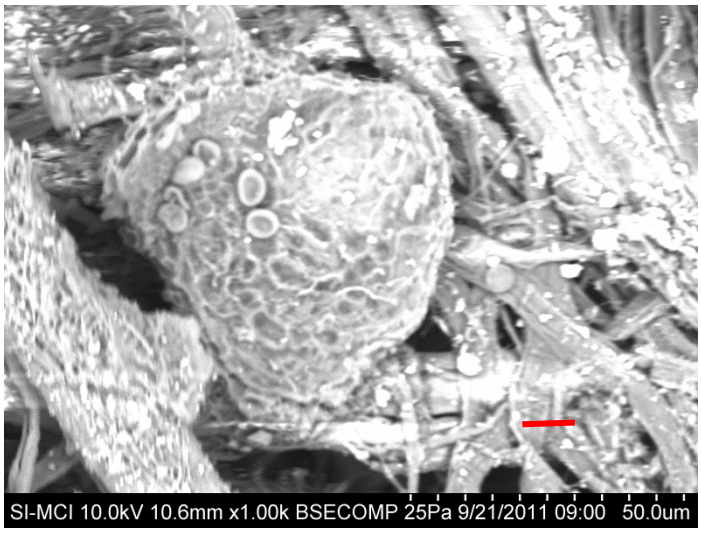


Fig. 9.22 Fruiting structures, pycnidium in the location 1-L20. Size of individual structures: 50-60µm. SEM-VP micrograph, 1000x; Scale bar: 10 µm

Shape and size of the fungal formations guided settings of surface metrology instruments such as light profilometer used in surface topography.

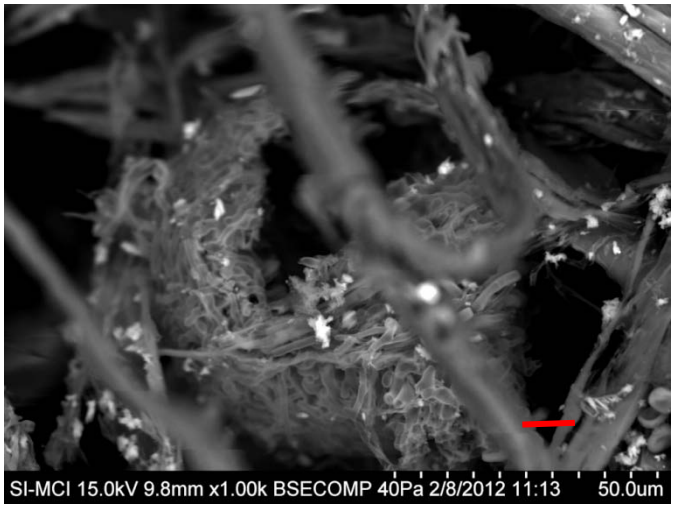


Fig.9.23 Fruiting body partially embedded into paper matrix. SEM-VP micrograph, 1000x; scale bar: 10µm

This form that differs from one in L20 may indicate yet another type of fugal specie. Depth of its location in fiber matrix, see fig. 9.30 and 9.31.

9.1.5 Surface topography of paper with bio-deposits Fig.9.24; 9.25 H.Szczepanowska

Stain A: Surface topography of paper with bio-deposist

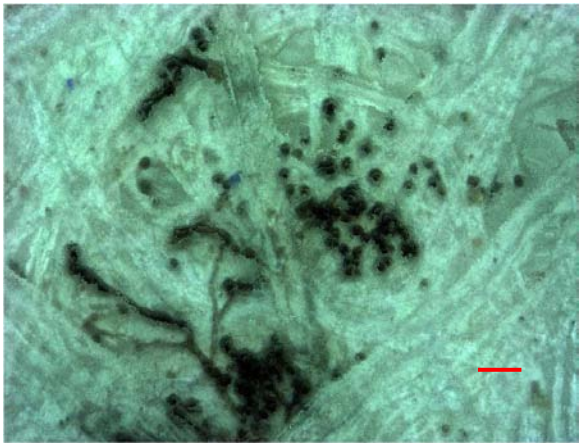


Fig. 9.24 Three-dimensional imaging of fungal deposits in paper matrix using confocal scanning laser microscopy (CLSM). Scale bar: 20µm

Fig. 9.25 Using short-wave lasers and pinhole optics, a lateral resolution of 0.13 µm 0.01 mm can be obtained. Scale bar: 20 µm

Depending on the used wavelength, the fugal deposits are depicted as black or, as visible on adjacent image, orange particulates. Collected XYZ measurements serve for creating profile of the measured area.

The VK-9700 scans across a target in the X and Y directions using a laser with a 408 nm wavelength.

Surface topography of paper with fungal stains

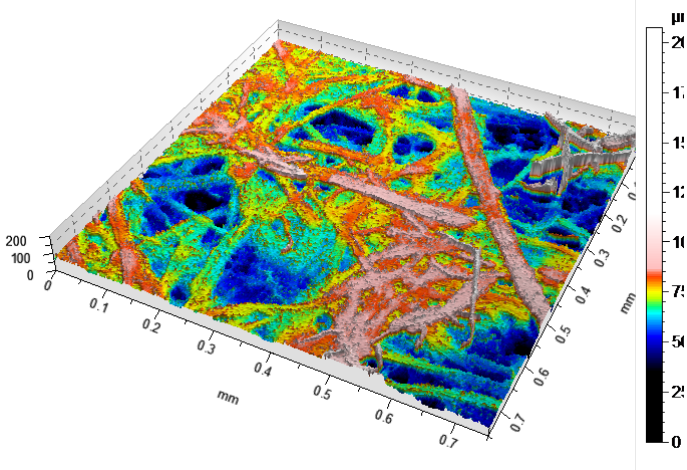
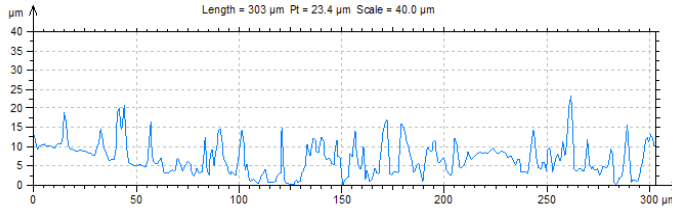


Fig. 9.26 3D topography map generated from by measuring the surface with CLA. Deposits along paper fibers reach a uniform, 75 µm height in Z direction, appear to be fungal residue.

White light profilometer

Non-measured points were filled using a smooth shape calculated from the neighbors.)Created on: 3/3/2012 3:56:07 AM)
Measure duration: 6 h 4 m 48.000000 s
Axis: Axe X: Length: 0.750 mm
Size: 751 points
Spacing: 1.00 µm
Offset: 43.7 mm
Axis: Axe Y: Length: 0.750 mm
Size: 751 lines
Spacing: 1.00 µm
Offset: 49.4 mm
Axis: D: Length: 236 µm
Z min: 543 µm
Z max: 778 µm
Size: 25725 digits
Spacing: 9.16 nm



Length: 303 µm ; Scale: 40 µm; The highest point: 22.4 µm

Fig. 9.27 Profile of the fiber area with fungal deposits. Deposits represented as peaks correspond with fungal reside captured on 3D topography map.

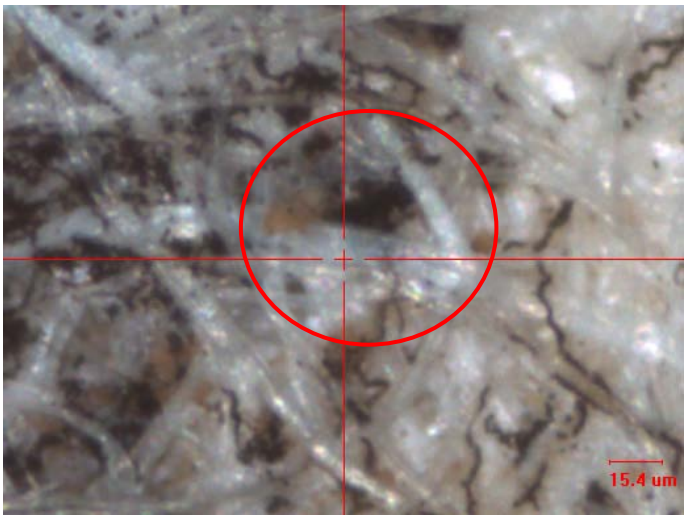


Fig. 9.28 Aerial view of the measured stain. Note accumulation of dark pigmented fungal cells and corresponding void of data in 3D mapping, fig. 9.29 H.Szczepanowska

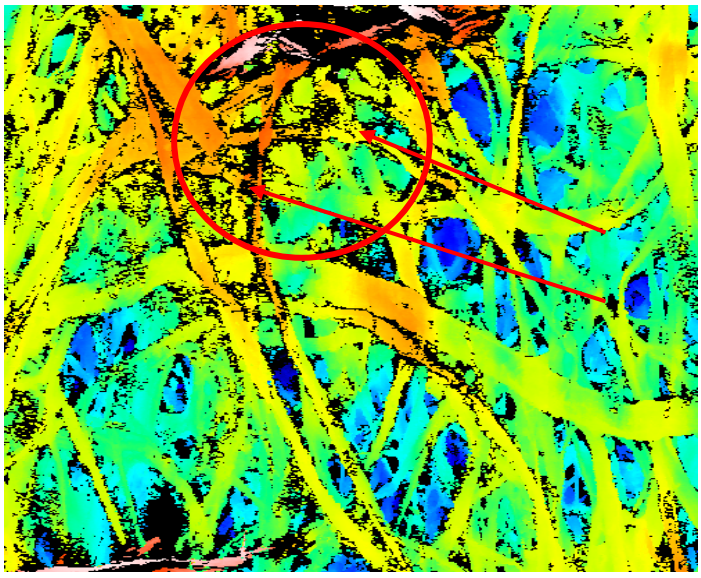


Fig. 9.29 3D map generated from measuring the surface with optical, non-contact white light profilometer, AltiSurf500.

Even the individual cells can be depicted by the profilometer, however, showing as voids of data. H.Szczepanowska

Fig. 9.30-9.32 H.Szczepanowska

Stain B: Surface topography of paper with bio-deposits	**Noncontact white light profilometer,** **AltiSurf 500**

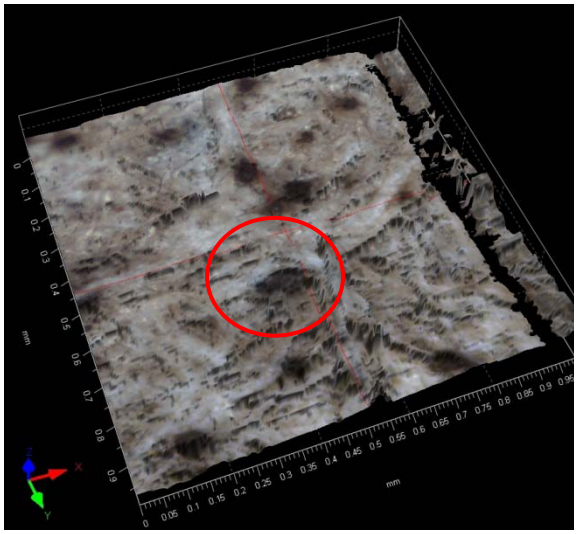


Fig. 9.30 3D view of the area with spherical fruiting inclusions in stain B.; Area measured: 5mm x 2mm

Profile spacing: 5µm ;Data acquired: 1µm; Sensor tip: 2 µm

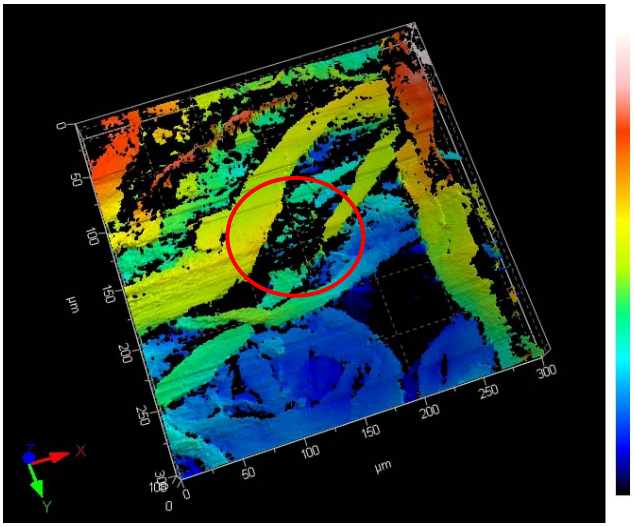


Fig. 9.31 3D topography map of a fragment of the same area. Fruiting structures seemed to have absorbed light, resulting in voids of data. Shape of the voids corresponds with the shape of fungal inclusions.

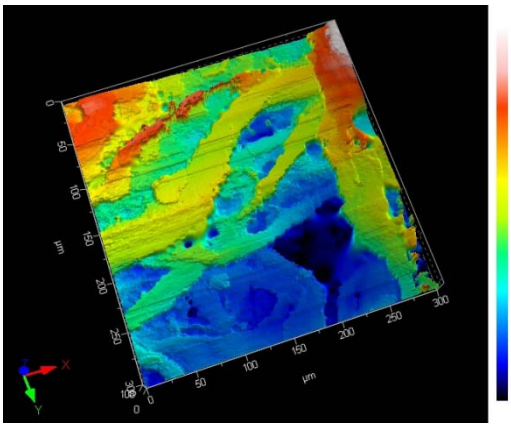
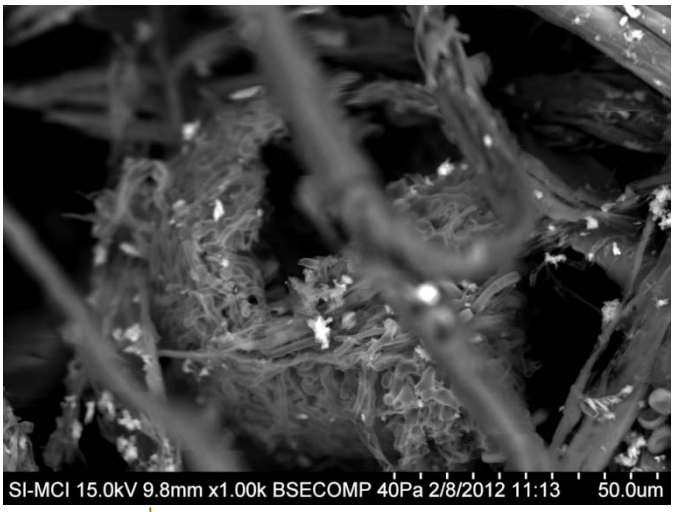
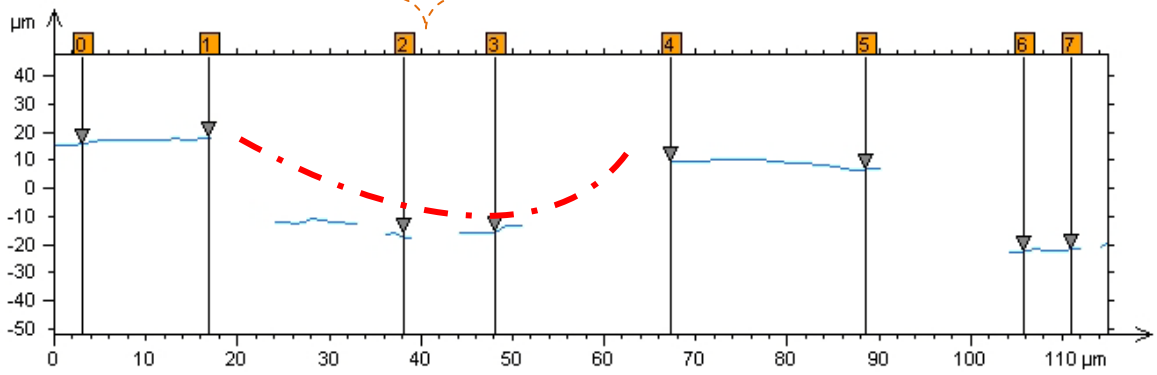


Fig.9. 32 The same 3D map with filled-in missing data. Note the area of fungal deposit barely visible as outlines.

Profile of the inclusion even though shows missing point of data corresponds with the shape of the fungal fruiting structure. Filling-in the missing points will obscure information provided by raw data measurement, as shown on fig. 9.32.





	0-1	2-3	4-5	6-7
Horizontal distance	13.813 µm	9.974 µm	21.301 µm	5.246 µm
Height difference	2.635 µm	0.208 µm	-2.941 µm	0.755 µm
Left abscissa	2.875 µm	37.989 µm	67.152 µm	105.722 µm
Right abscissa	16.688 µm	47.963 µm	88.453 µm	110.968 µm
Left height	15.882 µm	-15.771 µm	10.007 µm	-22.627 µm
Right height	18.518 µm	-15.563 µm	7.067 µm	-21.871 µm

Fig. 9. 33 (SEM-VP micrograph above) and 9.34 profile. Profile obtained with non-contact, white light profilometer AltiSurf 500, corresponds with the shape and size of the fruiting structure. The diameter of the fruiting body was 50-60 µm, and the 'void area' indicated by lack of data is very close to 50µm. The depth of fruiting body 'immersion' in paper matrix is ca 25µm.© H.Szczepanowska

Each technique used in the investigation of fungal stains and fungal interactions with paper substrate provided supplemental information upon which further investigation relied. However, familiarity with the material that has been measured is essential for proper interpretation of results.

Three-dimensional view of the fungal interaction with paper matrix was obtained by data acquisition from synchrotron. Fig. 9.35.

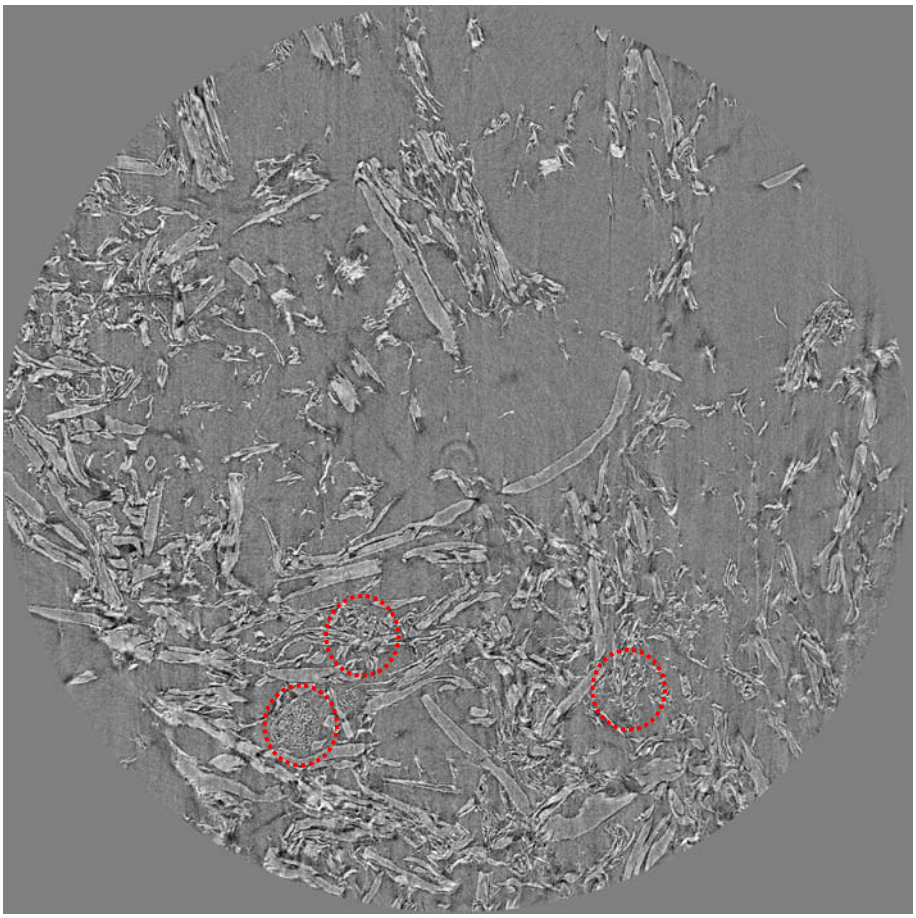


Fig. 9.35 Slice-image of the paper from 1mmx1mm sample obtained from X-microtomography at the European Synchrotron Facility, Grenoble France. Fungal structures located in the paper matrix are well defined and marked here with red circle.

9.2 The Etching on paper, 1920

Paper's provenance: Private collection, Annapolis MD. Date of the artworks, 1920, signed in pencil.
Paper size: H:35.2mm W: 27.5mm

9.2.1 Context of biodeterioration

The etching was displayed in a window mat and backing material placed on reverse. Black stains produced by fungi were located along the top edge and penetrated through all layers of the assemblage, mat and backing board. Fig. 9.36-9.39 H.Szczepanowska.

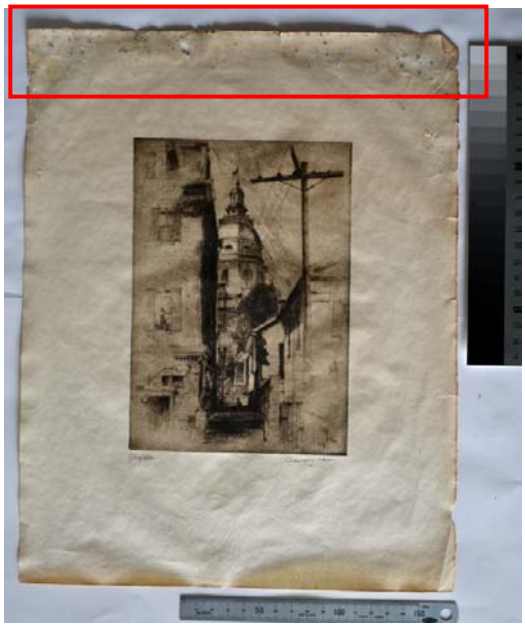


Fig. 9.36 The Etching released from the mat. Recto is the front side of the paper with on image. Black fungal deposits are prominent and their distribution in similar to that on verso.

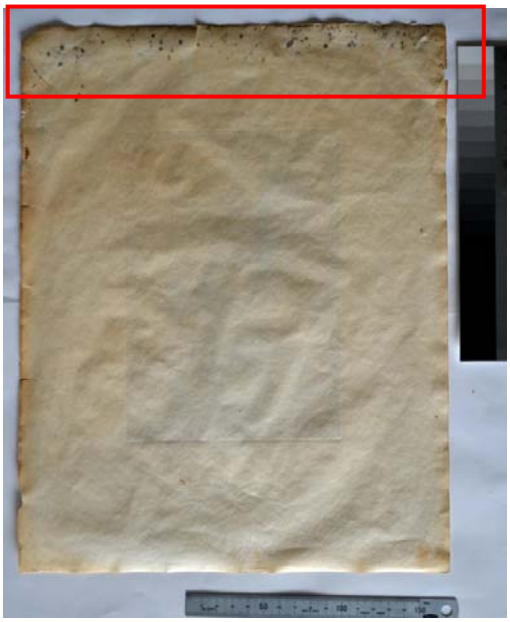


Fig. 9.37 Verso is an opposite side of the artwork. Black fungal stains are located along the top edge, distribution corresponds with their location on the backing board. Intensity of pigmentation is identical on both sides of paper.

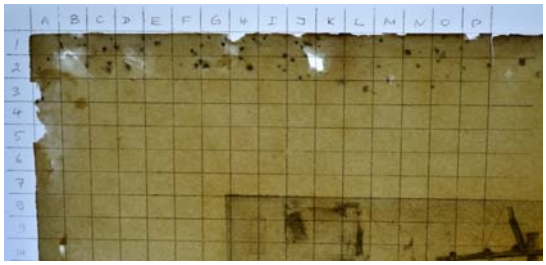


Fig. 9.38 Left side of the artwork, top, was documented on a transmitted light table. Grid served as guidance in stains' location.

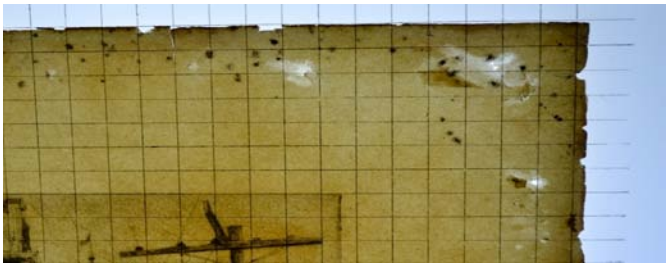


Fig. 9.39 Right side of the artwork, top, examined on a light table. Combination of both parts provides a base for documenting the distribution of stains.

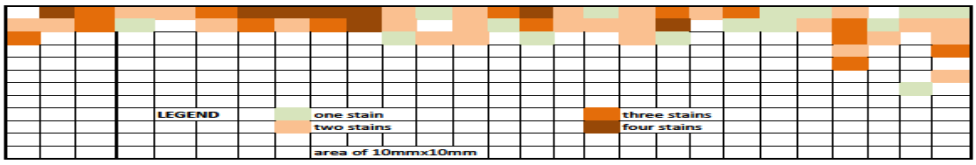


Fig. 9.40 In search of a pattern in stains distribution their location was marked according to number of stains per 10mm x 10mm area. No particular pattern was found. However, more stains were accumulated along the very edge of paper. H.Szczepanowska

9.2.2 Examination protocol

1. Physical characteristics of paper, fibers and sizing analysis results are summarized in chapter II.2 Materials and Methods.
2. Investigation of fungal deposits followed the examination protocol designed for all samples in II.1 Investigation strategy:

- Biodeteriorated paper: surface characteristics of areas with fungal stains
- Transmitted light microscopy (fibers and fungi identification)
- SEM-VP surface and cross-section examination
- Surface morphology of biodeteriorated paper, with fungal deposits, investigation of both sides of paper with Confocal Scanning Laser Microscope
- Surface topography, using white light confocal scanning profilometer
- Interaction of fungi with substrate, viewed with X-ray microtomography synchrotron

9.2.3 Surface characteristics and morphology of bio-mass; Fig. 9.41-9.44 H.Szczepanowska

Fig. 9.41 Upper part of the etching sheet with those areas which underwent further investigation marked in red.

Biostains, surface characteristics		
Stain characteristics, recto of artwork	**Stain characteristics, verso of artwork**	**Configuration of fungal deposits on paper**

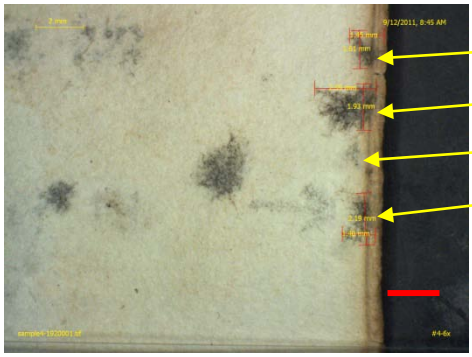


Fig. 9.42 Location of stains, counting from the bottom: 4.1; 4.2; 4.3 were selected for further investigation. Stereo-micrograph, scale bar: 2mm

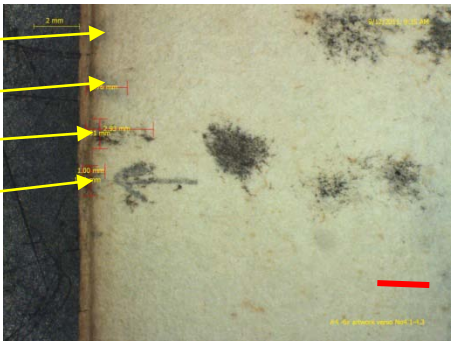


Fig. 9.43 Some of the stains were barely visible on the opposite side. Their juxtaposition configuration is marked on fig. 9.42. Stereo-micrograph, scale bar: 2 mm

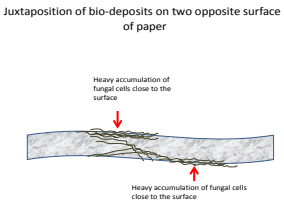


Fig. 9.44 Bio-mass is accumulated near the surface and just under the surface on each side of paper.

Size of spots on recto:
4.1: 2.19mm x 1.40mm
4.2: 2.59mm x 1.93mm
4.3: 1.45mm x 1.60mm

Size of spots on verso:
4.1: 1.31mm x 1mm
4.2: 1.76mm x 1mm
4.3: no measure, no residue

Minor residue is visible on the exactly opposite side of paper. Accumulation of cells is not in parallel position, but in juxtaposition. Visualization not to scale.

Examination method/area	Optical surface analysis; stereo-surface microscopy; 6x-50x; Wild microscope	Transmitted light; range: 50x-400x Leica DMLM

Fig. 9.45 Location of stains; Scale

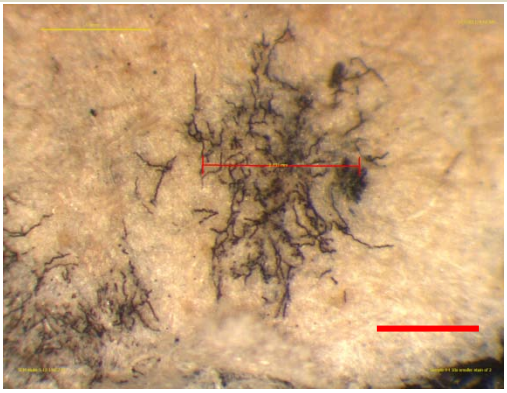


Fig. 9.46 Stereo-micrograph, scale bar: 0.5mm. Black fungal deposits appeared spreading outwards, from the center. Size of this stain: 0.73 mm

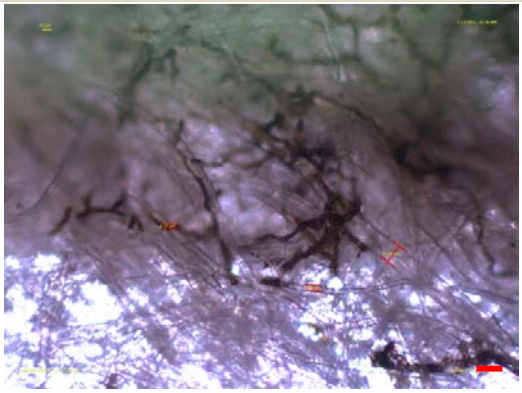


Fig.9.47 Transmitted light micrograph, Scale bar: 20 µm. Cells' size: 5.17 µm Paper fibers size varied 6.91 µm - 22.34 µm.

Fungal cells are in chains and in clusters, characterized by heavily melanized walls, with cell interior semi-transparent. Cells are attached to the paper fibers exterior and

10mm

branch out to connect with another fiber.

Transmitted light microscopy

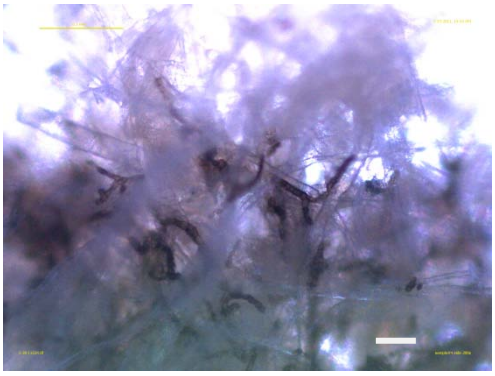


Fig. 9.48 Transmitted lithgt micrograph, showing a pattern of cells chains attachment to the paper fibers. scale bar: 50 µm

Most likley paper fibers are utilized as anchorage.

Transmitted light microscopy

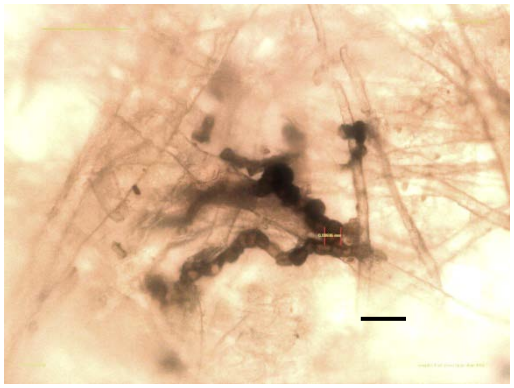


Fig. 9.49Transmitted lithgt micrograph, scale bar: 25 µm Cell chains are patrtially attached to paper fiber and branch out to air-pockets in paper structure.

Cells are arranged in chains. Size of individual **cells: 9.36µm.**

9.2.4 Morphology of microorganisms Fig.9.50 -9.53 H.Szczepanowska

SEM-VP paper surface-chains of fungal cells

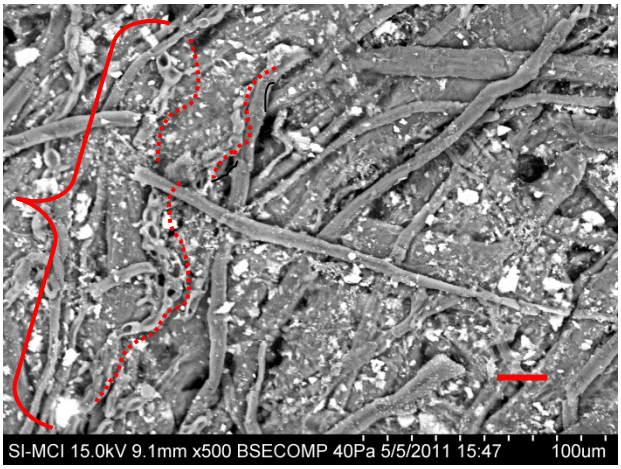


Fig. 9.50 SEM-VP micrograph, scale bar: 20 µm; 200x; Chains of connected cells are following the paper fibers, partially attached to the surface at others areas only closely positioned to the fibers' surface.

SEM-VP paper surface- clusters of cells

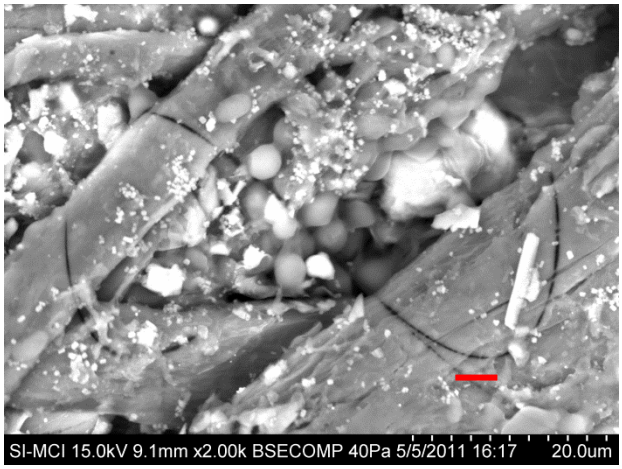


Fig. 9.51 SEM-VP micrograph Scale bar: 4 µm; 2,000x; Clusters of cells are nested close to paper fibers. Their average dimensions are greater than cells in chains, ca 8-9 µm.

Surface topography of paper with bio-deposits Keyence CLSM, VKX

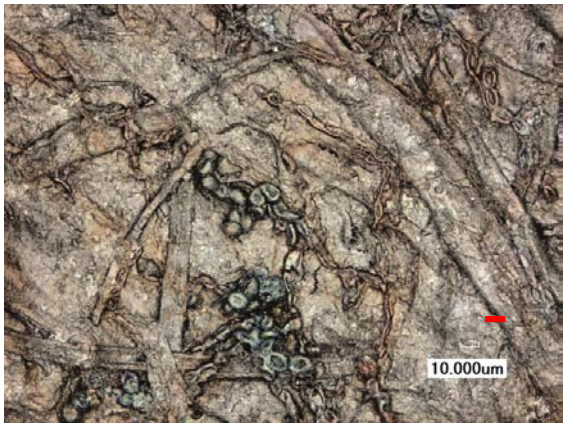


Fig. 9.52 Surface topography of fungal stain -3D visualization using CLSM.

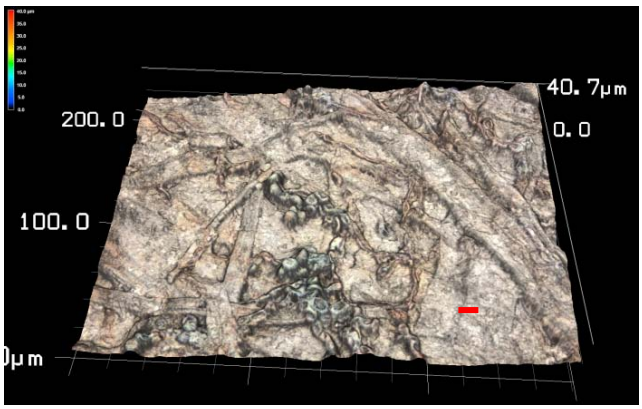


Fig. 9.53 The 3D mapping of the fungal cells spatial distribution on paper surface. Scale bar: 10 µm; 1000x

Two types of fungal cells formations (chains and clusters) and their interactions with paper fibers are clearly defined. Clusters, visible as bluish cells, larger than cells in chains are congregated in paper matrix. Cells in chains are following and wrapping the paper fibers. Scale bar: 10 µm; 1000x

The image was generated from the measurements with CLSM. It emphasizes the fungal bio-mass distribution in paper matrix.

9.2.5 Surface topography of paper with bio-deposits Fig. 9.54 -9.56 H.Szczepanowska

Surface topography of paper with bio-deposits

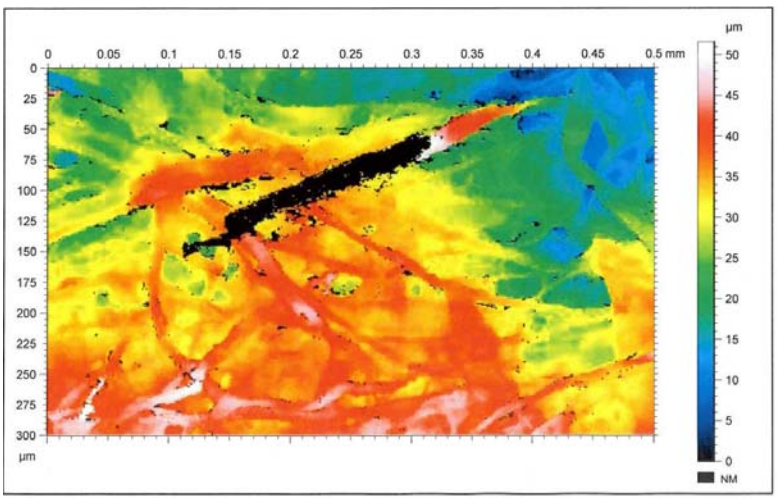


Fig. 9.54 3D topography map shows lack of data along the fibers, between in range 0.1mm-0.3mm.

Intensity map below indicates accumulation of mass along that fiber. Combination of both provides information that most likely it is biomass along the fiber.

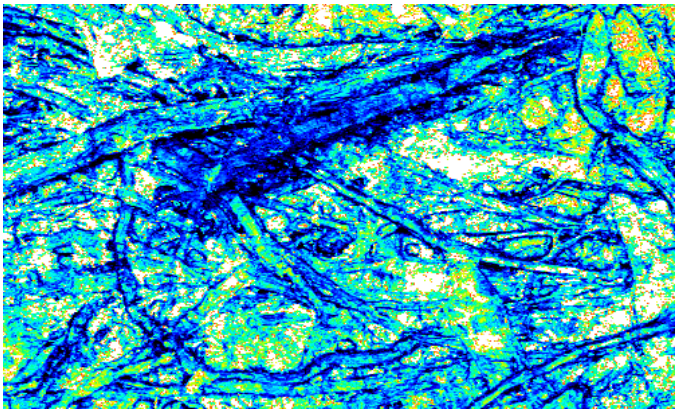


Fig. 9.55 Intensity map shows accumulation of mass along the horizontal fiber. Correlated with 3D map provides additional information aiding n proper interpretation of the surface topography.

Scale the same as of 3D map, area measured: 0.5mm x 300mm.

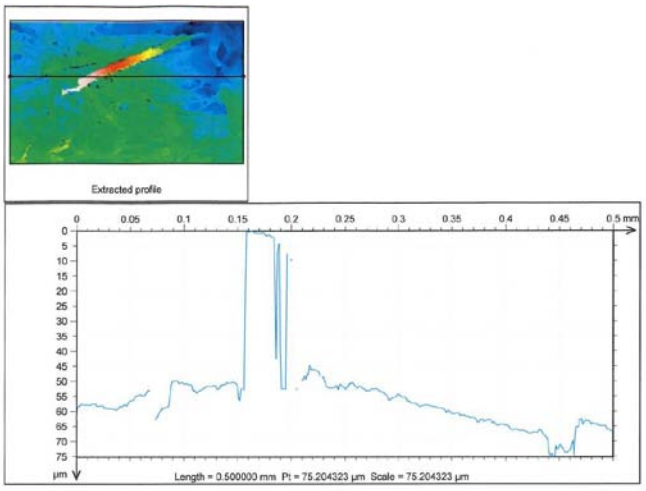


Fig. 9.56 Profile of the measured area confirms elevation in the area where data was not collected and appears as vacant, black 'fiber'.

Most likely that is the area of fungal deposits.

9.2.6 Summary points

Similarly to the investigation of the 17th century Japanese print each employed technique provided supplemental information. Interpretation of the results, especially in case of surface topography using white light profilometer requires careful analysis and repetitious number of measurements on similar surfaces to draw meaningful conclusions. The difficulty is compounded by lack of uniformity of the measured area; paper is highly heterogeneous. Each point that is measured represents different features, either due to density of fungal deposits or due to their different pattern of growth or thickness of biomass.

9.3 Japanese woodcut print, 1847

Artist: Kunisada, **Date:** ca 1847; **Size:** H: 34.3mm W: 24mm

9.3.1 Context of biodeterioration

The artwork was covered with fungi on recto and verso. According to the artwork's owner fungi grew over two-months period. Visual inspection indicated at least three different types of fungi, dark brown fruiting structures, brown powdery aerial growth and light-gray web –like filaments. A representative area for each type of bio-mass was analyzed. Each one indicated a different type of fungal species, characterized by their unique morphology, pigmentation of the aerial growth and/or pigmentation secreted to the substrate.

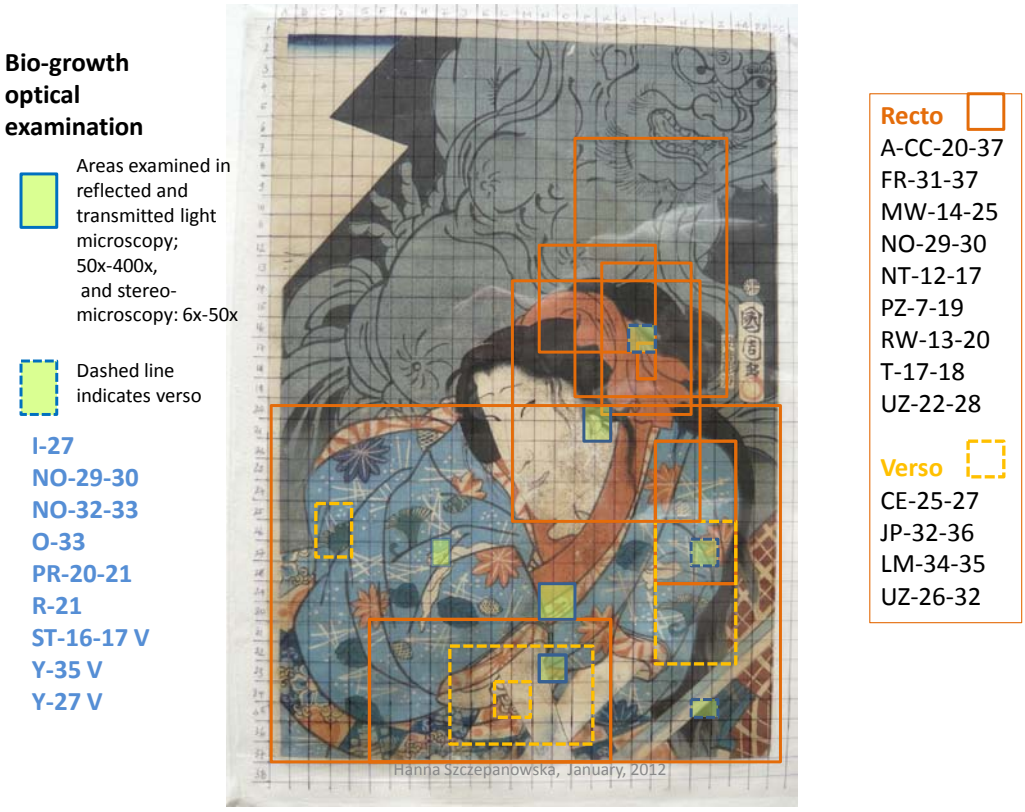


Fig.9. 57 Japanese woodblock print, diagram of the areas selected for examination.

The areas selected for the examination represent three different types of fungi growing on the print.

1. Dark brown (nearly black) vertically protruding perithecia, *Chaetomium sp.* Location: throughout the center of the print.
2. Light white—gray, web-like aerial mycelium; most likely *Pennnicillium* sp. Location: upper part.
3. Light brown, growing over perithecia, most likely *Aspergillus sp.;* Location: bottom lower portion.

Examination protocol of bio-mass and stains followed the methodology outlined in chapters 5 and paper was characterized according to the protocol outlined in chapter 6.

9.3.2 Surface characteristics and morphology of bio-mass fig. 9.58-9.63. H.Szczepanowska

Biodeterioration	Side view of bio-growth and aerial mycelium	Types of bio-deposists

Fig.9.58 Overall view of fungi-infested recto of the 19th century woodblock print. Macro-photograph, Leica Delux C-2.

Fig.9.59 Side view of the central portion of the print showing vertical trend of perihtecia growing on paper. Macro-photograph, Leica Delux C-2. Scale bar: 50mm

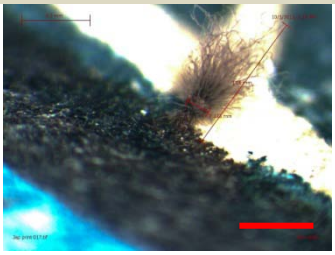


Fig.9.60 Stereo-micrograph of the surface. Single standing, dark brown fruiting structures of Chaetomium (perithecia). Average size of fruiting structures: 1.2mm x 0.2mm. Scale bar: 0.5mm.

Fig.9.61 Fungal bio-mass covered also verso of the artwork. No correlation was observed between patterns of growth on either side.

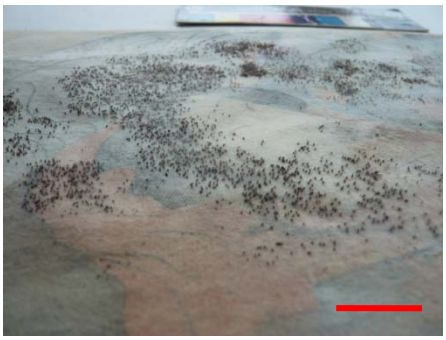


Fig.9.62 Side view of the bio-growth on verso. Perithecia are protruding vertically from the surface. Scale bar: 50mm

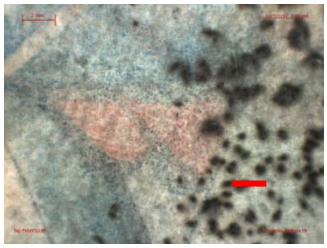


Fig.9.63 Close up of Individual perithecia that covered the artworks on recto and verso. scale bar: 2mm

9.3.3 Surface morphology of fungal stains fig. 5.c .9 -13, H.Szczepanowska

Fig.9.64 Location of the examined area on the Japanese print: PR-20-21

Macro photograph, Leica Delux C-2. ©H.Szczepanowska

Optical surface analysis

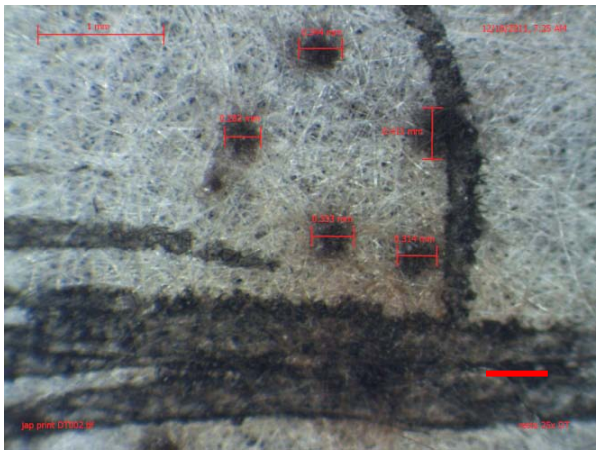


Fig. 9.65 Black pigmented stains were formed under each perithecium; stains' diameter: 0.28 to 0.41mm; scale bar: 0.5mm

Reflected- light microscopy

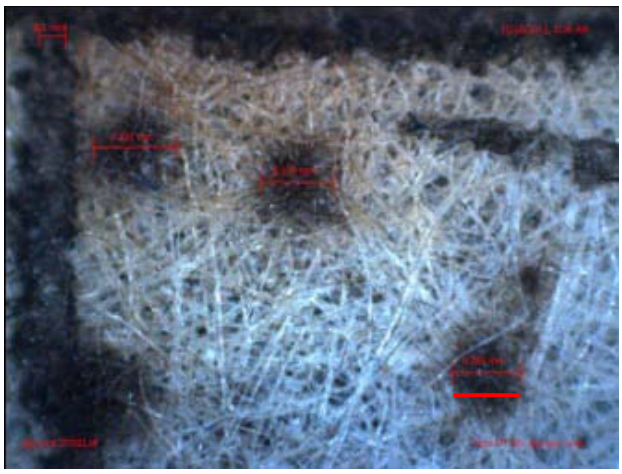


Fig.9.66 Black deposits in paper fibers matrix were visible once perithecia were removed; scale bar: 0.1 mm

Transmitted light microscopy

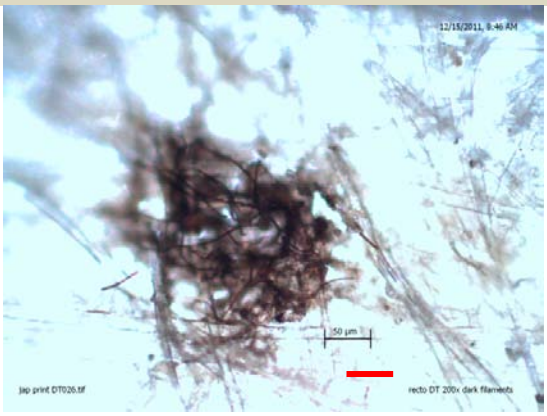


Fig. 9.67 Overexposed image emphasizes the contrast of pigmentation secreted into substrate and black fungal filaments. Perithecium has been removed. Transmitted light micrograph. Scale bar: 50 µm

Transmitted and polarized light microscopy

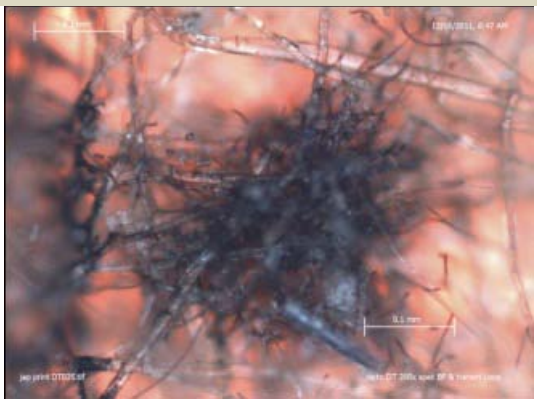


Fig. 9.68 Black fungal stains were uniform in size, ranging from 0.26-0.42mm in diameter. Black pigmentation was limited to fungal filaments amassed in clusters. Filaments diameter size, average: 2µm . Scale bar: 0.1mm; detail see fig.9.69.

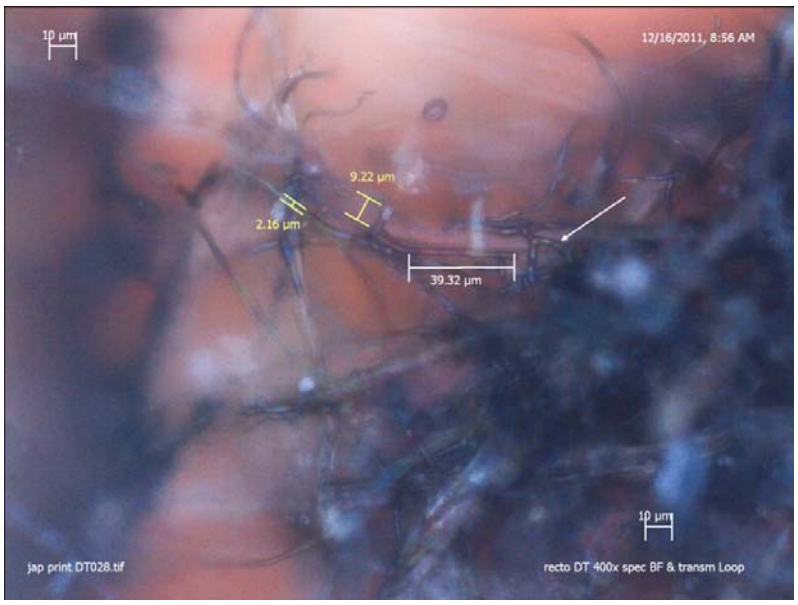


Fig. 9.69 Black fungal filaments are trailing the surface of the paper fibers. On this micrograph, the area of attachment extends over 39.32 µm. In other places, that attachment was longer. 400x Transmitted light microscopy; DMLM Leica Microscope. Scale bar: 10µm.

The above figure is an example of fungal tropism, most likely autotropism and chemotropism. **Authotropism** is a response between neighboring hyphae, partially responsible for spacing of hyphae at the colony margin. **Chemotropism** is the reaction to a chemical, such as for example oxygen or nutrients. (Dic. of the Fungi 2008).

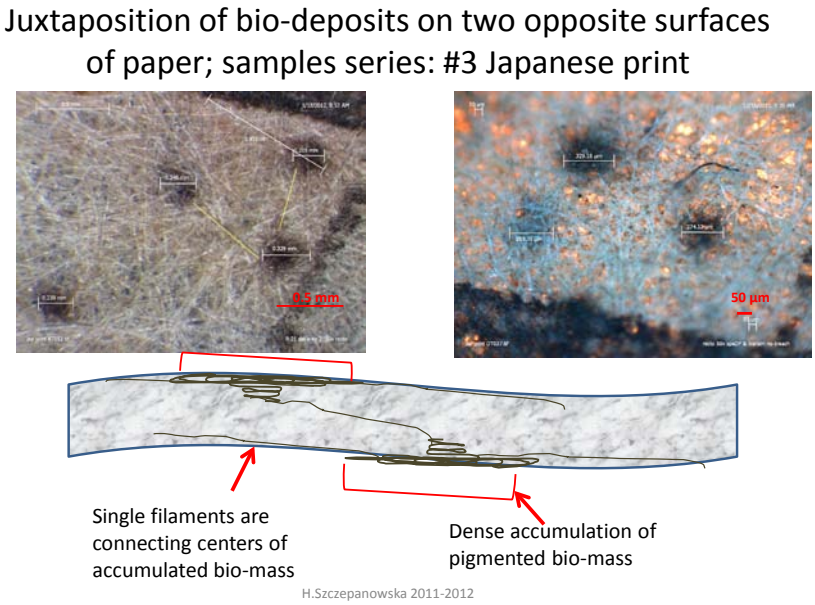


Fig. 9.70 Schematic diagram illustrating configuration of fungal filaments penetrating paper matrix and intensity of deposits on both side of the Japanese paper.

9.3.4 Morphology of microorganisms fig. 9.71-9.77. H.Szczepanowska

Optical microscopy

Optical surface analysis; stereo-surface microscopy; 6x-50x; Wild microscope

Transmitted light , range: 50x-400x Leica DMLM

VP-SEM

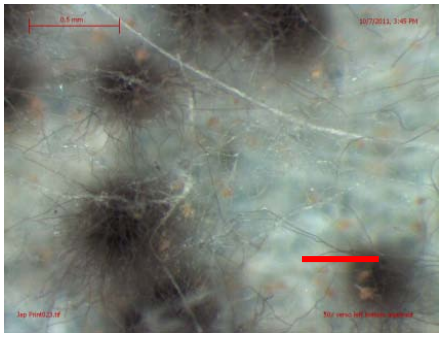


Fig.9.71 Perithecia and smaller brown chains of conidia on conidiophores growing over perithecia. Stereo-micrograph. scale bar: 0.5mm

The conidiophores reached 100µm length. Size of individual perithecia av.: 300µm (233.29µm, 254.05µm, 279.46µm.

Hairs extended over 800µm length;

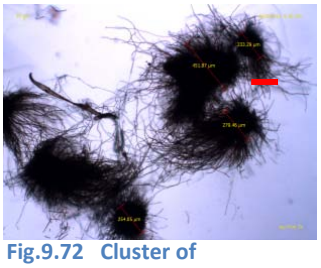


Fig.9.72 Cluster of perithecia, 200x .Scan bBar: 20µm.

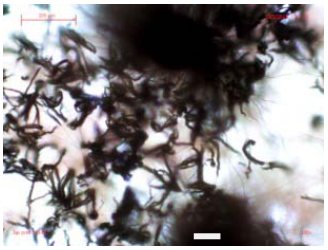


Fig.9.73 Conidia growing over perithecia, formed a second layer of deposit. Scale bar: 100µm.

The morphology indicated two distinctly different fungi.

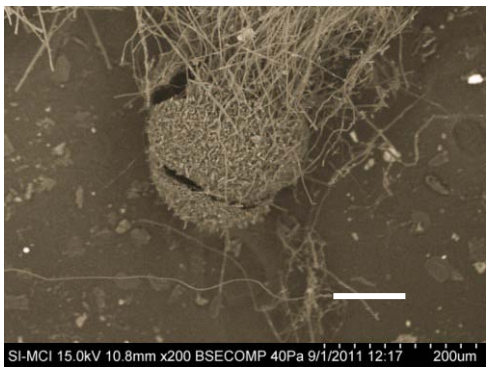


Fig.9.74 An isolated fruiting structure, perithecium. Size of individual perithecia, ca 300µm. SEM-VP micrograph. scale bar: 100 µm

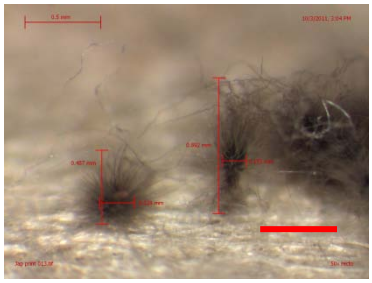


Fig.9.75 Varied sizes of perithecia vertically growing from the surface of paper. Some were suspended in the air. Scale bar: 0.5mm

Fig.9.76 Ornamentations on the surface of perithecial hairs. Hairs' filament diameter: 2.10µm-3.60µm. scale bar: 50µm

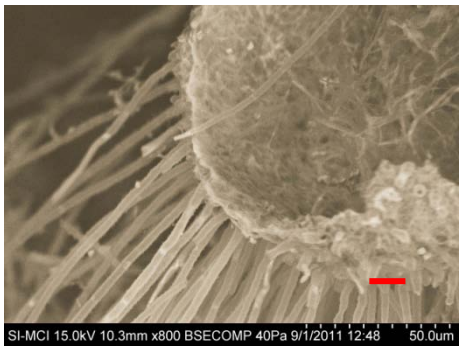


Fig.9.77 Morphology of the perithecial shell. Interior was filled with spores. SEM-VP micrograph. Scale bar: 10µm

9.4 Fungal stains patterns in studied artworks

9.4.1 Interfaces of fungi and paper in examined artworks

The interfaces of fungi and cellular substrate, namely paper, in all three examined artworks were investigated using various analytical instruments to explore correlations between paper structure and composition and formation of fungal growth and pigmentation. The observations on macro- and microscopic level permitted preliminary conclusions, as follows:

Trends in macro-structures

1. Fungi grew in areas where paper was exposed to water. That is supported by evidence by biodeterioration of paper No 2 (1920 Engraving) and No 3 (1847 Japanese print). In case of biodeterioration of paper No 1 (17th c paper) must be assumed that it occurred as a result of exposure to water.
2. Distribution of fungal elements was random without any particular pattern on all papers.
3. Concentration of fungal deposits was observed on recto (top-side) and verso (reverse) surface of paper in all three studied cases.
4. Fungal stains are caused by pigmented cells congregating in colonies, or more precisely in groups of cells (see definition of colony in glossary); large pigmented fruiting structures on the surface of paper or partially embedded into matrix; or as secretion of pigments to the substrate.

Trends in microscopic formations

5. Microscopy of fungal interactions with substrate indicated tendency of fungi to grow towards air pockets and pores in paper.
6. Fungal elements used paper fibers as support of their attachment, similar to anchorage. That applies to single cells, chains of cells or clusters of cells.
7. Penetration of fungal filaments through the paper bulk leads to growth and stains formation on both sides of paper. However, mycelial growth in bulk is less prominent than on the surface of paper.

Investigation of such complex problem as biodeterioration of paper requires multitude of instruments and multidisciplinary approach. Each technique and methods shed light on some aspects of fungal interactions with the substrate. Although uniform conclusions are not possible due to variations of outcome resulting from changes of even one variable within that interrelated and co-depended set of systems, some similarities can be observed in fungal interaction with paper substrate, as illustrated by include images.

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 fibrous structure of paper enabled expansion of hyphae.

9.4.2 Observed patterns of stains' formations

1. **Paper No 1: the 17th century Study Sheet.**

Two types of stains: A- scattered, black stains, B - fungal inclusions.

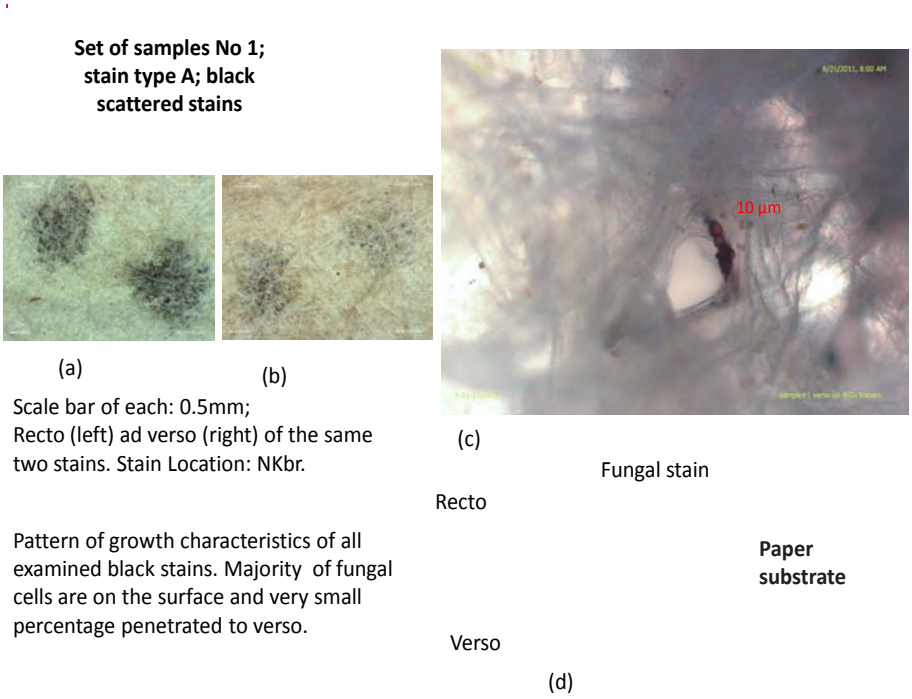


Fig. 9.77 Deposition of pigmented fungal elements in the black, scattered stains type A, on the 17th century Study Sheet.

The stains are formed by congregation of dark pigmented cells interacting in different ways with paper fibers and paper matrix (bulk). (a) recto, (b) verso, (c) micrograph of fungal cells migrating from recto to verso, (d) a diagram illustrating pattern of stains' formation on recto and corresponding stains on verso by migration of mycelium from one side to the other. H. Szczepanowska

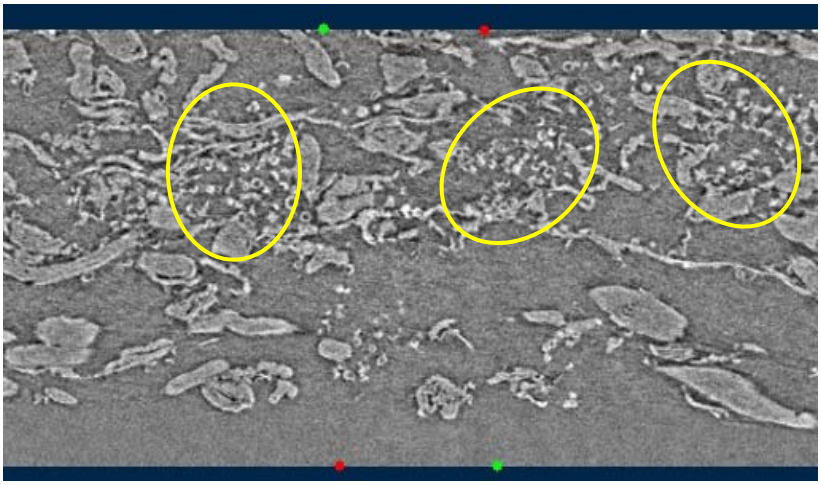


Fig. 9.78 Cross-section of stain type A obtained on X-ray microtomography.

Fungal cells are congregated near the surface, as it was predicted. Cells are scattered among paper fibers and only the areas of their large accumulation are marked.

Paper No 1: the 17th century Study Sheet

Stain Type B- fungal inclusions

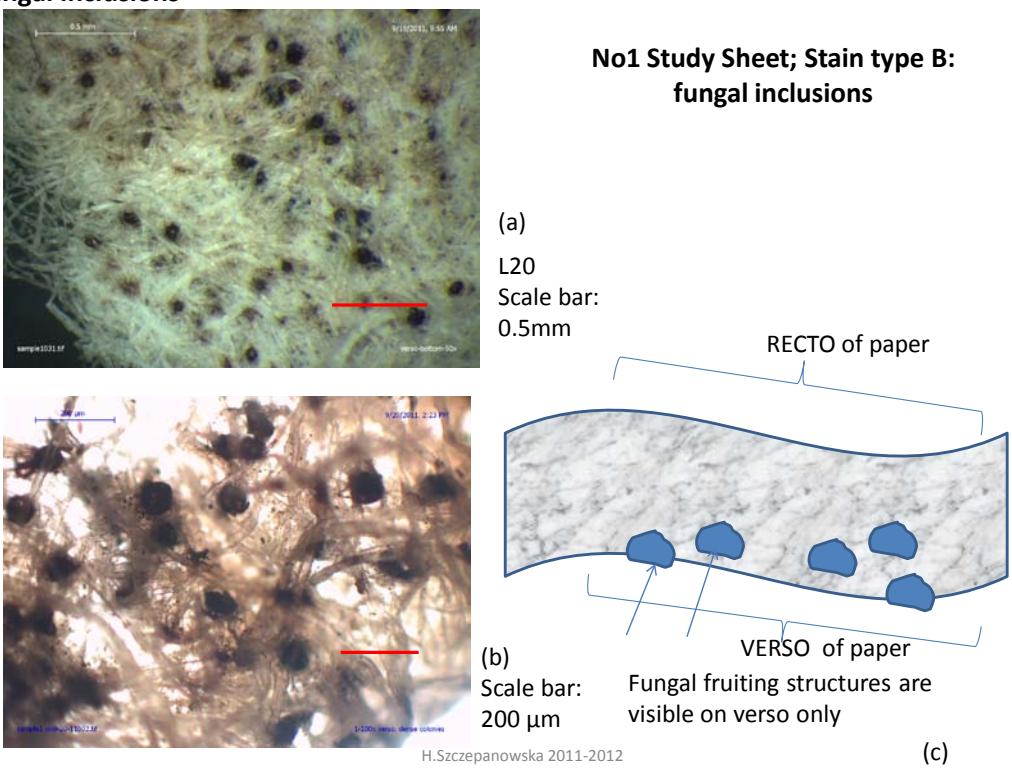
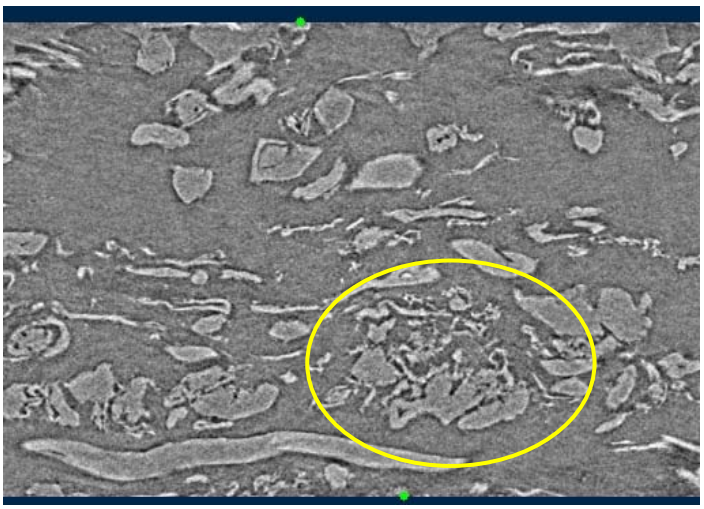


Fig. 9.79 Individual fruiting structures in paper matrix formed another type of staining in the 17th c paper , by clustering of pin-hole like black inclusions. (H. Szczepanowska 2011)

Fig. 9.79 (a) stereo-micrograph of individual black fruiting structures as they appear on paper, (b) transmitted light micrograph of the structures showing their location in paper matrix, (c) diagram derived from investigation of stains, which indicated their location near the surface of paper.

Fig. 9.80 Cross-section of L20 area with fungal inclusions obtained on X-ray micro-tomography shows accumulation of cells in round formations. However, there presence is not as clearly captured as in other cases.



Black stains, Type B-individual inclusions; fig. 9.81-9.84 H. Szczepanowska

Interactions of fungi with paper matrix

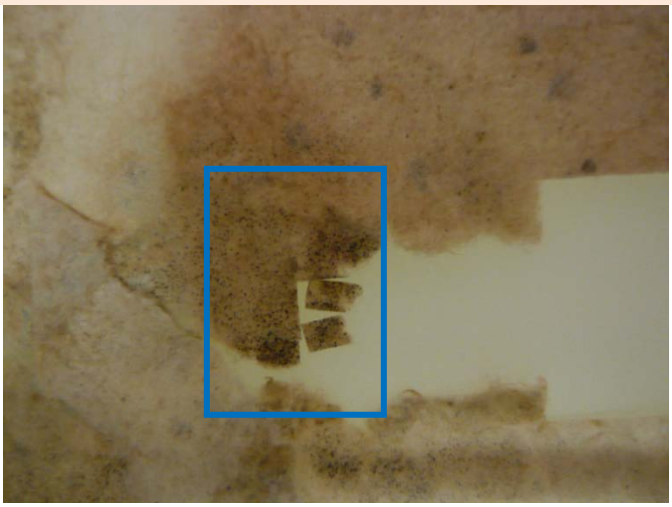


Fig.9.81 Black inclusion of individual fruiting structures in paper visible as pin-hole –like spots.

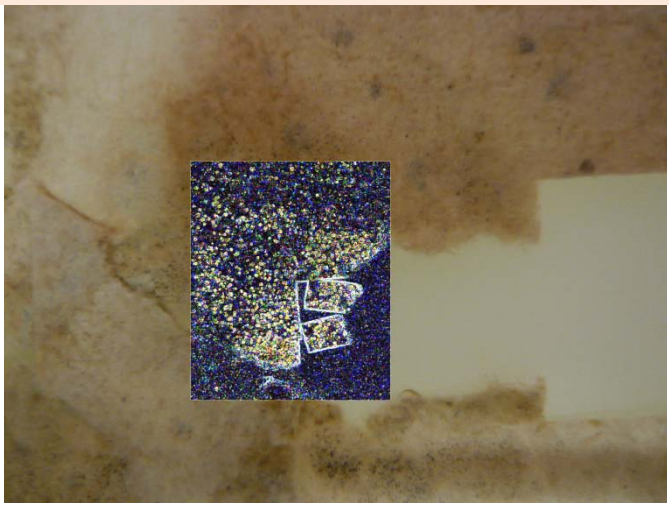


Fig. 9.82 Enhanced image, processed in imageJ program emphasized the fungal stains. Processing in 0-8Gama in 10pixel radius spots.

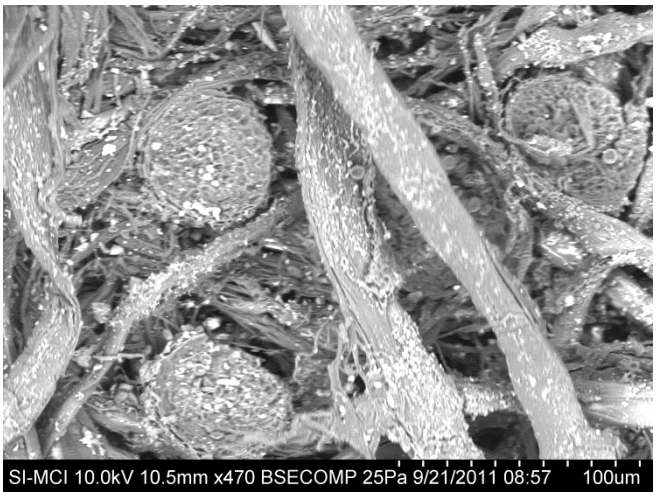


Fig. 9.83 Fruiting structures are anchored to paper fibers and embedded in the paper matrix.

Depositions of the fruiting structures, pycnidium is near the surface, yet protected by fiber matrix from removal by external forces. The attachments are facilitated by mycelium, which differs these stains from the scattered stains type A, formed by individual cells.

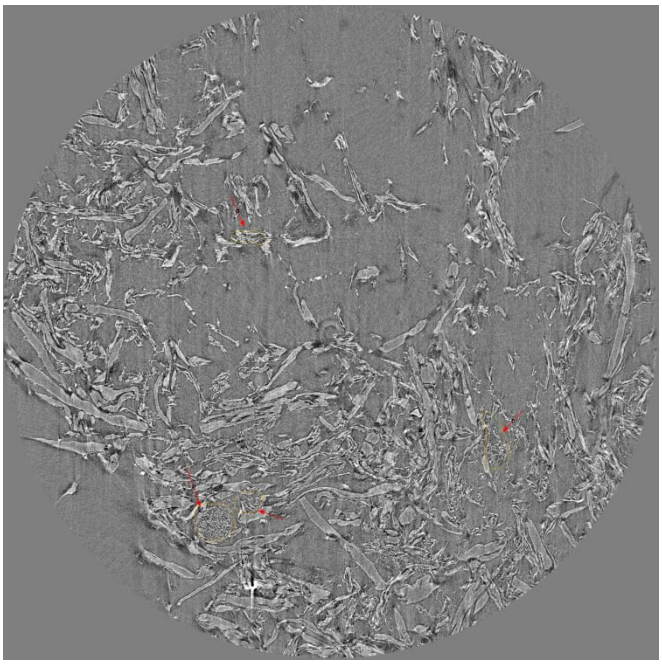


Fig. 9.84 Tomograph image obtained in X-ray micro-tomography, showing clearly spherical inclusions, marked here with red arrows.

Paper No 2: 1920 Etching; Fig. 9.85, 9.86 H.Szczepanowska

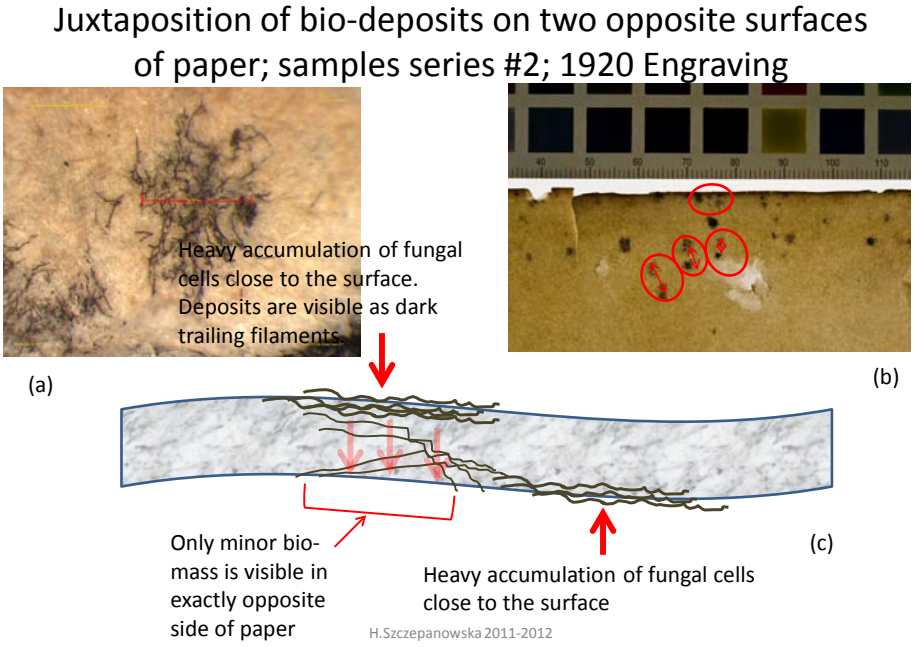


Fig.9.85 Formation of stains differs on each side of paper as a results of fungal growth pattern when migrating through the bulk.

In this case, fungi grew in juxtaposition on both sides, as illustrated on (b) and diagram (c).Consequently one side of paper shows more prominent stain that the other. However, that combination of juxtaposition pairing of stains applies equally to both sides of paper.

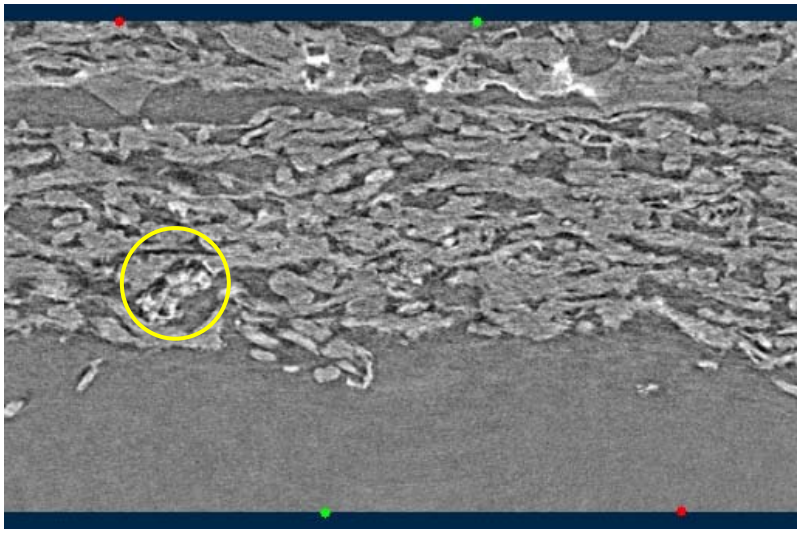


Fig. 9.86 In a cross section of No2 paper only clusters of cells were recognized, marked here in yellow circle. Chains of cells were not detected.

Paper No 3: 1847 Japanese woodblock print

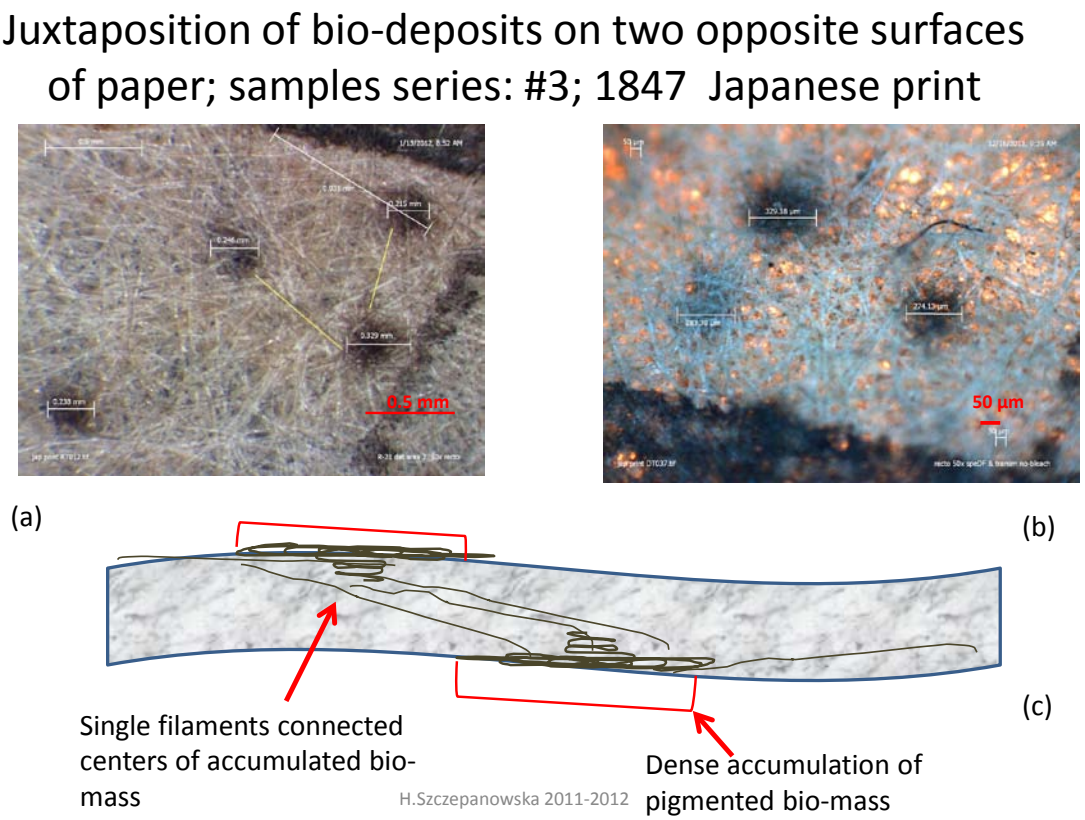


Fig. 9.87 Black deposit underneath of each fruiting body that grew on the Japanese print resulted from deposition of each structure. Interconnecting filaments were also dark pigmented. Path of the connecting filaments is marked on (a) in yellow. Stains, similarly to those on paper 2 were forming nearby, but in juxtaposition on each side of paper as illustrated on (b) and in diagram (c).

9.4.3 Summary points

1. Deposition of black fungal pigmentation appears to be more intense on the surface of paper rather than in bulk.
2. Pigmented stains show preference to form in juxtaposition on both sides of paper, rather than forming next to each other or in a parallel position.
3. The pattern of stain formation is directly related to fungal growth requirements, their preference to grow on the surface and securing enough space around each colony or fruiting structure.

10. Removal of fungal deposits from paper

10.1 Currently used methods of fungi eradication

Reports dealing with biodeterioration of cultural material are primarily focused on describing the observed forms of biodeterioration and very limited number of researchers suggests removal of fungal deposits. The most important publications are listed in Table IX.

Table IX A review of selected publications dealing with biodeterioration of cultural heritage

Year	Reference
1981	Rose, A.E. (ed) *Microbial Biodeterioration.* Economic Microbiology. AcademicPress, London.
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.
1993	Gorbushina, A.A., Krumbein, W.E., Hamman, C.H.,. Panina, L, . Soukharjevski, S., and Wollenzien, U. Role of black fungi in color change and biodeterioration of antique marbles. *Geomicrobiology Journal,* Vol.11, Issue 3 &4: 205-221.
1994	Szczepanowska, H., Moomaw H.W. Laser stain removal of fungus induced stains from paper.*Journal of the American Institute for Conservation* (JAIC) 33: 25-32.
1997	Sterflinger, K. Krumbein, W.E. Demactiaceous fungi as a major agent for biopitting on Mediterranean marbles and limestone. *Geomicrobiolgy Journal*, 14 (219):210-230.
2000	Karbowska-Berent, J., Strzelczyk A.B.,T*he Role of Streptomycetes in the Biodeterioration of Historic Parchment*. Wydawnictwo Universytetu Mikolaja Kopernika., Torun. Poland.
2000	Cifferi, O., Tiano, P., Mastromei, G. *Of Microbes and Art; The Role of Microbial Communities in the Degradation and Protection of Cultural Heritage*. Kluver Academic Press, Plenum Publishers.
2000	Szczepanowska,H. Cavaliere, A. R. Fungal Deterioration of 18th & 19th century documents: A case study of Tighlman Family collection, Wye House, Easton MD". *International Biodeterioration and Biodegradation*, Elsevier 46: pp.245-249.
2002	Florian, M-L. Fungal Facts. Solving Fungal Problems in Heritage Collections. Archetype, London.
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.,(2003) 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 models study with laser wavelength of 532nm. 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 Neetherlands. (no treatment papers included)

2004 Allsopp, D., Kenneth, S., and Gaylarde, Ch. *Introduction to Biodeterioration,* Second Edition. Cambridge University Press.

2002 Szczepanowska, H. and Cavaliere, R. A. Tutankhamen tomb – a closer Look at Biodeterioration, Preliminary Report", pp., in: *Schimmel –Gefahr fur Mensh und Kultur durch Microorganismen; Fungi, A threat for People and Cultural Heritage through Micro-Organisms*, A.Rauch, S.Miklin-Kniefacz, A.Harmsse (Eds). Thesis, Verband der Restauratoren: 42-47. (5 contributors out of 37 suggested treatment strategy).

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.

2008 Caneva, G., Nugari, M.P. Salvadori, O. (eds) *Plant Biology for Cultural Heritage. Biodeterioration and Conservation.* The Getty Institute, los Angeles.

The focus in the majority of the listed references is on the study of biodeterioration symptoms and description of the effect it has on materials. The recommendations call for preventive actions such as control of the relative humidity in the environment. The actual treatments are restricted to mechanical removal of the bio-mass. Laser removal of fungal stain which I proposed in 1994 was replicated in 2003 by Pilch et al, using the same wavelength irradiation (532 nm)and the set of microorganisms. The obtained results conformed my original findings. Those two actual treatment methods seems mechanical and laser irradiation are the actual conservation methods.

Considering that fungal structures are intricately intertwined with paper matrix, the mechanical removal from artworks seems like the only realistic option at this time. An illustration of such a treatment is included in the section 10.2. Understanding better the pigment secretion by fungi and associated secretion of acids one has to accept the limitations and irreversible damage that accompanies biodeterioration.

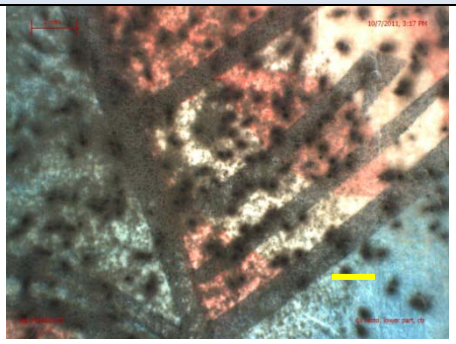
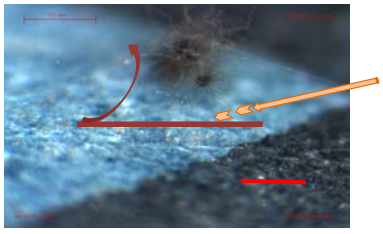
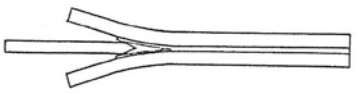
10.2 Proposed methods of bi-mass removal derived from this study

Considering that the fungal mass is embedded in paper matrix, it is proposed to remove the aerial mycelium by application of physical forces. Scalpel, tweezers and brush are suggested as removal tool and breaking the weak bond between fungi and paper substrate.

Mechanical removal of the bio-mass (aerial mycelium and perithecia) fig. 10.1-10.7

H.Szczepanowska

Fig. 10.1 Location: NO-29-30 area overgrown by several different fungal species.

Perithecia-solid particles; bio-mass before treatment	Mechanical removal with tweezers and brush	Result of mechanical removal
 **Fig.10.2 Perithecia-solid particles; bio-mass before treatment. scale bar: 2mm.**	 **Fig. 10.3 Heavy accumulation of perithecia was removed with tweezers.**	 **Fig.10.5 Underneath of each perithecial head black residue was left embedded in paper fibers. Scale bar: 2mm**
The objective of mechanical (vs chemical removal) was to prevent spores from spreading out on paper and/or smudging pigmented fruiting structures on the surface.	 **Fig.10.4 Perithecia in less densely accumulated areas were lifted with a brush. (This image shows verso).**	
Mechanical forces involved in the separation can be compared to the Boeing wedge test, used for adhesion assessment. Metal wedge is forced between two adherent surfaces. Forces applied in microbial separation are incomparably smaller.	 **Fig.10.6 Application of mechanical force in the removal of perithecia. scale bar: 0.1mm**	 **Fig.10.7 The Boeing wedge test for adhesion assessment. (*J.Comyn, Adhesion Science, 1997, fig.9.11, p.123).***

PART III. 11. CONCLUSIONS

The biodeterioration of heterogenous cellular material such as paper is an enormously complex subject. This research was designed to advance the understanding of some of the phenomena of biodeterioration, with foci on interfaces of fungi and paper, surface modifications caused by fungi and role of the environment in these processes. In spite of the known deleterious impact of biodeterioration on cultural heritage material no such investigation has been reported. This, therefore, is an initial attempt at mapping areas of further research leading to the prevention and the treatment of biodeterioration. A systematic protocol of the examination proccess was formalized and new techniques were utilized to explore substrate-fungi interfaces. Conclusions follow. They were derived from the findings and suitabity of new techniques used in this investigation. Suggestions regarding prospective research are also provided.

This list of conclusions outlines areas which have the greatest impact on the understanding of biodeterioration process, arranged in three catagories:

I. Methods and techniques of investigation
II. Findings derieved from the experimental work
III. Challanges of working with biodeterioration of heterogenous cellular material

11.1 Methods and techniques of investigation

- **Interdisplinary nature of investigation** is the key towards achieveing a better understanding of biodeterioration processes. Among the main disciplines which lend their experties are mycology, physics, biological physics and optics, tribology, biochemistry, engineering especially mechanical engineering.
- **Multiscale and multivariant types of investigation** provide complementary informaton about responses of microorganisms, characteristics of materials on which they grow and interactions between materials.
- **Traceology is a novel approach** in the investgation of biodeteriorated paper and living systems on paper. It reveals interdependences between surface topography and microbial responses, such as patterns of growth and pigmentation.
- **New technqiues** which were involved in this investigation include: confocal laser scanning microsopy, white light confocal profilometry and X-ray microtomography. Each one supplements the traditional techniques with more accurate and repeatable measurements of living systems' interactions with paper substrate. None of these techqniequs were reported as being used in the investigation of biodeterioration of cultural heritage materials.

11.2 Results of the experimental work

- **All factors involved in biodeterioration work in synergy:** the environment, types of microorganisms and characteristics of substrates undergoing biodeterioration. Alteration of a single condition changes the outcome.
- **Monolayer of liquid water is the single essential factor** that initiates new life of microorganisms and, consequently, biodeterioration of artifacts.

- **Presence of sizing in paper plays a much lesser role** in supporting fungal growth that has been reported in literature.
- **Light** conditions under which fungi grew affected their growth patterns and intensity of pigmentation.
- Response to light of the study organism (*Aspergillus niger*) indicated that **intense growth and pigment production in daylight light** (versus cultures grown in dark) were associated with **physical deterioration of paper.**
- **Light alters chemial composition of paper** as it was exemplified by formation of calcium oxalate; reaction of oxalic acid with calcium carbonate filler (Winsor&Newton paper). Crystals formations were different in light from those formed in dark-grown cultures. Calcium oxalate, although known to be associated with fungi, has been reported only in one case as occuring on paper. No report was found as to light effect on crystals formation on paper.
- **Interfacial interactions** between paper and fungi revealed **fungal tropism,** growth towards or away from an environmental stimulus. The following examples were found: thigmotropism, a reaction of fungal hyphae to topography of surfaces; phototropism, a response to light, and chemotropism, a growth towards airy pores in the paper structure. These fungal behavioral trends attributed to tropism were recognized in historic papers and on cultured stains. No reference has been found so far in literature to tropism of fungal growth in the context of cultural heritage material.

It is important to note that the microbial responses to the environment differ and are species specific, therefore the above observation derived from the experimetal work (Light Experiment) can be cited only in the context of the studied microorganism, *Aspergillus niger.*

11.3 Challanges of working with biodeteroration of heterogenous cellular materials

- The most important factor affecting biodeterioration is interaction of paper and water. It is still largely unknown phenomenon fom a physics stand point.
- Limitation of sampling material from original artworks provides scarce material to analyze.
- Continous changes in living systems of fungal communties result in observations of a specific point –in-time in life of a microorganism.
- The mechanisms of microbial adhesion to surfaces, the most crucial event in microbes life and essential for initation biodeterioration, are still largely unknown.

11.4 Implications of finding towards preservation of cultural heritage material.

Some aspects of the above findings have practical implications relating to the preservation of cultural heritage materials, specifically paper-based. The most important is the ability to predict the course of deterioration ratio of paper collections that were exposed to water, during flooding or usage of water in fire- rescue operations. Paper which is more absorbent will be affected by fungi before this which is sized, such as watercolor papers. Intensity of pigmentation will be initially

greater on the absorbent papers. However, the longer paper remains wet, the greater the pigmentation and stain intensity will be. Therefore, time is critical in designing rescue operations of flooded collections and those wetted during fire suppression.

Regarding the removal of fungal deposits from infested objects of cultural value the evidence of this investigation pointed to intricate interrelations between fungi and paper matrix. The removal of aerial mycelium from the surface by mechanical means, as illustrated in one case of the Japanese woodblock print, improved aesthetics of the print by revealing the image obscured by fungi. However, the fungal structures remained in the paper matrix. A better understanding of the interrelationships between light and fungal response may provide another option for deactivation of fungi in the matrix.

11.5 Prospective new line of research

Many aspects of fungal interactions with cellular material in the context of cultural heritage remain unexplored. As pointed earlier, a concerted multidisciplinary effort is necessary to resolve one of the most important phenomena in the biodeterioration processes: **physics and dynamics of water transport in paper.**

Research of any aspect of environmental factors altering fungal response, in particular production of bio-pigments, will benefit preservation efforts of cultural heritage material. Considering restrictions of sampling of historic and artistic works, and the protenacious nature of pigmented fungal secretions, the application of **proteomic investigation** appears to be a suitable choice. It is an attractive method because it requires minute amounts of material to obtain meaningful results.

Instrumental traceology techniques used in this investigation should be explored further to realize their full potential. However, light absorption phenomenon in fungal species needs to be studied first and in conjunction with the usage of these instruments to properly interpret the obtained data.

The current state of knowledge of biodeterioration processes in cultural heritage material is very limited, therefore, further investigation of any aspect of material interaction with fungi is likely to advance and benefit the field of preservation.

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"Winsor & Newton Artists Watercolour Paper was developed to the highest specifications of Winsor & Newton in the early 1980s. Each sheet of fine art paper is brilliant white, acid-free, 100% cotton, buffered and mould made with four deckled edges. Winsor Newton watercolor paper is available in cold pressed, hot pressed and rough surfaces and weights of 140lb. or 300lb. In addition to the internal sizing, the gelatin surface sizing produces unsurpassed brilliance from the watercolor paint that is applied to it."

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www.pnas.org.
www.ars.usda.gov/services/site_publications.htm; accessed on 7/5/2011
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Doc Kaiser's Microbiology Home Page Copyright © Gary E. Kaiser; Updated: July, 2008
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www.ars.usda.gov/services/site_publications.htm
www.mycobank.org
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http://www.microbelibrary.org/
http://paper.lib.uiowa.edu
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APPENDIX

I. Glossary of terms
II. Paper rigidity measurement
III. Paper Tests Protocol: pH, gelatin sizing and starch sizing in paper
IV. Light Experiment: fungi culturing, experiment protocol and set up
V. SEM-EDS report; paper surface altered by fungi ; and SEM-EDS report; red pigment on Study Sheet No1.
VI. XIII. Set of recently presented and published papers on bio-deterioration:
    a. ICSM Annecy, France 2012 (Conference Proceedings); Bio-aerosols, Saratoga Springs NY,
    b. 2011 (Conference Proceedings, in press); MRS 2011 (published)

Appendix I. Glossary of terms

1. Glossary of fungal terms
2. Glossary of paper terms

Appendix I.1Glossary of Fungal Terms

Compilation from the following sources:

http://www.fungionline.org.uk/6asexual/7thallic.html
WordNet 3.0, Farlex clipart collection. © 2003-2008 Princeton University, Farlex Inc)
http://bugs.bio.usyd.edu.au/learning/resources/Mycology/Glossary/glossary_a_b.shtml

http://botanydictionary.org/pycnidium.html

http://www.britannica.com

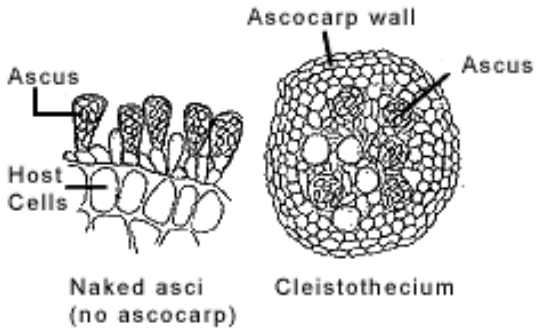
McGraw-Hill Dictionary of Scientific and Technical Terms, 6th edition, published by The McGraw-Hill Companies, Inc. 2003

Ascus: A sac-like structure containing ascospores. Karyogamy and meiosis results in the formation of 4 but usually 8 spores (ascospores) per ascus.

Acervulus (pl. Acervuli): a mat of hyphae giving rise to short conidiophores closely packed together forming a bed-like mass.

Ascomycota is the largest division of fungi. The orders suggested by Schoch et al (2009) include: Sordariomycetes, Laboulbeniomycetes, Leottiomycetes, Lichinomycetes, Lecanoromycetes, Eurotiomycetes, Dothidiomycetes, Arthoniomycetes, Orbiliomycetes, Pezizomycetes, Saccharomycetes, Schizosaccharomycetes, Pneumocystidiomycetes and Noelectomycetes. This hypothesis is based on 434 taxa. As the division contains many fungi, the classification will, inevitably, be expanded and refined as further information is included.

Ascocarp: a fruiting body containing ascospores produced within more asci. (See cleistothecium, perithecium).



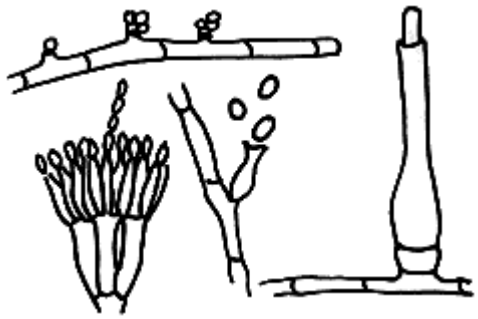
Cleistothecium: A type of ascoscarp characteristic of fungi in the Plectomycetes. A globose structure containing asci with no specialized opening to the exterior.

Haustorium: an organ produced by a parasite that absorb snutrients from its host. Most commonly the term refers to the hyphal extensions formed by obligate fungal parasites which invade individual host cells.

Ostiole: a neck-like structure terminating in a pore surmounting an ascocarp or pycnidium

Perithecium: a closed ascocarp with a wall and an ostiole . Examples of perithecia are illustrated 1-3.

Phialide: a particular type of flask-shaped cell that gives rise to conidia in basipetal fashion.



Examples of phialide forms.

Pycnidium (pl. pycnidia):

An asexual fruiting body partially lined inside with conidiophores. It is produced by fungi in the class Deuteromycetes. Differences in conidiomatal structure traditionally have been used to separate three orders of Deuteromycetes, the Melanconiales, the Sphaeropsidales, and the Pycnothyriales. However, differences in the ways that conidia are produced are now used in classification and identification. **(*McGraw-Hill Dictionary of Scientific and Technical Terms*, 2003).** Pycnidia bear

spores (conidia) variously known as pycnidiospores, oidia, or spermatia. The spores are liberated through an opening (ostiole) in the pycnidium.

Rhizomorph; a thick strand of organized hyphae resembling a fine root used for anchorage.

Saprophyte: an organism which obtains its organic nutrients in solution from dead or dying tissues of any other organism.

Seta (Setae) a bristle-like hair, exemplified by Chaetomium

Sporangium(a): A specialized enlarged cell containing sporangiospores. It is a sac-like structure in which cytoplasm is mitotically divided by walls to form thousands of spores.

Spore -One-to several-celled propagule of totipotent cytoplasm with cell walls, produced by cell division. (Thomas Volk Encyclopedia of Biodiversity, Vol.3, 2001)

Thallic conidia: Conidia formed as a result of the septation and fragmentation of a hypha.

Thigmotropism is a movement in which an organism moves or grows in response to touch or contact stimuli. The prefix **thigmo-** *θιγμος* comes from the Greek for "touch".

Appendix I. 2. Glossary of Paper Terms

D**ielectric** In general physics a substance or medium that can sustain a static electric field within it, or a substance or body of very low electrical conductivity; insulator. Source: The American Heritage® Dictionary of the English Language, Fourth Edition copyright ©2000 by Houghton Mifflin Company. Updated in 2009. Published by Houghton Mifflin Company.

Grammage The weight of paper expressed as grams per square meter.

Imbibitions *Chemistry* Absorption of fluid by a solid or colloid that results in swelling. Source: The American Heritage® Dictionary of the English Language, Fourth Edition copyright ©2000 by Houghton Mifflin Company. Updated in 2009. Published by Houghton Mifflin Company.

Stochastic is an adjective that refers to systems whose behavior is intrinsically non-deterministic, sporadic, and categorically not intermittent. Stochastic character- pertains to a process involving a randomly determined sequence of observations each of which is considered as a sample of one element from a probability distribution. *Online Etymology Dictionary*. Retrieved July 23, 2012, from

Dictionary.com website: http://dictionary.reference.com/browse/stochastic This adjective was used in relation to randomness of paper matrix.

Surface area It is the measure of how much exposed area a solid object has, expressed in square units.

Surface energy quantifies the disruption of intermolecular bonds that occur when a surface is created. In application to solids, surfaces must be intrinsically less energetically favorable than the bulk of a material. The surface energy may therefore be defined as the excess energy at the surface of a material compared to the bulk.

Turgor Pressure It is a force per unit area exerted outward on a plant cell wall by the water contained in the cell vacuole.

Sizing An additive to paper which purpose is to modify paper's characteristics, usually to make it more resistant to water .

Appendix II. Paper rigidity measurement

Date: February 26, 2012

Purpose: Evaluation of paper directionality, anisotropy, behavior in the same RH conditions.

Environment: The environment in which papers were conditioned was monitored for over 24 hrs. Readings are included in a separate graph.

Principle: Measurements are taken in both directions when paper is bent under its own weight.

Procedure:
Each paper was weight at one side , length and width at a time, and the distance from the plane was measured in cm. Measurements were taken at 1cm off the top edge, at 10cm and at the bottom of the sheet.

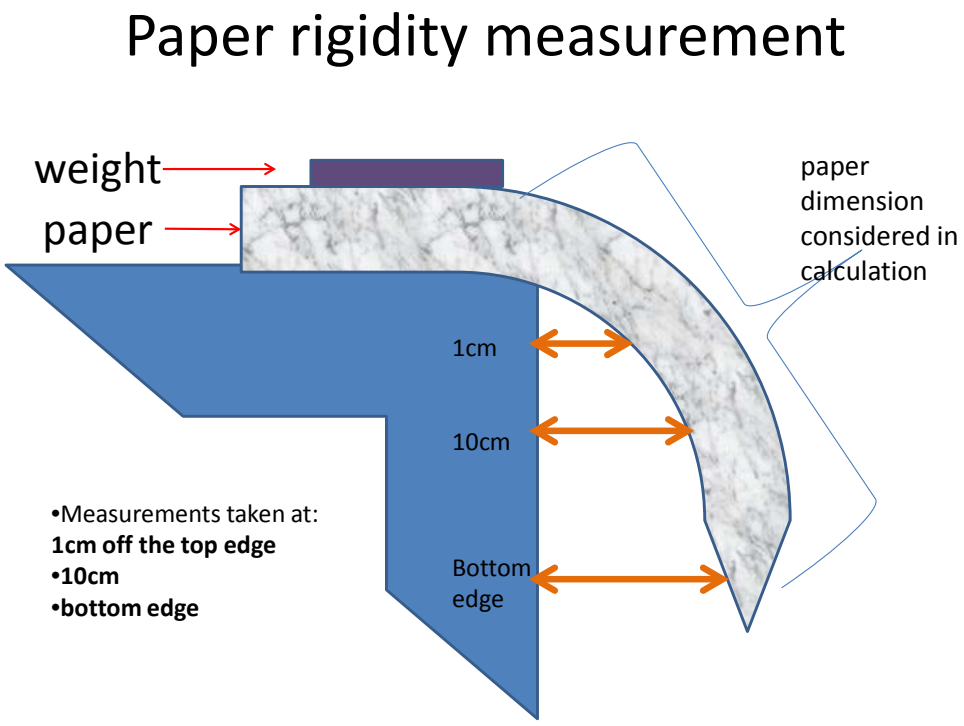


Fig. A.1 An illustration of the method of measuring rigidity of paper. ©H. Szczepanowska, 2012

Measurements of each sheet were taken in cm.

Sample/ direction	W4-C	WN-C	No1:17th c paper	No 2: 1847 Jap print	No 3: 1920 Etching
dimensions	H: 35.5 W: 27.5	H: 36 W: 28	H: 23.5 W: 17	H: 34.3 W: 24	H: 35.2 W: 27.5
Effective length in test	H:33.5	H: 32.5	H: 22	H: 32	H: 33
Bent along short edge	@1cm from top : 1.5 slop @ 10cm: 3.5 @ bottom, both sides: 3.5 @ctr: 3.5	@1cm: 1.2 @ 10cm: 10 @ bottom both sides: 22 @ctr: 22	@1cm: 1.5 @ 10cm: 3.5 @ bottom L: 3.5; R: 3 @ctr: 3	@1cm: 2 @ 10cm: 3.5 @ bottom, both sides: 4 @ctr: 55	@1cm: 0.7 @ 10cm: 1.5. @ bottom L: 4 R:5.5 @ctr: 5
Effective width in test	W: 25.5	W: 26	W: 15.5	W: 22.5	W: 26
Bent along long edge	@1cm: 2 @ 10cm: 5..5 @ bottom, both sides: 7 @ctr: 7	@1cm: 1.5 @ 10cm: 3.5 @ bottom, both sides: 3.5 @ctr: 3.5	@1cm: 1 @ 10cm: 5.5 @ bottom L: 6; R: 4 @ctr: .5	@1cm: 1.5 @ 10cm: 3.5 @ bottom, L: 5 R: 4 @ctr: 3	@1cm: 1. @ 10cm: 2.5 @ bottom, L: 4; R: 1.5 @ctr: 3

Discussion

Bending or folding pattern is affected by additives to paper, such as hinges in case of Japanese print and general paper characteristics. The 1920 Etching showed different behavior at the edge infested with fungi. That change was caused either due to the presence of adhesive, or due to additional bio-deposits in paper. The 17th century paper has been sampled extensively, is uneven in thickness, some areas are skinned others are intact; it is also severely creased. The creases affected the way paper was bending, unevenly at each end of paper.

Appendix III. Paper Tests' Protocols

1. Test of starch sizing
2. Test of gelatin sizing
3. Measuring pH level of paper

Appendix III.1 Test for starch sizing

Test date: February 23, 2012 **Purpose:** **test for presence of starch sizing.**

Principle: A solution of iodine (I_2) in potassium iodide (KI) will turn dark blue in contact with starch, thus indicating its presence.

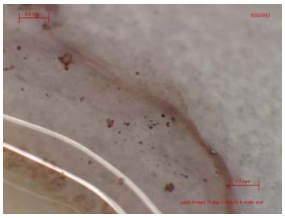
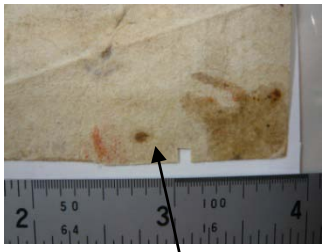
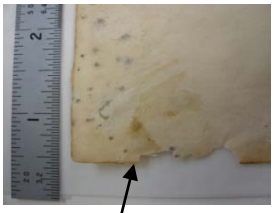
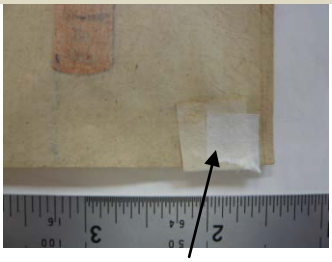
Equipment:

- Micropipette
- Test wells (for paste used as standard)
- Balance
- Reagents

Reagent preparation:

1. Prepare iodine/potassium iodide (KI_3) solution: add 2.6g potassium iodide to 5mL laboratory water and add 0.13g of iodine (I_2).
2. Wait until iodine is fully dissolved and add 100mL laboratory grade water.

This test is very sensitive and a few fibers of paper are sufficient to use as indicators of starch, if present.

Control-paste	No1	No 2 1920 Engraving	1847 Jap print
 	 Change of color became evident quickly, however not purpose but dark, brownish-red.	 Color change was semi-quick and not very intense. The area of adhesive residue should be starch-based.	 Hinge which most likely was attached with paste was tested for starch. The results did not show as color change immediately. The color was dark red, the same as the control paste.
conclusions			
Paste that was cooked at 50C and 75C and uncooked starch were tested as controls. The results were not consistent with instructions, or anticipated outcome.	Starch is most likely is present as sizing. Areas of glue splotches did not react which indicates that splotches are non-starch adhesive.	Starch should be present, and most likely is. Slow color change may indicate decomposition of starch during cooking.	Starch should be present, and most likely is. Slow color change may indicate decomposition of starch during cooking.

Appendix III.2 Test for gelatin sizing

Date: February 24, 2012 **Purpose:** **Protein test, to determine presence of gelatin.**

Principle: The reagent BCA forms a purple-blue complex with Cu^{1+} in alkaline environment providing basis to monitor the reduction of alkaline Cu^{2+} by proteins.

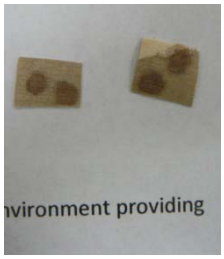
Reagents: BCA is Bicinchroinic Acid Solution. Source: Sigma BCA1 B9643. The selection of the reagent was made based on comparing the features of reagents offered by Sigma.. The test procedure follows recommendations of Sigma.

Reagent A: 1,000ml solution of BCA mixed with sodium carbonate, sodium tartrate and sodium bicarbonate o in 0.1 N NaOH. Final pH 11.25.
Reagent B: 25ml solution of 4% Copper (II) Sulfate Pentahydrate .
Protein standard: Bovine serum albumin –BSA

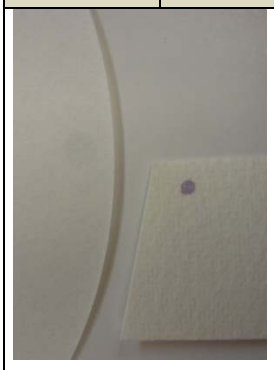
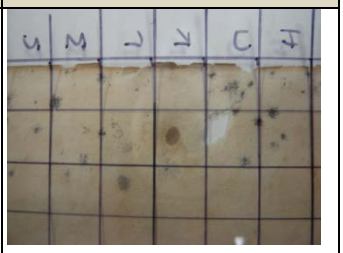
Reagent preparation:
Mix 50 parts of Reagent A with 1 part of Reagent B, until it is light green (left test tube).

Procedure:

1. Mix I part of protein sample with 20 parts BCA working reagent (A mixed with B).
2. Environment: 37°C , color develops immediately and can be accelerated at higher temperature.
3. The absorbance at 562 nm is recorded and the protein concentration is determined by comparison to a standard curve.



Test. Prepared reagent was applied on tested papers initially in the amount of 0.1µ L. It was reduced to 0.025 µL. Papers were very absorbent and test leaves colored spot on artworks.

W4-C	WN-C	No1:17th c paper	No 2: 1847 Jap print	No 3: 1920 Etching
				
W4-no sign of spot discolorati on- no presence of gelatin sizing.	Distinctly purple spot appeared immediately after test was applied.	(Reverse grid corresponds with verso of No 1 sheet). Distinct purple discoloration appeared, however not as strong as on WN paper.	Purple spot appeared immediately after reagent was applied.	(Reverse grid corresponds with verso of No 1 sheet). Distinct purple spot was visible right after application of the reagent.

Discussion

The test is very sensitive. It detects small amount of protein present, consequently the ration of false readings is greater than with low-sensitivity reagents.

On March 2, 2012, 7 days after the test was performed, Whatman 4 tested area kept in the laboratory book as evidence, showed discoloration, not exactly purple, however gray-purple. Knowing that Whatman paper should not contain any sizing, it is filter paper, I consulted with the protein expert, Dr. Moini at MCI what could be the reason behind the discoloration. He explained that proteins are everywhere, therefore in case of sensitive test; their presence will be indicated on the surface.

In light of the above the test can be reliable only is aseptic conditions. Such conditions are not realistically possible for artworks to meet.

Appendix III.3 pH Test

Test Date: March 3, 2012 **Purpose:** assessment of acidity level in papers

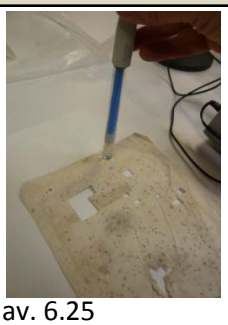
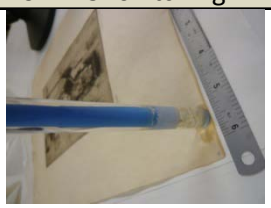
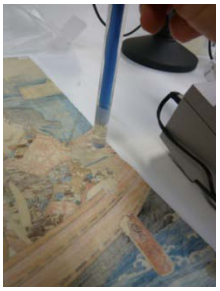
Principle: A measure of acidity or alkalinity of a substance, in solution. The negative of the base -10 logarithm of the hydrogen ion concentration in solution. For measuring paper surface, flat electrode was used., to ensure contact with the surface.

Equipment:

- pH meter Thomas Scientific TS 625 pH
- buffer solutions for calibration; pH 4 and pH 7
- pH of water used for test: 7.41

Procedure:

1. The pH meter was calibrated with buffer solutions.
2. Drop of 1 µl -2 µl was applied in four different places on the tested papers.
3. Reading as collected, as soon as the pH meter stabilized.
4. Temperature in the room: 23° C

W4-C	WN-C	No1	No 2. 1920 Etching	No3. Jap print
 av. 5.7	 av. 6.9	 av. 6.25	 Av.6.8	 av.5.55
5.84 5.80 5.99 5.64	6.80 6.83 7.05 7.01	6.33 6.21 6.32 6.27	6.55 6.95 6.89 6.91	5.74 4.77 4.81 6.91

Note: The Japanese woodblock print in this test was substituted by another from the same period. The original 1847 print was no longer available for study. It was infested by fungi, yet to much lesser extent than the original 1847 print.

Discussion

Representative areas were tested on each artwork:

1. with and without fungal residue
2. with and without adhesive, recto and verso

Based on the measured levels of acidity, there was no correlation between level of acidity and fungal infestation. In fact, the least infested artwork, made of good quality hand-processed paper fibers, the Japanese print showed the lowest pH indicating that it was the most acidic. Although acidity promotes fungal growth, other factors, such as quality of paper fibers and length of time of water availability will determine the intensity of infestation.

Appendix IV. Light Experiment

1. The experiment set-up
2. Fungal growth in relation to light exposure, summary of observations

Appendix IV.1 The experiment set-up

Experiment set-up

- **Two plates were inoculated with *Aspergillus niger*, along edge of two different papers**
  - **Whatman # 4 (W4)**
  - **watercolor paper, Windsor and Newton (W&N).**
- **Three samples from the study papers were placed on Petri dish to check their viability; # 1, # 3, # 4.**
- **Media used: agar-agar; source: Carolina Biological Supplier**
- **Light conditions:**
  - **Day Light DL (window seal)**
  - **Minimal Light ML, almost dark (fume hood)**
- **Period of observation: 13 days; 1-9 data collected on daily basis, last one collected on the 13th day.**
- **Observation began: June 23, 2011 ended: July 5, 2011**
- **Data collected:**
  - **Optical (images of growth progression) ; light microscopy of colonies**
  - **Environmental data: Elsec 764**

#Elsec 754 Environmental monitoring unit, collected envrio data throughout the duration of experiment

RH Radiation detection: Twin silicon photodiodes connected to single chip microprocessor.

Visible wavelength range 400-700nM (CIE response).
The available visible power range 0.1 - 200,000 Lux (0.1 - 20,000 Foot-candles).
UV wavelength range 300 - 400 nM.
UV proportion range 0 - 10,000 mW/Lumen.

RH range 0-100 %RH.

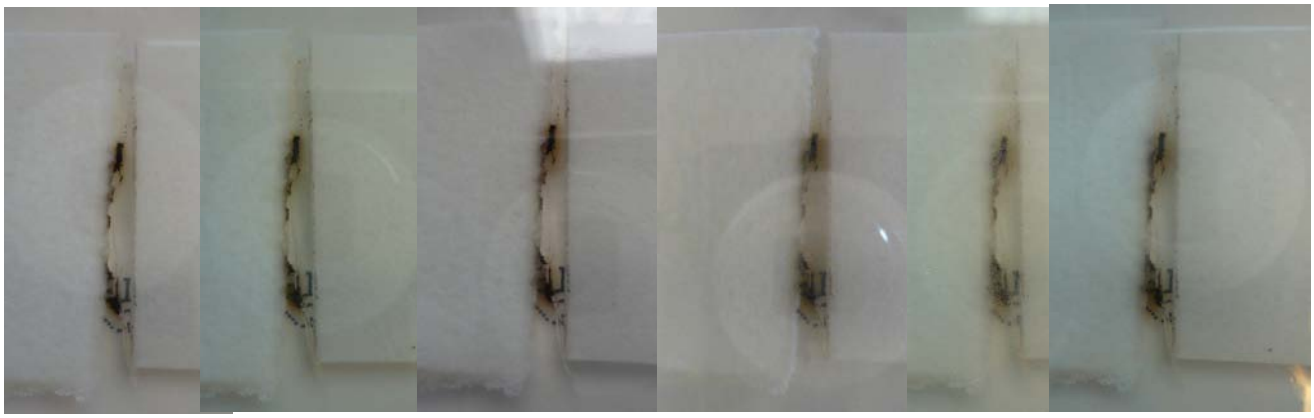
Temperature: 0.1 in Celsius or Farranheit

According to the manufacturer, accuracy is as follows:
•Light: 5% +1 displayed digit.
•UV: 15%
•Temperature: 0.5oC (0.9oF).
•RH: 3.5%.
range 0-100 %RH.
Temperature: 0.1oC or oF.

Appendix IV.2 Fungal growth in relation to light, summary of observations

Ex.No.2: Day 1; data collected every 2 hrs from the time of inoculation

DL1- (daylight) Petri dishes' centers, recto



9 am 10 am 12 14 16 18

Production of pigment by ***As.n***. was obvious after the first 2 hrs. Pigment was secreted to agar and migrated to paper. Note that water condensation began after 6 hrs of exposure to daylight, at 14 hr.

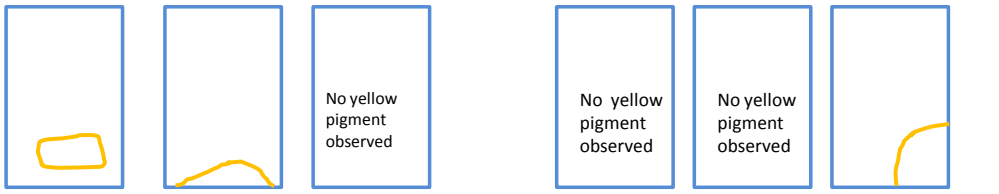
48 hrs cultivation (day 2)

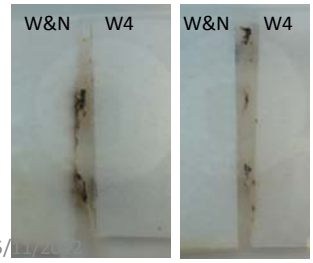
- Aerial mycelium grew along all inoculated areas.

To show the light gray growth images were taken on black background.

- Yellow pigment was formed with varied intensity on DL and ML samples of W&N papers only
- Black pigment migrated into paper, also on W&N samples

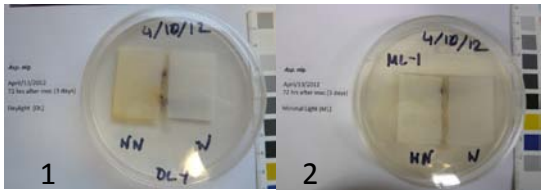
- DL1 DL2 DL3 ML1 ML2 ML3





5/[illegible]/2[illegible] 2 H.Szczepanowska, Research Conservator

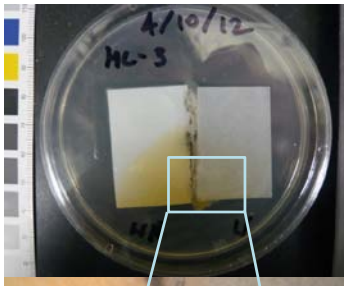
72 hrs cultivation (day 3)



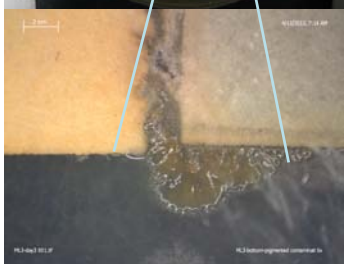
1 2

- Aerial mycelium developed on all plates with varied intensity (fig1, 2)
- Black pigmentation was only slightly more prominent on daylight cultures.
- Yellow pigmentation expanded on WN paper; it became clear that it was caused by a different organism than *As. n*. The most intense discoloration and microorganism development was observed on ML3 showing slimy consistence. (fig.3,4)That indicated actinomycetes.

3



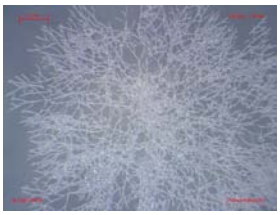
4



- **Actinomycetes**
- Actinomycetes are a specific group as bacteria. Morphologically they resemble fungi because of their elongated cells that branch into filaments or hyphae. (fig.5)
  During the process of composting mainly thermophilic (adapted to high temperatures) and thermotolerant actinomycetes are responsible for decomposition of the organic matter at elevated temperatures. In the initial phase of composting the intensive increase of microbial activity leads to a self heating of the organic material.
  Actinomycetes live predominantly aerobically, i.e. they need oxygen for their metabolism. Generally, actinomycetes grow on fresh substrates more slowly than other bacteria and fungi. During the composting process the actinomycetes degrade natural substances such as chitin or cellulose.
  Natural habitats of thermophilic actinomycetes are silos, corn mills, air conditioning systems and closed stables.

  Some thermophilic and thermotolerant actinomycetes are found to be responsible for allergic symptoms in the respiratory tract (e.g. "extrinsic allergic alveolitis", EAA).
  Source: http://www.uni-giessen.de/~gh1484/actino.html

5



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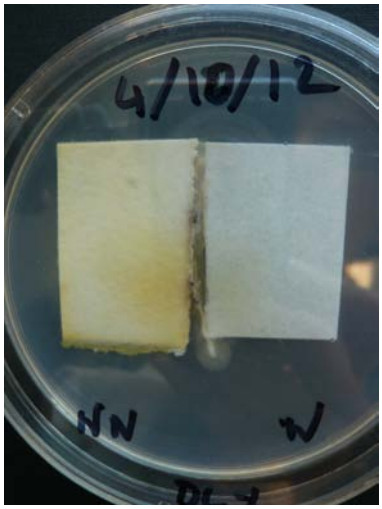

Exp No.2: 96 hrs cultivation (day 4)

DL

Pigmentation on DL and Ml plates was halted by expansion of A*ctinomycete* which produces antibiotics. It is a microorganism with features of bacteria and filamentous fungi.

ML

Act prefers higher pH in contrast to fungi who dominate at lower pH; WN pH was 6.7 when W4 pH 5.7; growth of *Act* began on WN and later spread on W4.

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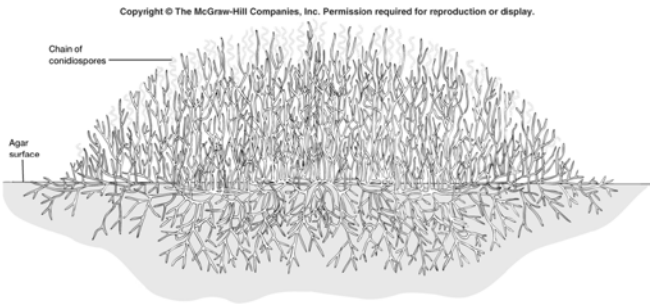
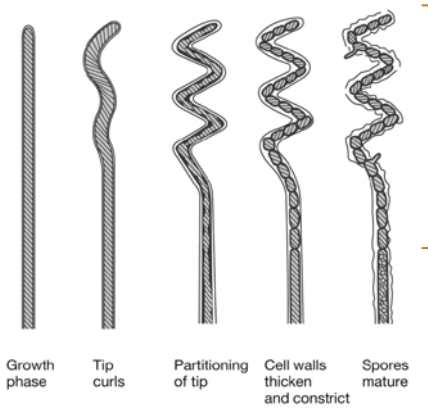
Actinomycete



Left: Actinomecetes growing on exp. 4-2012.

Below: Act. On verso of WN-DL exp. June 2012. SEM micrograph of that side beneath.



ML-WN; exper.#1

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Experiment No 2; 10 days observation; 4/10-2012-4/20/2012 (DL1)

DL1-4/10: 9am | DL1-4/10: 18am | DL1- 24hrs; day1 | DL1- day2 | DL1- day3 | DL1- day4 | DL1- day6

DL1- day7 | DL1- day8 | DL1- day9 | DL1- day10

Experiment No 2; 10 days observation; 4/10-2012-4/20/2012 (ML1)


ML1-4/10: 9am


ML1-4/10: 18am


ML1- 24hrs; day1

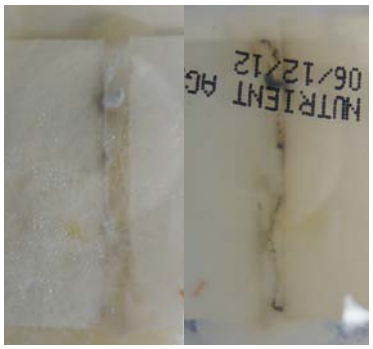

ML1- 24hrs; day2


ML1- 24hrs; day3

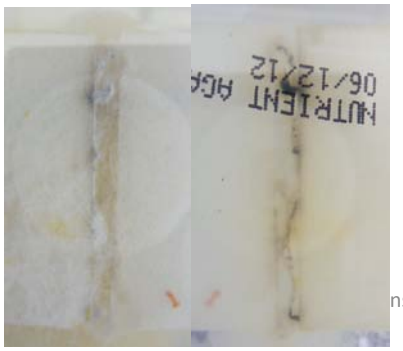

ML1- 24hrs; day4


ML1- 24hrs; day6

ML1- 24hrs; day 7



ML1- 24hrs; day 8

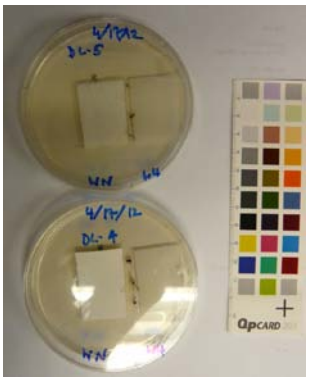


ML1- 24hrs; day 9

ML1- 24hrs; day 10

Experiment No 3; set-up; date: 4-17-12

- To eliminate contamination, time of papers' sterilization was extended to 48 hrs.
- WN and W4 paper samples were arranged in a sterilization container separately to reduce time of searching and length of opening the container.
- Inoculation needle and tweezers were sterilized in flame and in ethyl alcohol



Two plates of repetitious inoculations were set up for each light conditions

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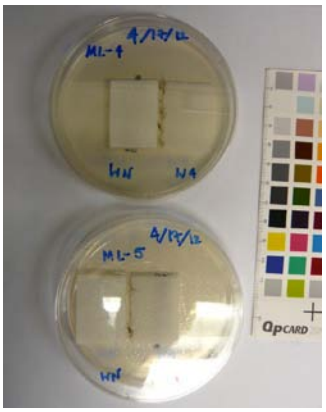
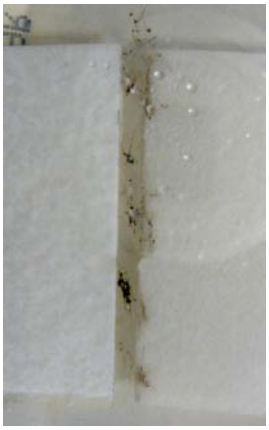


Experiment No 3; set-up day observations

DL4-setup; 4/17/12

DL5-setup; 4/17/12



ML4-setup; 4/17/12

ML5-setup; 4/17/12

•WN paper unevenly wetted once placed on Petri dish agar-agar. DL5 shows very differently response from DL4- note water penetration visible shortly upon contact with nutrient.
•ML5 plate showed some water accumulation prior to inoculation. The seal on the plate was broken during set up of experiment No 2, originally planning to use all the available plates from that batch.

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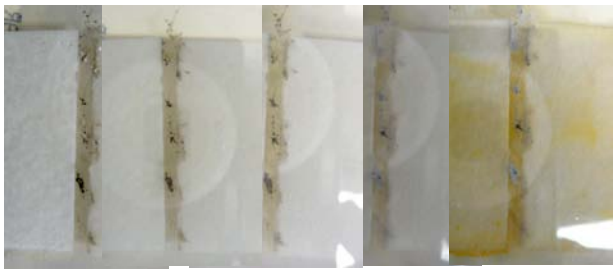
Experiment No 3; 6-day culturing observations

DL4

4/17/setup 4/18 day1 4/19/ day2 4/20/ day3 4/20/ day6

Infestation became evident in 3rd day

ML4



4/17/setup 4/18 day 1 4/19/ day2 4/20/ day3 4/20/ day6

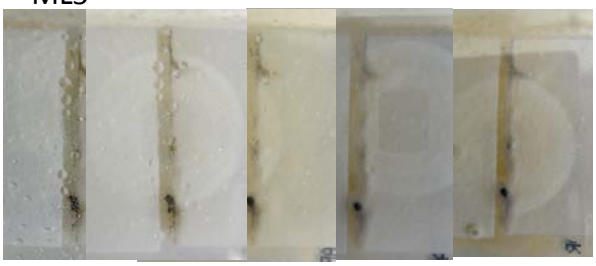
Infestation by *Actinomycete* producing antibiotics halted any development of *As. n.*

Intense yellowing by actinomy-cete-day6

DL5

4/17/setup 4/18 day 1 4/19/ day2 4/20/ day3 4/20/ day6

ML5



4/17/setup 4/18 day1 4/19/ day2 4/20/ day3 4/20/ day6

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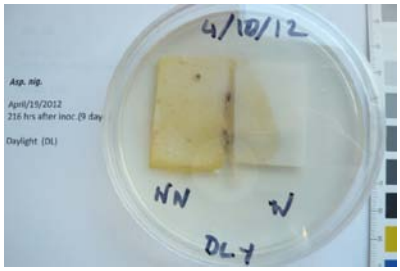
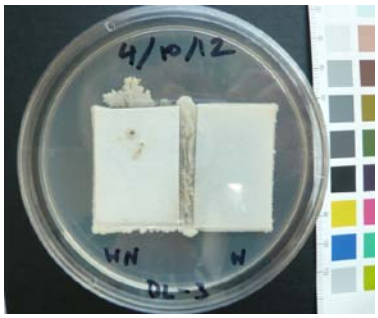
Summary of observations; experiment # 2 and # 3

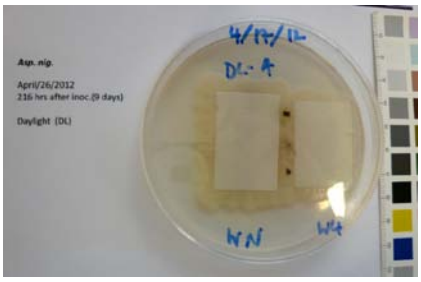
DL1 & DL3 4-10-12 setup; 9 days cultivation

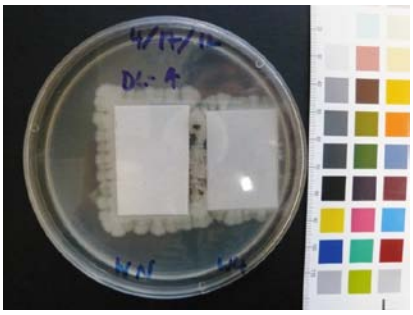




- Actinomycetes infested all plates, DL and ML.
- The infestation became obvious after 2nd day of cultivation. And was manifested by strong pigmentation characteristic for Act. and abundant aerial growth in the early stage. Of Act Development.
- Growth began on more acidic paper WN, and a couple of days latter transferred onto W4.
- It is not clear why Act took over, possibly due to lower temperatures in the environment.

DL4-4-17-12 setup; 9 days cultivation





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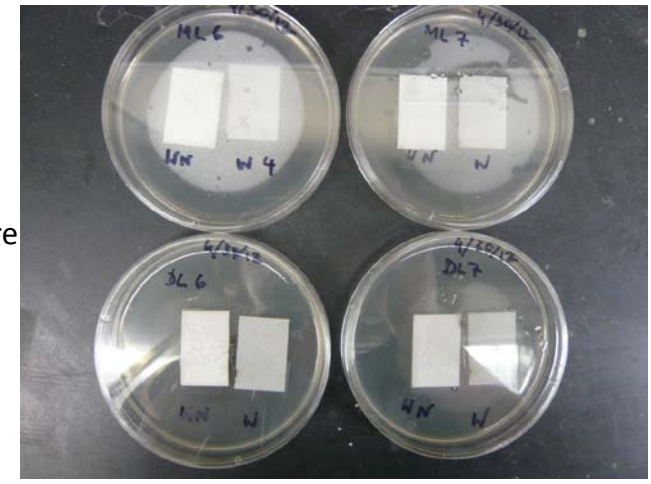
Experiment No 4; set-up date: 4-30-12

- To eliminate contamination, time of papers' sterilization was extended to 62 hrs.
- Inoculation needle and tweezers were sterilized in flame and in ethyl alcohol

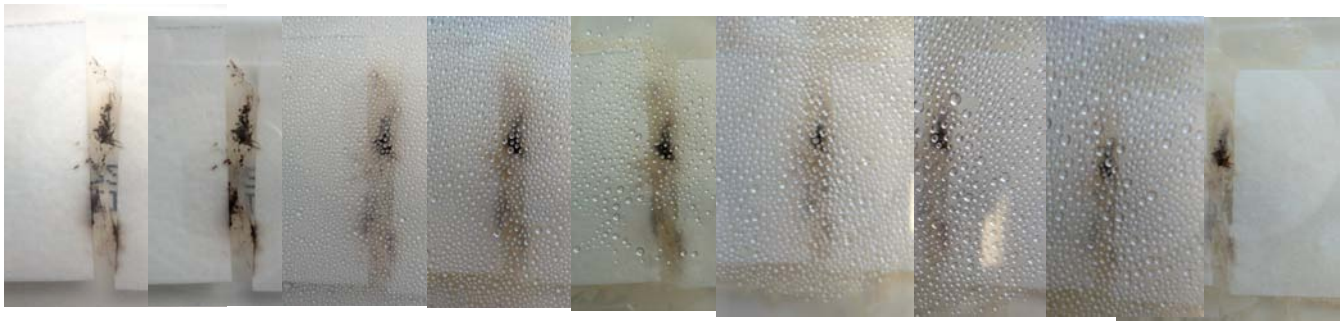
WN and W4 paper samples were arranged in separate sterilization containers to reduce time of searching and length of opening the container.

Two plates of repetitious inoculations were set up for each light conditions



Experiment No 4; (setup 4/30-5/11) 11-day culturing, DL recto

DL6-recto



5 hrs inoc.

Day 1 Day 2 Day 3 Day 4 Day 7 Day 8 Day 9 Day 11

DL7-recto

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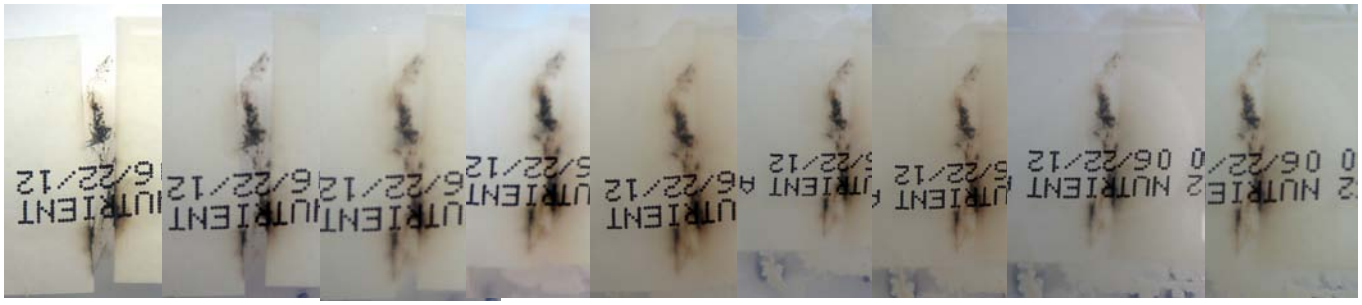
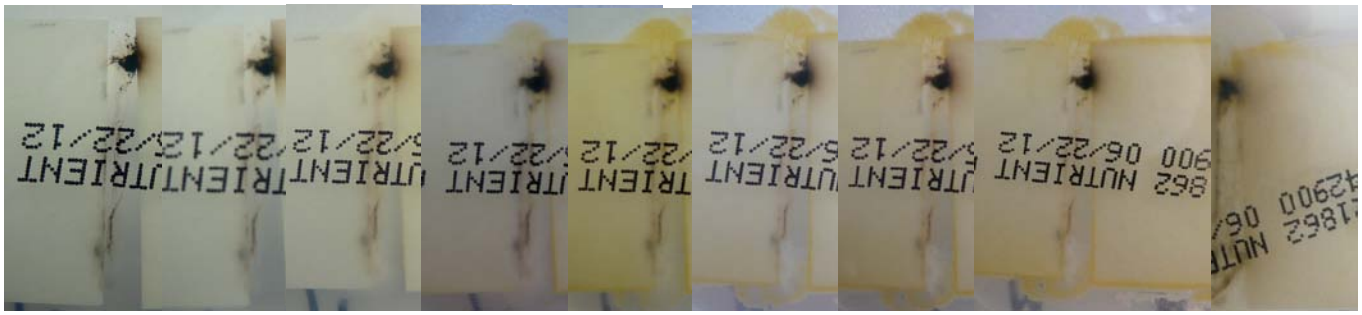
Experiment No 4; (setup 4/30-5/11) 11-day culturing, DL verso

DL6-verso

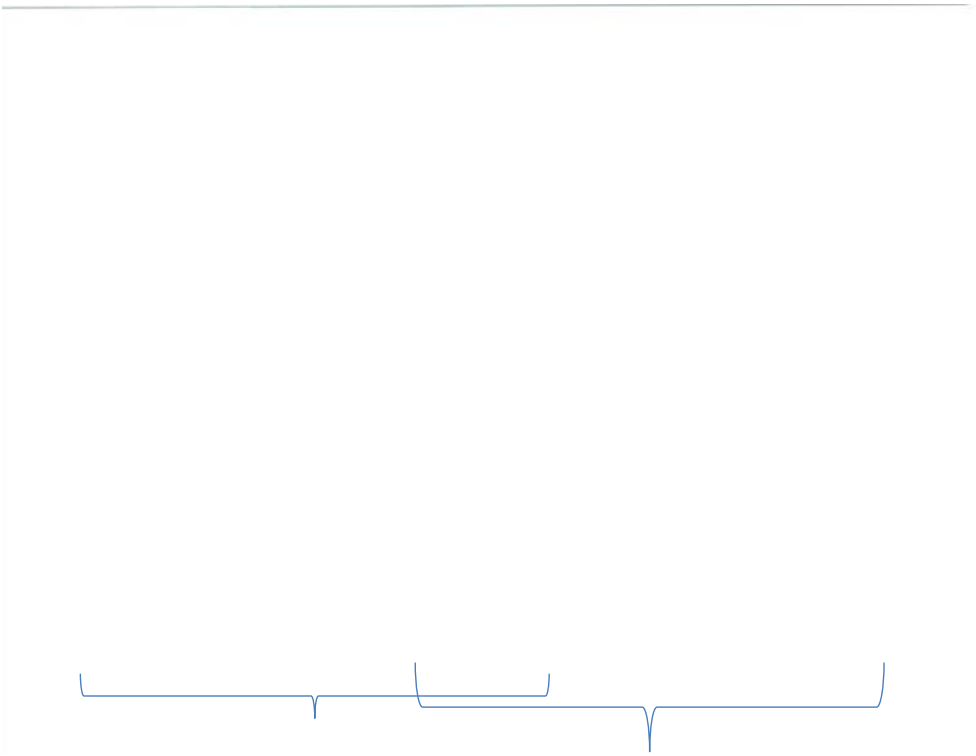


Day 1 Day 2 Day 3 Day 4 Day 7 Day 8 Day 9 Day 11

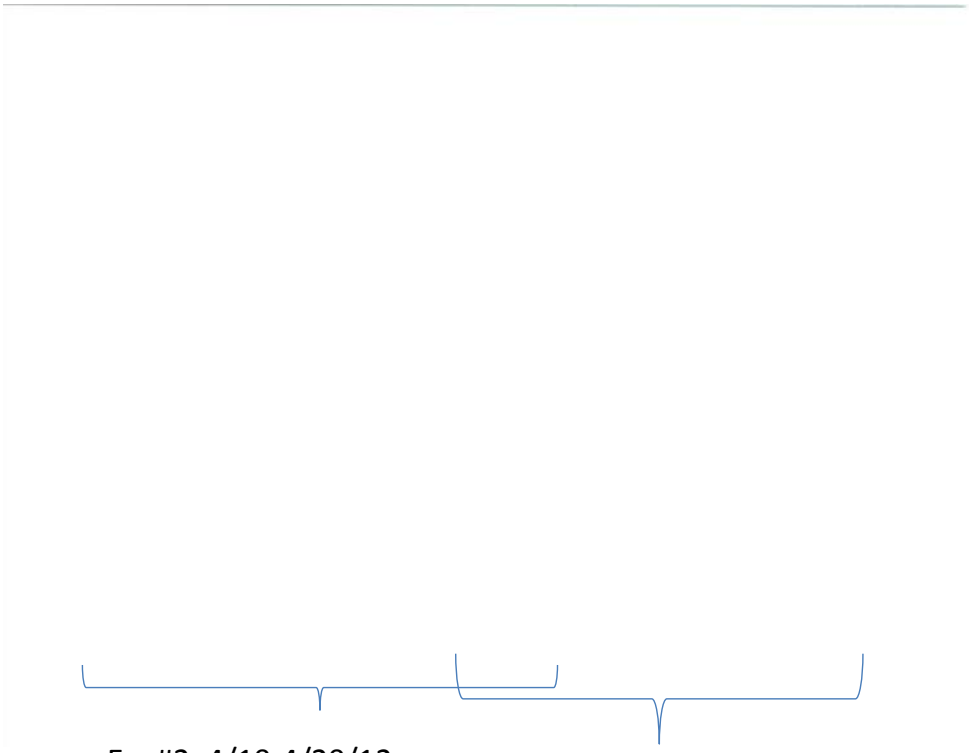
DL7-verso



Environmental conditions; exp. # 2 and # 3; DL (light)



Environmental conditions; exp. # 2 and # 3; ML (dark)

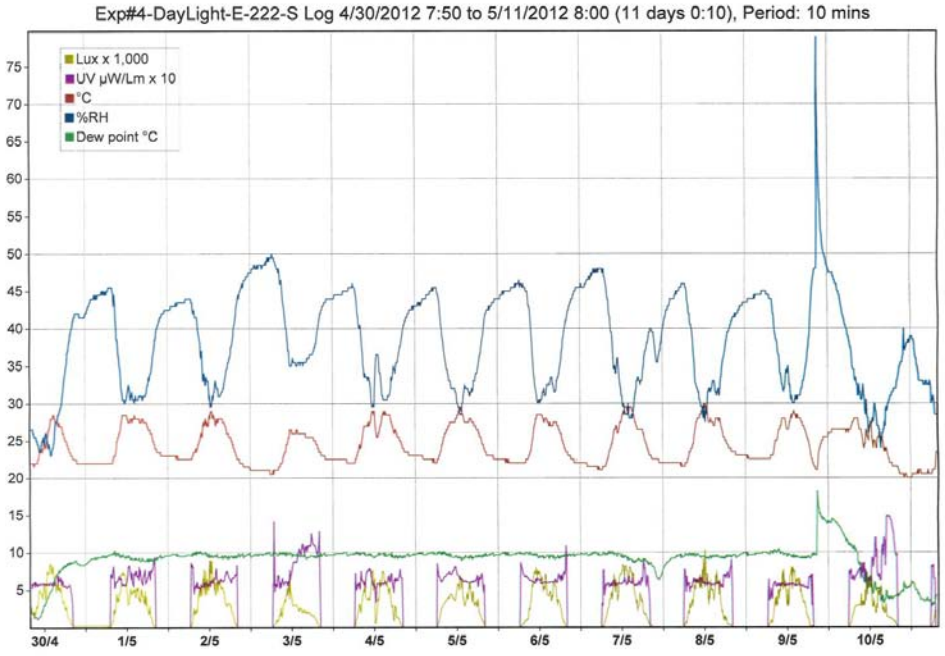


Vis light: 0-60 Lux*
UV: 0 µW/Lm
T: 20°C
RH: 25-51%
Dew point: -1-1°C

*** Vis light only when imaging was taken, or one entered the room**

Above values for period of exp.# 2 & #3

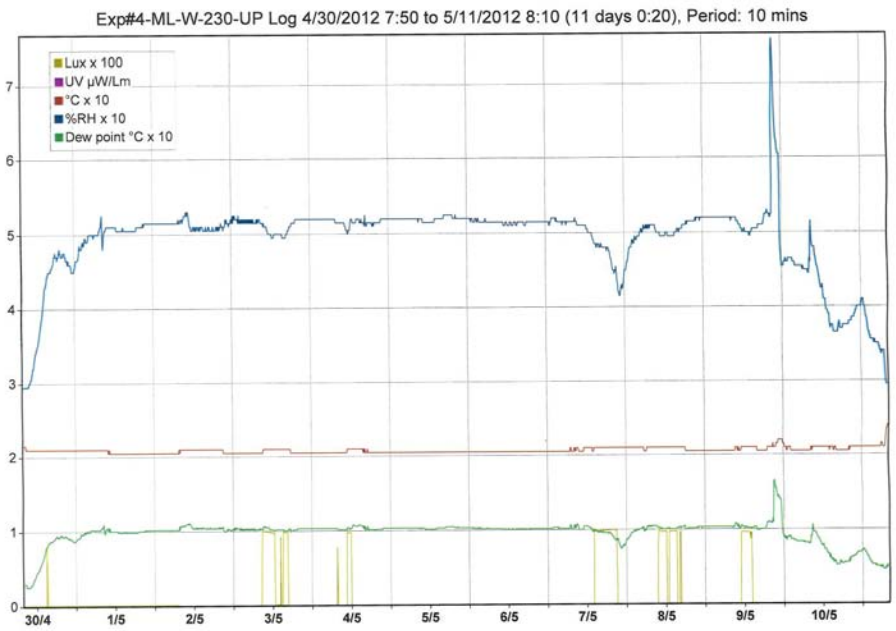
Environmental conditions; exp. # 4; DL culturing period: April 30 through May11, 2012



Vis light: 0- 8000 Lux
UV: 0-150 µW/Lm
T: 22-28°C
RH: 25-50%
Dew point: 2-10°C

Above values for period of exp.# 4; April 30 through May11, 2012

Environmental conditions; exp. # 4; ML culturing period: April 30 through May11, 2012



Vis light: 0- 100 Lux
UV: 0
T: 20°C
RH: 30-52%*
Dew point: 2-10°C

***RH: note unusual spike on 5/9 midnight. Building system malfunctioned .**

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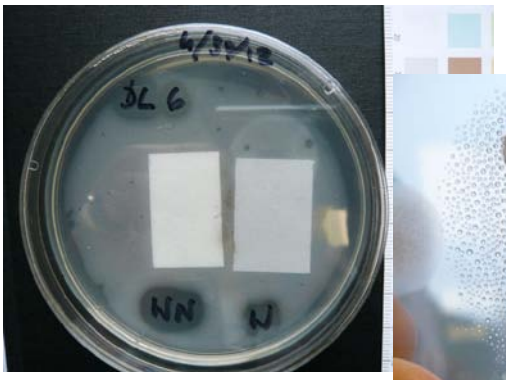
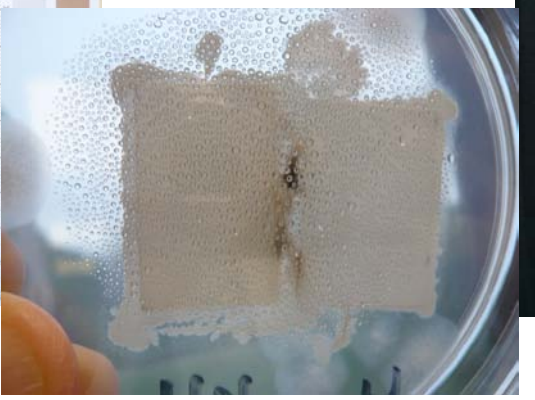
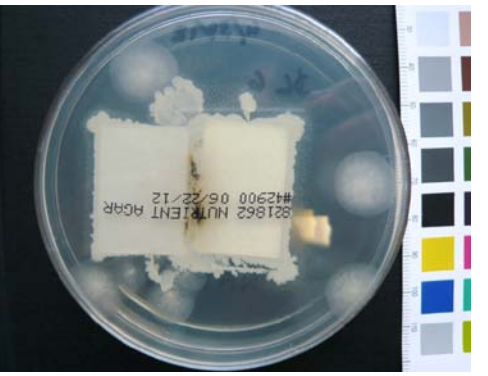
Exp. #4: Comparison day1-and day 8

DL6-verso

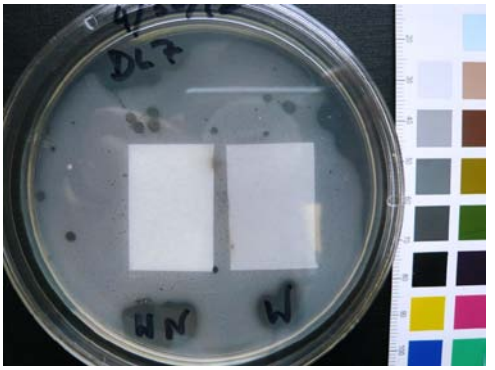




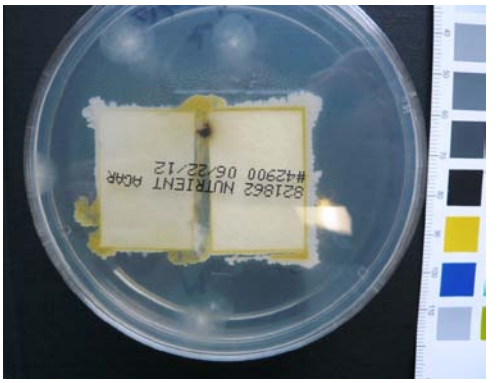


Day 1

Day 8



DL7-verso



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conclusion

- The purpose of his experiment was to induce pigmentation by *Aspergillus niger.*
- The experiment did not produce anticipated results, because the microorganism selected for this study (A. *niger)* was overpowered by Actinomycetes. The situation was repeated in all replicated plates in the course of three trials. It is possible that light conditions, amount of UV light in particular had some impact on Actinom. growth.
- The last trial will be repeated in June, in light and external temperature conditions similar to that during experiment #1, which was successful.

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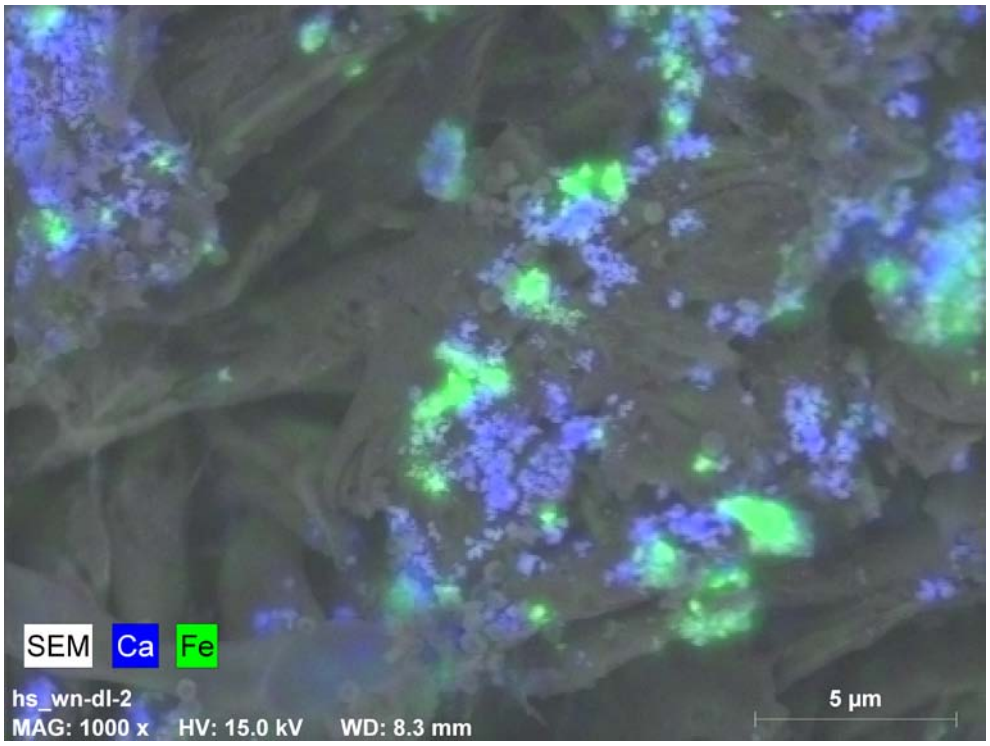
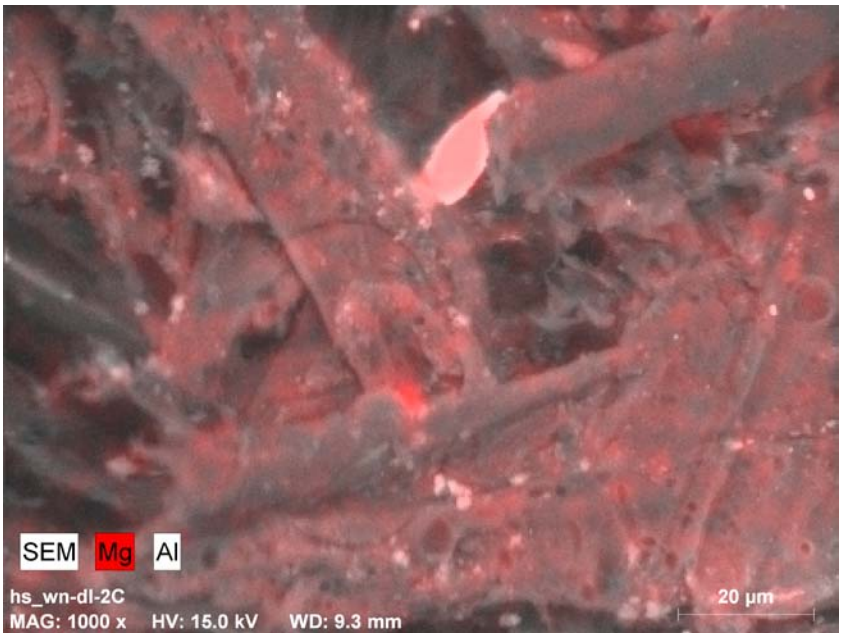
Appendix V. SEM-EDS report

1. Chemical alteration of paper surface caused by fungi
2. Analysis of red pigment on Sheet Study No1

Appendix V.1 Chemical alteration of paper surface caused by fungi





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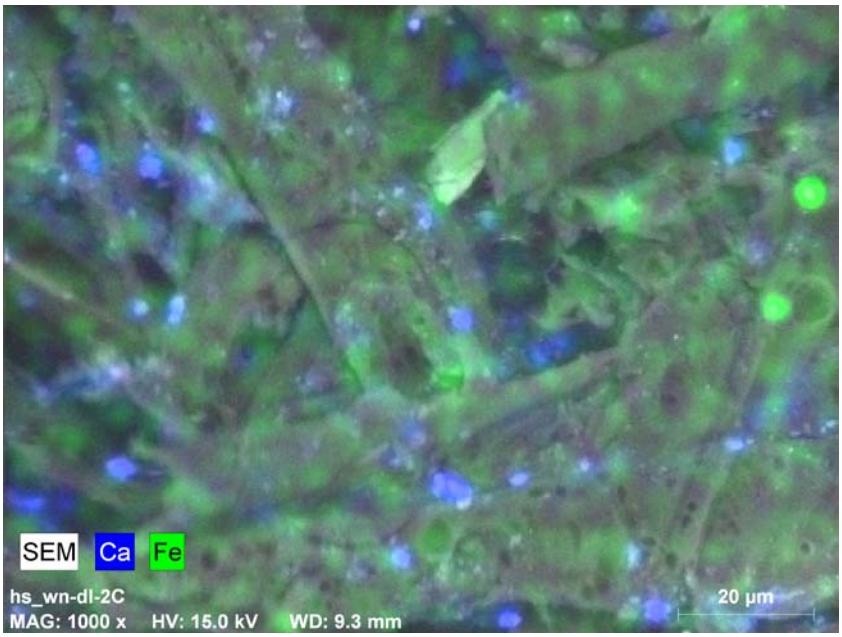

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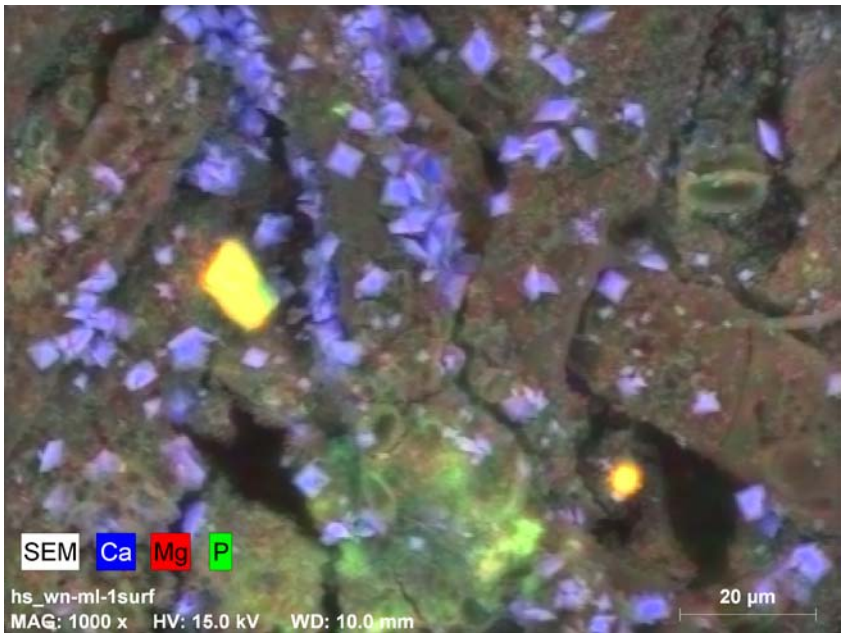

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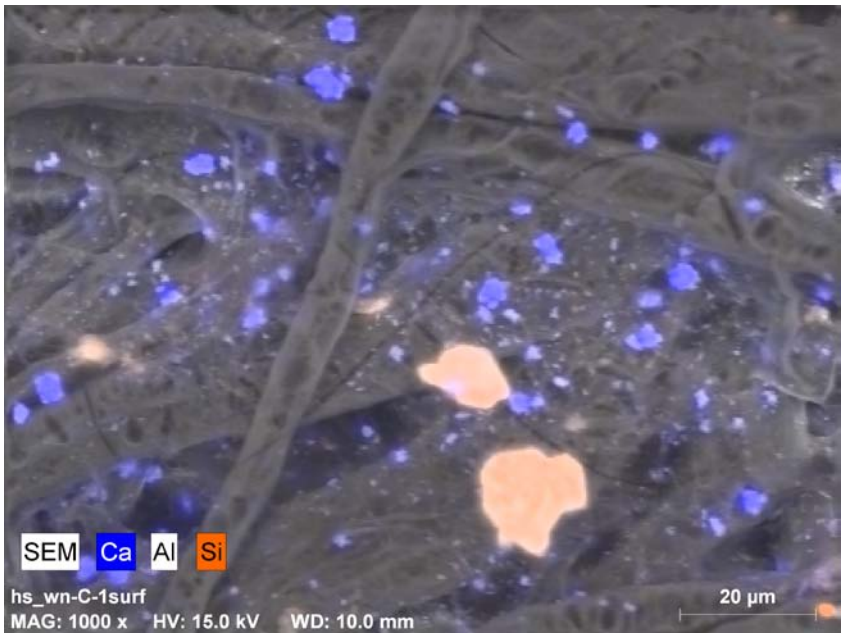

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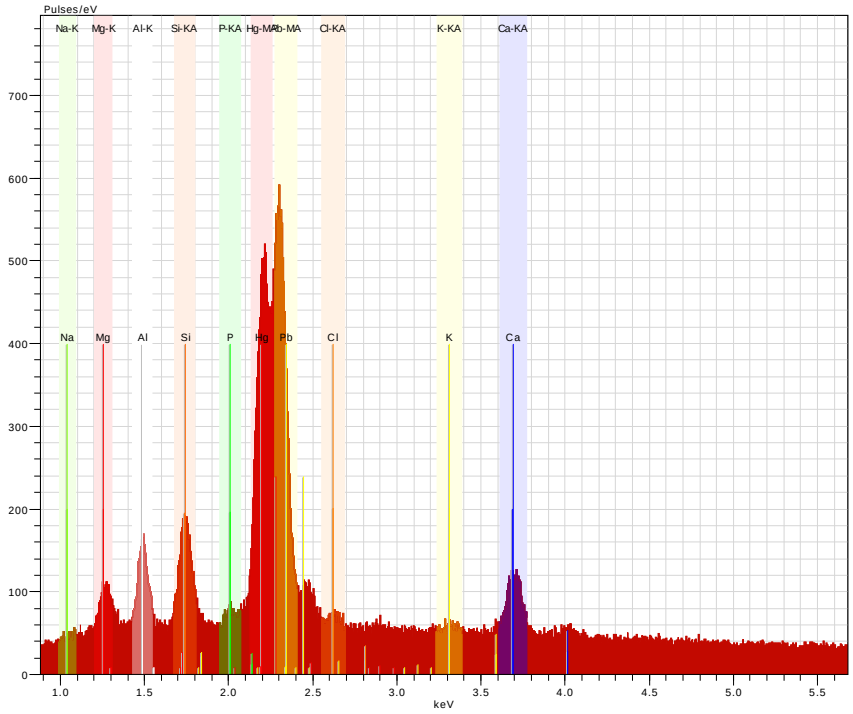
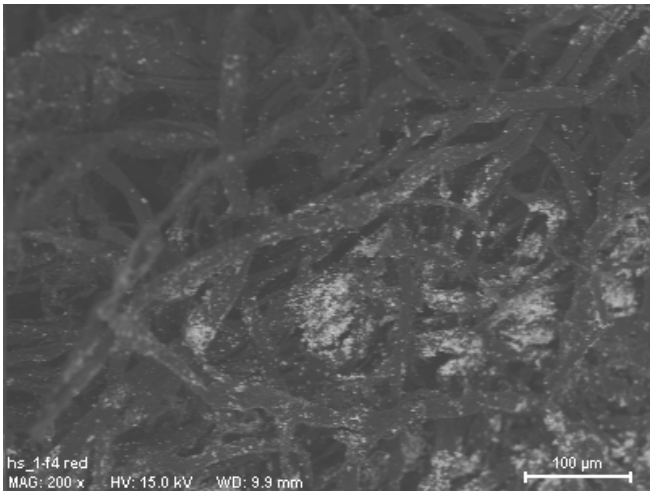
Appendix V.2 SEM-EDS Red pigment analysis, Study Sheet No.1





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Puls th.:1.67kcps

hs_1-f4 red
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HV:15.0kV ;

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