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DISSERTATION

Foxing of paper caused by fungi and molecular monitoring of conservation treatments

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To my family.

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Chapter I

General Introduction

1. Biodeterioration and Cultural Heritage

Science has proven to be crucial not only in deciphering cultural artefacts with visual, literal or ideological content, it has also evolved into a key-player in maintaining and restoring items of cultural value to preserve them for us and for the future. Cultural heritage like buildings, statues, artefacts, textiles, paintings and books are subjected to destructive conditions imposed on them through the chemical structure of their substrate, environmental conditions and ageing. The continuous increase of industrial and urban activities and use of resources over the last three centuries alone resulted in more aggressive environmental conditions through changes in the atmosphere (Chapin *et al*., 2000).

In addition to changing environmental conditions, most artefacts have been exposed to different forms of biological attacks, summarised as biodeterioration. Growing organisms or biodeteriogens cause complex natural physical or chemical spoilage processes of the substrate available. We harvest those activities during composting or food production, but they are unwanted on objects of art and documents. Biodeterioration can be a structural as well as an aesthetic threat to artefacts and the type of damage is dependent on the type of organism and substrate involved. The extent of the damage caused is again dependent on environmental conditions that favour biological growth, physical parameters like moisture, temperature, and light, and accessibility of the object.

Whilst insects and rodents can be associated with some forms of damage (Harvey, 1992; Florian, 1997; Adamo and Maggauda, 2003), it is mostly microorganisms that are responsible for the biodeterioration of cultural items. Different phylogenetic groups of microorganisms have been described as biodeteriogens, e.g. archaea, lichens, algae, cyanobacteria and chemolithotrophic bacteria, as well as chemoorganotrophic bacteria and fungi (Ranalli *et al.*, 2009). From fungal attacks of Palaeolithic cave drawings and bacteria destroying ancient Maya' sites, to microbial communities growing on synthetic polymers of contemporary art, the 'appetite for culture' can have a significant impact on the economy of many nations (Rinaldi, 2006). The loss or destruction of important cultural heritage is predicted to be even faster for some modern synthetic materials used in contemporary art (Cappitelli *et al*., 2006b; 2008).

2. Foxing, a biodeterioration of paper

Cultural reports and intellectual developments of humankind have long been recorded on paper and related objects like parchment or papyrus. These graphic supports are composite, organic materials that are recognised as a favourable environment for microbial growth in general and for microscopic fungi in particular (Cappitelli and Sorlini, 2005). The raw materials of paper and its production have changed since it was invented in China in about 105 AD (Hart, 1978). Spreading west together with the arts of writing, by the twelfth century paper had become the most common writing material. Before the nineteenth century, paper was hand-made and consisted mainly of cellulose from rags of linen, hemp and cotton with few impurities, but this process gave way to a mechanical and chemical process when the demand for paper grew (Hunter, 1999). Wood became the main raw material and hence the components of paper included significant amounts of lignin thereon, shorter cellulose chains as well as many polymers, non-fibrous material and impurities, making recent paper more vulnerable to microorganisms than older paper (Kowalik, 1980).

Cellulose fibres are linear polymers of glucose monomers that are linked via β-1,4-glycosidc bonds, and the cellulose chains are held together by strong intramolecular hydrogen bonds into a highly oriented structure (Laguardia *et al*., 2005). The difference in chain lengths of cellulose from wood pulp, with between 300 and 1700 units, and cotton and other plant fibres with 800 to 10,000 units, make wood derived cellulose easier to break down (Klemm *et al*., 2005). Clays and chalks are added to fill pores and to give the paper an opaque appearance, and bulk or sizing materials like starch, gelatine or various synthetic polymers help to even out the surface and to reduce the spread of ink (Cappitelli and Sorlini, 2005). Dust particles get caught in the fibrous surface of the paper, human handling and use can transfer oil to the paper, and both provide further food to colonising microorganisms. Taken together, paper offers a variety of nutrients for heterotrophic organisms, supported by the hygroscopic nature of its chemical structure that helps capturing water.

Paper only offers an environment with low moisture content when stored following the recommendations for libraries and archives for the storage of paper, set around a relative humidity (RH) of 45-55 % at 18-22 °C to prevent microbial

growth (Florian, 1997). However, microclimates in books or even within the paper's structure can differ vastly from the overall RH of a room, and many old books contain unsized tissues that interleave the pages and are capable of absorbing water and to retain it over a long period (Derow and Owen, 1992).

This thesis focuses on a certain type of stain that appears regularly on paper, called foxing or foxing spot. The term is derived from Reynard the fox, an anthropomorphic fox and trickster figure, on the basis of his rusty red fur and was first mentioned as early as 1848 (Iiams and Beckwith, 1935). The Japanese language has a more poetic word for foxing, *hoshi*, which means stars and refers to the shape of the stain (Zaremba and Wirth, 2009). Foxing spots appear on paper dating from the sixteenth century to recent times but seem to increase on paper from the eighteenth century on, coinciding with the change in paper manufacture and contents. Different types of foxing spots on various paper types are shown in Figure 1.

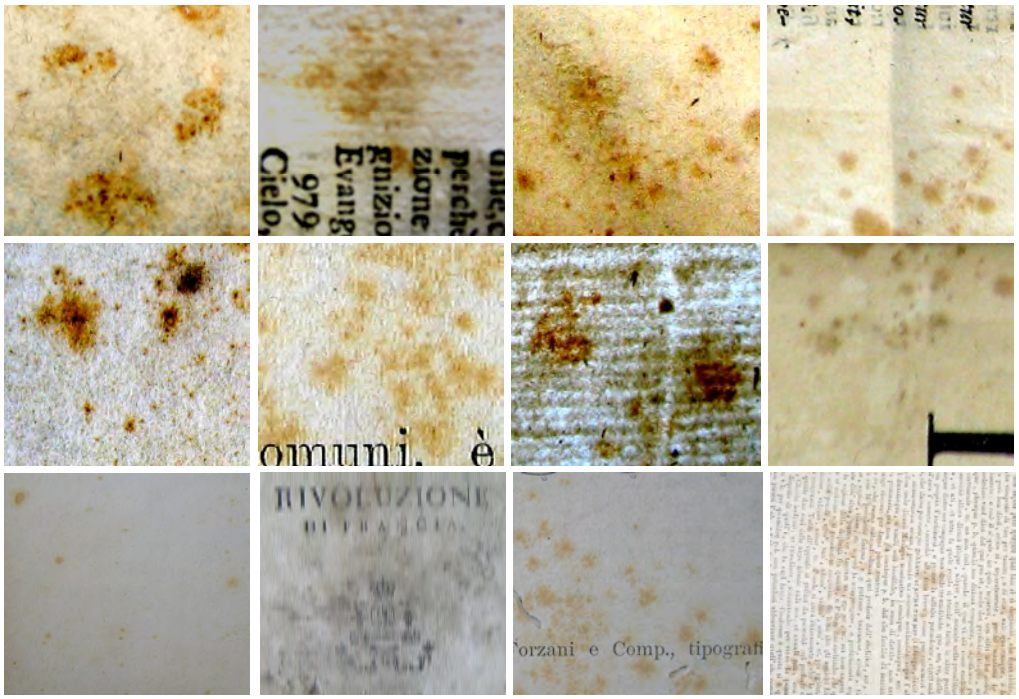

Figure 1. Foxing spots on different paper types. Foxing appears in colors ranging from yellowish to black and the spots are irregularly shaped. A three-dimensional distribution or migration pattern often observed and foxing spots are common in paper of all ages and quality. Photos of the foxing spots were provided by Flavia Pinzari, ICPAL, Rome, Italy.

The variety in colour and shape of foxing spots ranges from yellow to reddish and brown to black with sharp or irregular edges. They are mostly small (1-10 μm), roundish and scattered, but there are no specific criteria or system for description and identification hence depends on the viewers' subjectivity (Choi, 2007).

Cain and Miller (1982) proposed a classification method by shape, colour and UV light examination, but it has not been accepted or used by librarians, conservators and scientists. Foxing spots should be distinguished from offset or acid stains from secondary material, other pages or printing ink, which may accelerate foxing but represent a different alteration (Derow and Owen, 1992).

3. Mechanisms underlying foxing spot formation

Research on foxing has been performed for more than 60 years but there is still confusion as to what is or are the causes that lead to foxing spots. Three major explanations are under review with a forth explanation recently being proposed. Fungal activity, metal-induced degradation and a sum of multiple causes are the most frequently reported catalysts of foxing as summarised in Figure 3.

However the disputed formation process, some mechanisms underlying the formation of foxing spots and components of it have been identified. The analysis of the constituents of foxing spots has mainly concentrated on organic components and amino acids with the main organic acid identified as malic acid (Arai *et al*., 1990; Chen and Xie, 2002). Malic acid is a fungal metabolite and its concentration is considerably higher in foxing spots than in unaffected areas. No inorganic factors involved in foxing were identified by Chen and Xie (2002) but the concentrations of twenty amino acids that were identified amongst foxing spots were distinctly lower in the unaffected areas, with some of the amino acids not detectable at all. The authors considered that acid types including aspartic acid, glutamic acid, aminobutyric acid, ornithine, and serine originated from fungal bodies, proving a fungal origin of foxing spots. Saccharides, including cello-oligosaccharide, were also observed in foxing spots and they were considered degradation products of cellulose (Arai *et al*., 1990). This points

towards biologically active components involved in the formation of foxing.

Another hypothesis about the formation and source of the colour of foxing spots is a browning reaction, called Maillard reaction. Glucose and amino acids deposited as residues by fungi react leading to the browning of paper and therefore foxing (Arai, 2000). A process proposed by Rebrikova and Manturovskaya (2000) suggests that the reddish colour is the result of amino-carbo reactions, with the carbonyl group coming from cellulose oxidation, and the amino group from pollutants or paper impurities.

Another model proposed by Florian (1997) suggests the colour is inherent to the fungal structures and metabolites that fade and discolour over time and are absorbed by the paper. Choisy and co-workers (1997) also grouped foxing spots according to colour formation, assuming that flouorogenic compounds appear in a first step, followed by chromogenic compounds in conjugated chromophores and the final full colour is reached with free ketones.

In addition, UV fluorescence of foxing spots has long been reported and interpreted either as proof of the existence of fungal mycelium or of free radicals formed by cellulose oxidation (Florian, 1997; Rebrikova and Manturovskaya, 2000). In both cases, fluorescence is interpreted as indicative of stages in foxing development, increasing from the initial and early stages of formation through either increasing biological growth or increasing cellulose oxidation, and decreasing with time accompanied by an increase in the colour of the foxing spot but reduction or stagnation in growth.

The relationship between light and foxing has not been studied in detail yet, but it appears that light is not needed for the formation of foxing. Cain and colleagues (1987) found foxing spots distributed on paper that had not been exposed to light or human usage since it was assembled in a book. They concluded that the potential for foxing can be created very early in the paper manufacture and the visible foxing spot develops later during storage independent of light. Ligterlink *et al*. (1991) also pointed out that foxing appeared during storage without light exposure, but insisted that the spots originated before binding as identical patterns could be followed through sheets that were not bound adjacent in the book. As a logical consequence, books that display matching foxing pattern on several adjacent pages would indicate that the foxing

began after the book was bound (Derow and Owen, 2002).

3.1. Foxing as a consequence of fungal colonisation of paper

The kingdom of fungi includes yeasts, moulds, mushrooms and toadstools and comprises of a great diversity of heterotrophic organisms that mostly decompose organic matter for their metabolism. In a mainly saprophytic lifestyle, fungi absorb the necessary nutrients from the decomposing material and they are able to utilise water contained in the air. Fungi have a unicellular or a pluricellular filamentous thallus or mycelium and generally display fruiting bodies that produce spores. The filamentous mycelium can be regarded as the whole fungal body with the spore bearing fruiting body or visible mushroom being only a temporary spore dispersal unit (Alexopoulos and Mims, 1979). Both sexual and asexual reproduction occurs. With few exceptions, cell walls of fungi contain chitin, a long carbohydrate polymer also found in insects. Chitin adds structural support and rigidity to the fungal cell.

Fungal spores and early growth on paper are not visible to the naked eye, posing problems with the early detection of a colonisation, as damage can already be done when the fungi proliferates to become visible hyphae or mycelium. Whereas foxing spots are clearly an alteration of the colour of the paper, they do not appear mouldy. Visible mould colonies on paper surfaces may also lead to stains of the paper substrate, but this is distinct from foxing. According to Meynell and Newsam (1978), mould differs from foxing in the degree of damage to the paper substrate as foxing is described as non-destructive irregular stained patches, whereas mould comes with structural damage, stains and destructive pigmented lesions.

In the fungal life cycle, the young fungal structures up to a few years in age have the inherent colour of pigmented mycelium, conidia or spores, which only lead to discoloration of the paper or foxing through ageing (Florian, 1997). The authors observed that a major group of foxing spots seemed to display limited or aborted fungal growth with conidia that germinated in the first place but died before proper vegetative growth. This absence of vegetative growth is again consistent with the distinction between foxing and mould.

All different life stages of fungi can be observed on paper: spores,

germinated young hyphae or adult mycelium and spore forming structures. A schematic presentation of fungi living on paper is shown in Figure 2 emphasising the different fungal structures that belong to the stages in the fungal life cycle. Through the release of extracellular enzymes or pigments, fungi can cause direct and indirect damage to paper. The variety of exo-enzymes includes cellulases, glucanases, laccases, phenolases, keratinases and mono-oxygenases consolidates their potential as biodeteriogens (Sterflinger, 2010).

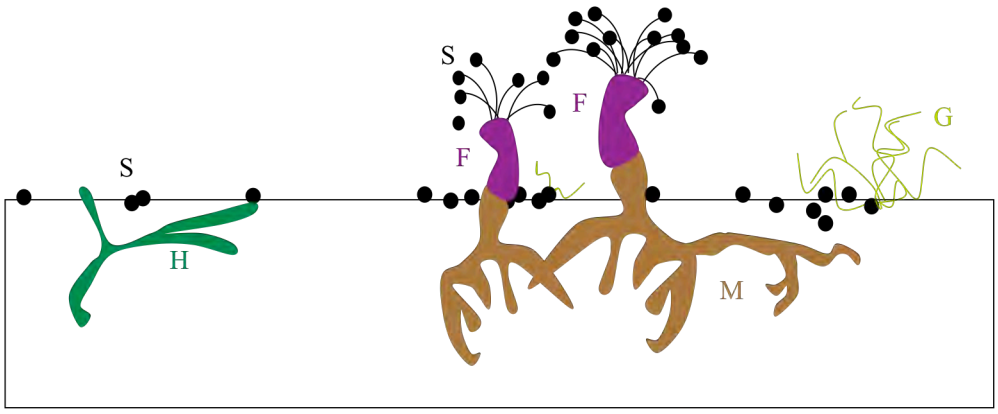

Figure 2. Illustration of fungi colonising a paper surface. Mold fungi possess segmented hyphae (H) that grow into long, intertwined masses to create a mycelium (M). The vegetative mycelium grows beneath the surface to differentiate into reproductive forms or fruiting bodies (F) above the surface, which produce the spores (S) that germinate (G) into new hyphae. Yeasts do not produce mycelia and are considered more primitive in their unicellular or pseudohyphae form. Fungi can form structures to anchor themselves into the paper fibers, they can damage the paper through cellulolytic processes and produce stains by metabolising organic material or traces of metal in the paper. Fungal spores can survive prolonged periods of unfavorable conditions on paper and are easily distributed through the air.

Following Iiams and Beckwith (1935) early observations, Meynall and Newsam (1978) suggested fungi growing on paper cause foxing after performing fluorescence microscopy. Since then the number of fungi isolated or identified in relation to foxing has continuously increased. The involvement of fungi in foxing is now broadly accepted, but it is still not clear how and when they initially came onto the paper or which fungi are responsible.

The raw materials used in paper production can carry some of the fungi

that are found on paper, they can originate from airborne spores or they can be introduced through handling of the paper or in the printing process (Florian, 1997). Florian and Manning (2000) concluded that foxing spots could develop through fungal attack either during manufacture of the paper product or through handling of the final product, as they could find fungi both above and below the printing ink. Fungi can remain latent in unfavourable conditions for a long time, and their spores are able to proliferate years later.

Amongst the 30,000 or more species of fungi, many more than the approximately 100 designated paper-attacking or cellulose-degrading fungi are capable of living on or from paper, but not all of these fungi are consequentially associated with foxing (Gallo, 1963). A study spanning 25 years of research on foxing was published by Arai (2000), which named xerophilic and facultative tonophilic fungi responsible for the formation of foxing. More fungi were identified by traditional and molecular methods and together they represent a great variety, out of which the genera *Alternaria*, *Aspergillus*, *Cladosporium* and *Penicillium* are found to be the most common (Choi, 2007). A list of fungal genera identified in relation with foxing is presented in Table 1 compiled from the publications indicated.

3.2. Metal-induced foxing

Starting with the raw material for paper, metals can be implemented into the paper in various ways. Iron is found in wood-pulp paper as raw wood naturally contains iron (Beckwith *et al*., 1940). Following the production line of paper there is metal equipment and machinery involved, both of which can induce metal particles in the form of abrasions. Metals like iron, copper and cobalt compounds directly catalyse or accelerate the oxidation of cellulose as found in foxing spots, whereas the rate of oxidation is dependent on the level of humidity (Tang, 1978).

Iron has been regarded as the most obvious abiotic catalyst in the formation of foxing spots, with the very colour of the spots indicating the presence of it (Iiams and Beckwith, 1935). Some foxing spots increase in darkness with increasing iron content, displaying a dark, iron-rich centre with metal and colour concentration decreasing towards the edges (Tang, 1978).

Fungal genera identified related to foxing	Reference	Fungal genera identified related to foxing	Reference
Acremonium sp.	zy	*Gleotinia sp.*	r
Acrothecium sp.	zy	*Gliocladium sp.*	n
Alternaria sp.	m, s, zy	*Gymnoascus sp.*	zy
Arthrinium sp.	c	*Helicostylum sp.*	zy
Aspergillus sp.	f, m, n, r, s, z, zy	*Menalospora sp.*	zy
Aureobasidium sp.	z, zy	*Mucor sp.*	s, zy
Bjerkandera sp.	r	*Oidiodendron sp.*	c
Botryotrichum sp.	zy	*Paecilomyces sp.*	s, z, zy
Botyris sp.	m	*Penicillium sp.*	c, f, m, n, r, s, z, zy
Cephalotrichum sp.	zy	*Peziza sp.*	c
Chaetomium sp.	c, r, s, zy	*Phlebia sp.*	m
Chloridium sp.	zy	*Phoma sp.*	c, zy
Chromelosporium sp.	m	*Pichia sp*	s
Cladobotyrum sp.	zy	*Polyporus sp.*	r
Cladosporium sp.	c, m, z	*Rhizopus sp.*	s, zy
Coprinus sp.	m	*Rhodotorula sp.*	zy
Cordyceps sp.	r	*Saccharicola sp.*	r
Crysopsorium sp.	zy	*Skeletocutis sp.*	m
Cunninghamella sp.	c	*Sordaris sp.*	zy
Curvularia sp.	zy	*Stachobotyrs sp.*	s
Doratomyces sp.	z	*Stemphylium sp.*	s, zy
Epicoccum sp.	c, zy	*Toxicocladosporium sp.*	m, s
Eurotium sp.	c, f, s	*Trichoderma sp.*	c, r, s, z, zy
Fusarium sp.	r, s	*Trichosporum sp.*	zy
Fusicladium sp.	c	*Ulocladium sp.*	c, r, s, zy
Geomyces sp.	z	*Verticlilium sp.*	zy
Geosmithia sp.	z	Yeasts (unidentified)	c
Geotrichum sp.	zy		

Table 1. Fungal genera identified related to foxing on paper. The list is compiled from studies on foxing in books, stamps and other paper-based material including zy: Zyska et al. (1997); f: Florian and Manning (2000), n: Nol *et al*. (2001), c: Corte *et al*. (2003), r: Rakatonirainy *et al*. (2007), z: Zotti *et al*. (2008), m: Mesquita *et al*. (2009) and s: Sterflinger (2010).

More recent research however is ambiguous about the role of iron in the establishment of foxing, as different observations were made related to low rather than high iron content. Press (2001) compared the distribution of foxing spots and unfoxed areas related to the iron content of the paper and found that foxing was linked to low rather than high iron content. Some authors call for the removal of metal-induced foxing from the foxing category altogether, as it displays metal-induced degradation (Hey, 1983). Bicchieri *et al*. (2002) also distinguished artificially induced brown stains from iron compounds on paper from biologically induced foxing in calling the effect foxing corrosion.

Another metal that can cause foxing spots on paper is copper. A mechanism for the formation was proposed by Daniels and Meeks (1988) who identified zinc, sulphur and chlorine to be coexisting with copper in the spots but not in unaffected areas. They concluded that the spots were due to a combination of black copper sulfide and brown copper that catalysed the degradation of cellulose.

3.3. Oxidation and condensation of paper

Interestingly, there is one common characteristic of foxing, independent of the suspected causes of formation, and that is the oxidation of cellulose macromolecules (Cain, 1983; Rebrikova and Manturovskaya, 2000; Bicchieri *et al*., 2001, 2002; Manso *et al*., 2009). Cellulose oxidation is the result of a reaction between cellulose and oxygen that leads to the formation of intermediate hydrogen peroxide radicals. Oxygen is absorbed at certain sites on the cellulose molecule and oxygen containing groups like carbonyls and carbocilic acids increase, leading to elevated levels of acid. Cellulose then hydrolyses, which results in a breakage of long cellulose chains into smaller ones, facilitating increasing water absorption of the paper.

Accumulating water in the heterogeneous areas of the paper can increase oxidation reactions, as can pollutants or impurities from the environment (Bicchieri *et al*., 2001). The hygroscopic nature of the paper and the presence of fungal metabolites or cellulose degradation products or other impurities can accelerate that reaction. Bicchieri *et al*. (2002) observed in all their samples that the cellulose substrate was strongly oxidised in the spots where they induced foxing with iron compounds. This preferential cellulose oxidation is related to the non-homogeneity of paper, as is moisture condensation.

Condensation of paper takes place at the interface of wet and dry parts of fibres, leading to a modification of cellulose visible as browning. The brown stains are result of paper or ink degradation products that are following the spreading moisture on the paper before being deposited during desiccation of the paper (Derow and Owen, 1992). Interaction of air, water and cellulose can lead to browning in areas of temporary moisture accumulation (Hutchins, 1983). Ligterlink *et al*. (1991) added temperature, paper porosity, irregularities in the

paper as folds, tears or dirt particles, and the presence of iron or fungi as influential on the induction of condensation. The presence of metal and/or fungi was not considered necessary for the browning of the paper, but fungal appearance could coincidentally represent sites on the paper with higher moisture content.

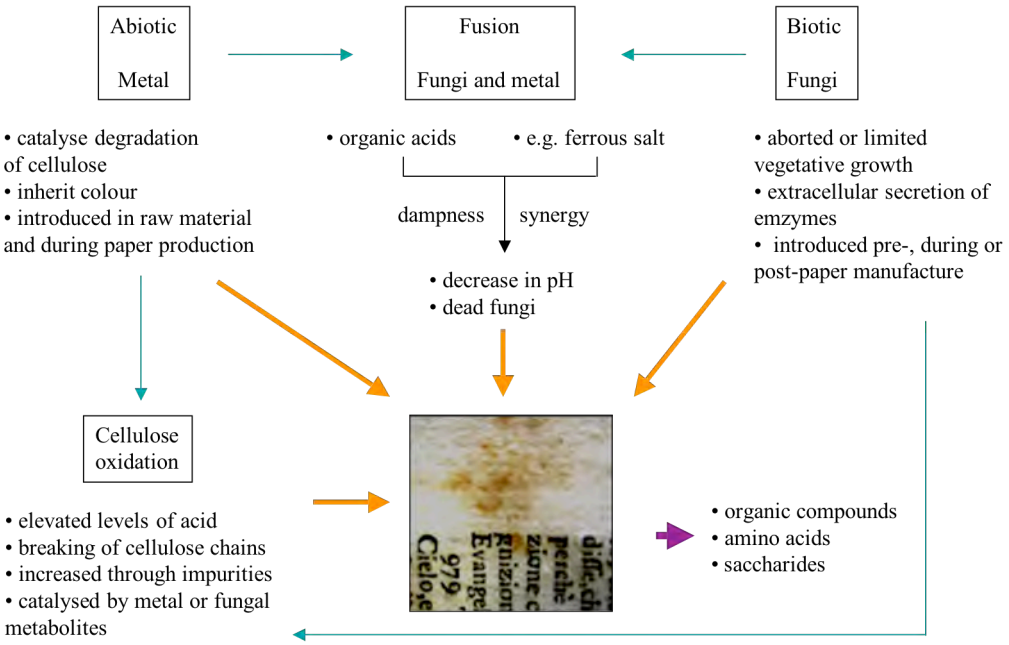

Figure 3. Schematic summary of theories about the formation of foxing spots. Both abiotic metal induced foxing and foxing induced by fungi, as well as a combination of both, lead to foxing spots that show locally oxidised cellulose. Cellulose oxidation is commonly observed in foxing spots. Green arrows indicate either catalysing relations or influencing components of the different theories. Organic compounds, amino acids and saccharides are common components found in foxing spots

3.4. Multiple causes for the development of foxing spots

Biases in theories of how foxing is generated can partially be explained with experiments that are often tailored towards one subject, either metal content or fungal colonisation, and not considering dual or multiple causes. Fungal infection of paper often correlates with iron or other metals present in the paper, and both fungi and iron are regarded ubiquitous in nature. In the early twentieth

century, Iiams and Beckwith (1935) proposed a duality of cause in the formation of foxing spots. They observed that organic acids that were secreted by metabolising fungi reacted with ferrous salt present in or on the paper. Iron oxides and hydroxides were formed as breakdown products that lead to brown or rusty coloration. They later found that iron also increased the intensity and degree of the discoloration of paper, which accompanies fungal growth and metabolic activity (Beckwith *et al*., 1940). Hey (1983) affirmed the duality of causes and concluded that a synergistic reaction between fungi and metal can occur in damp conditions, which lowers the pH of the substrate to the point where the existing fungi are killed.

Additionally, fungi use iron and copper as co-enzymes and need trace elements provided by metals for their growth. Any oversupply of these essential elements might be secreted after use in altered or activated form and accelerate the formation of foxing (Derow and Owen, 1992).

4. Methods for the identification and assessment of foxing on paper

4.1. Culturing

The identification of microorganisms from foxing spots classically required the isolation of a specific organisms by culturing and subsequent tests for physiological and biochemical properties as morphology alone, which is used to classify plants and animals, is not specific enough for reliable identification of microbes. Culture media are designed for maximal growth and contain nutrients in concentrations that are rarely found in nature and thus minimise the potential for discovering the whole microbial community in culturing attempts. Therefore, studies on biodeterioration relying on culturing techniques have considerable bias as the lists of species refers to those that are easily cultivable and omit slow growing, uncultivable or specialised microorganisms (Saiz-Jimenez, 2003).

Airborne propagules and fast-growing species can outgrow the active microorganisms functioning in the ecosystem. Organisms that live as parasites or symbionts or that entered a dormant physiological state also limit the culturing approach (Rölleke, 2003). Obtaining pure isolates however is invaluable if metabolic or physiological studies are planned to determine the properties of a

certain isolate or to link organisms to degradation or other activity observed in an environment. A combination of different culture medias will generate the best results as they represent different environmental conditions, but multiple tests also require multiple samples, which is a scenario avoided in relation to most items of cultural heritage.

Sampling valuable items of cultural heritage like books, paintings and textiles needs indirect or non-destructive methods like swabs or adhesive tape to avoid further destruction (Samson *et al.* 2002), but often no colonies develop after direct smearing of swabs or adhesive tape onto solid agar medium because the spores or mycelium fragments are often nutrient starved and dehydrated and are not able to grow without extra moisture provided (Pinzari *et al*., 2010).

Unspecific culturing results are obtained frequently especially with fungal species that cause damage to paper, as they are considered mostly xerophiles who require a growth medium with a low water activity in order to produce fruiting bodies or germinate, structures that are used in the identification of fungal species (Florian, 2002; Samson and Pitt, 2000). Culturing studies performed on library materials date back to the beginning of the twentieth century and mostly concentrate on fungi as biodeteriogens with more than 234 fungal species from 84 genera isolated from library materials, 57 of which were explicitly from books (Zyska, 1997).

More recent studies have focussed on the identification of fungal species specifically from foxing spots and the genera identified are amongst the listed in Table 1 (Florian, 1996; Florian and Manning, 1999, 2000; Arai, 2000; Nol *et al*., 2001; Press, 2001; Corte *et al*, 2003, Rakotonirainy *et al.,* 2007; Zotti *et al*., 2008; Mesquita *et al*, 2009; Pangallo *et al*., 2009). However, these studies did not succeed in providing a clear link between the presence of fungi on paper and the formation of foxing.

4.2. Visible and ultra-violet light examination

The color, shape and quantity of foxing spots can be determined under visible light together with characteristics of surface conditions of the paper in question. Successive pages can be checked for migration of spots and ultra-violet light can assist in determining the characteristics of the spots. Areas with light

foxing resulted in fluorescent spots under UV light as observed by Press (2001). He proposed that foxing is a consequence of growth of an organism that only emits fluorescence in its youth and early growth when they are practically unseen in visible light and browns with age, an observation also reported by Carter *et al.* (2000). This theory is supported by findings of Manso *et al.* (2009), who reported fluorescence around some foxing spots but also in unaffected areas.

4.3. Scanning electron microscopy and energy dispersive X-ray spectral analysis

Scanning electron microscopy (SEM) takes images of the sample surface by scanning it with a high-energy beam of electrons in a raster scan pattern to obtain a characteristic three-dimensional picture of a sample's surface topography or composition. SEM can be used to identify and characterise fungi on the paper substrate, and conidia with their different types of ornamentation, size, shape and colour are necessary for genus determination. Arai and co-workers used the morphology of hyphae and conidia they observed with SEM for the identification of fungi on paper, whereas other SEM studies examined iron and copper in foxing spots (Cain and Miller, 1982; Arai *et al*., 1988).

Florian and Manning (1999) applied SEM to foxing spots in a book from 1854 in order to establish the origin of contamination by the fungal species identified. They looked at the population of fungi and their distribution in the books as well as their location on the pages to determine how and when the paper was contaminated. Two main types of spots were identified, one with fungal structures growing on top of the paper and the other one containing dead fungal hyphae with holes between the fibres. The authors interpreted those as signs of lytic activity due to a bacterial infection of the fungi prior to the paper making process.

A combination of SEM with Energy Dispersive X-ray Spectral Analysis (EDS or EDX) enables detection of elements present in foxing spots, and this technique is mostly used to identify metals as the instrument is sensitive to elements with an atomic number higher than three. Arai (2000) checked for the existence of iron and copper in foxing spots by EDS analysis but could not reveal traces of them on the hemp paper used in the study.

Other optical methods that have been used to gain insight into foxing are Fourier Transform Infrared Spectrometry (FTIR), X-ray fluorescence or –radiography and Raman spectroscopy. FTIR was applied by Buzio *et al*. (2004) to reveal details about the molecular structure of foxing spots and sizing components, whereas X-ray fluorescence was applied to identify the elemental composition of foxing spots (Press 1974; Bicchieri *et al*., 2001; Castro *et al.* 2007; Manso *et al*. 2009).

4.4. Molecular microbiology techniques for the analysis of foxing

Molecular methods study the DNA, RNA and proteins of organisms to complement the more classical methods like cell counting and morphology based biodiversity analysis in order to deliver a better understanding of functions and activities of both culturable and unculturable organisms in a given environment. Sequencing of RNA present and its quantification can provide functional information of an organism, since the quantity of RNA in a cell is proportional to its metabolic activity (Molin and Givskov, 1999). Proteins are also used in functional studies, and they are detected by their amino acid sequence, their tertiary structure, by using antibodies that bind to surface structures, or direct activity measurements in enzyme assays (Gonzalez, 2003). But both RNA and proteins are rarely used in relation to studies of items of cultural heritage, which is possibly related to costs and expertise involved as well as the availability of sufficient sample material.

All molecular methods share the need for DNA or RNA extraction as a first step in the analysis. Extraction of nucleic acids from paper material has to overcome certain obstacles imposed by the importance of the samples. As the material analysed is usually precious and not available in a big quantity, the extraction has to work with small amounts of paper. When aiming for the identification of fungal species on paper, the presence of all metabolic states of fungi has to be taken into account, as fungi can exist as cells, hyphae, mycelium and spores with different levels of complexity for DNA extraction imposed by the structure.

The majority of DNA based experiments performed in the field of cultural heritage are based on ribosomal sequences that are widely used as phylogenetic

markers (Woese, 1987) and allow comparisons and phylogenetic affiliation of unknown microorganisms (Maidak *et al*., 1999). In contrast to using 16S ribosomal-RNA to perform phylogenetic classification as for bacteria, fungal species are predominantly identified to the species level by the use of the variable rDNA internally transcribed spacer (ITS) regions. The ITS regions as shown in Figure 4 are described as environmental barcoding marker for fungi (Bellemain *et al*., 2010), they are non-coding regions consisting of ITS1 and ITS2, which are intercepted by the 5.8S rDNA as part of the tandemly repeated nuclear rDNA. Non-coding regions benefit from a fast rate of evolution resulting in higher variation between closely related species and can provide greater taxonomic resolution than coding regions (Anderson *et al*., 2003; Lord *et al*., 2002). More than 100.000 fungal ITS sequences are deposited in databases to provide reference data for the identification of fungal taxa (Nilsson *et al.*, 2009).

The level of sensitivity and the outcome of molecular analysis rely heavily on the amplification of the small quantities of DNA available for analysis. ITS sequences are considered an appealing target for sequencing environmental samples with low DNA quantity present due to the large number of copies per cell (Bellemain *et al*., 2010). The most commonly used primers targeting the ITS region were published in the early 1990s and still remain the most used regardless of more recently published primers supposedly more specific (Martin and Rygiewicz, 2005). Some popular ITS primers and their target region are schematically shown in Figure 4.

Polymerase chain reaction (PCR) is the most established DNA amplification technique in the field of cultural heritage and it can be performed as a nested PCR to overcome the limitations of small initial sample material or DNA that is difficult to amplify (Al Nakkas *et al*, 2002). Quantification of specific DNA and RNA sequences has become possible with the implementation of real-time or quantitative PCR (qPCR) techniques but it has yet rarely been applied in the field of cultural heritage. Pinar *et al*. (2010) used qPCR to monitor the levels of *Myxococcus xanthus,* an organism used in the consolidation of historic ornamental carbonate stones.

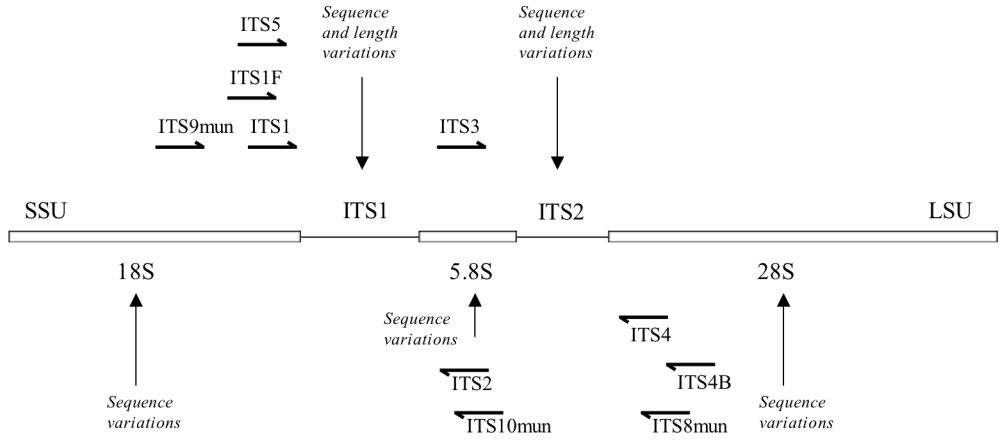

Figure 4. Schematic representation of the location of different ITS primers used for fingerprinting in the ribosomal RNA gene structure in fungi. The ribosomal genes consist of the small subunit (SSU) 18S, ITS1, 5.8S, ITS2 and the large subunit (LSU) 28S. The highly variable internally transcribed spacer (ITS) regions have sequence and length variations between fungal species and separate the rDNA coding subunits that show only sequence variation. Different primer sets are described in literature to amplify ITS1, ITS2 or both including the 5.8s rDNA sequences for fingerprinting analysis. Forward primers are positioned above their sequence position in the ribosomal cassette, and reverse primers below. Primers ITS1, ITS2, ITS3, ITS4 and ITS5 are taken from White *et al.(*1990), primers ITS8mun, ITS9mun, ITS10mun, from Egger *et al.* (1995) and primers ITS1-F, ITS4-B from Gardes and Bruns (1993).

In general, PCR amplified DNA contains a mixture of diverse but highly similar DNA fragments of unknown sequences. Further detailed phylogenetic information about the microbial communities is mainly obtained through cloning of the environmental DNA sample and sequencing of individual clones. A clone library is constructed to separate the multiple sequences and the resulting single template DNA fragments can subsequently be sequenced. A screening of the clone libraries prior to sequencing can help to determine redundancies or to estimate the relative abundance of particular cloned fragments in the environment (Muyzer and Smalla, 1998).

4.4.1. DGGE as a fingerprinting technique applied in cultural heritage

Genetic fingerprinting techniques visualise the biodiversity or species richness of a microbial community and ideally enable the differentiation of highly similar DNA fragments even if they show only minimal differences like single nucleotide disparities (Muyzer and Smalla, 1998; Gonzalez and Saiz-Jimenez,

2004). Denaturing gradient gel electrophoresis (DGGE) is amongst the most frequently reported fingerprinting techniques for the identification of microorganisms in cultural heritage studies (Gonzalez and Saiz-Jimenez, 2004).

DGGE is an electrophoretic method that melts and separates PCR-amplified double-stranded DNA fragments in the presence of a chemical gradient (Muyzer *et al*., 1993). The separation of a mixed sample is based on the decreased electrophoretic mobility of partially melted DNA fragments in polyacrylamide gels. Different melting behaviours of the DNA base pairs A-T and G-C result in significant changes of the running behaviour of DNA fragments in the gel.

The base configuration of the fragment hence generates different migration patterns, and nearly 100% of sequence variants can be detected in DNA fragments (Myers *et al*., 1985). G-C pairings are more stable and are added as a 'tail' to the fragments in question to prevent their complete dissociation into single strands. These GC-clamps are attached as part of a primer during PCR amplification and are usually about 40 base pairs long and produce a fork-like structure to anchor the fragment in the gel once the DNA has melted (Muyzer *et al.,* 1997). Numerous specific primer pairs are available for the generation of ribosomal DNA (rDNA) fragments that can be used in the phylogenetic identification of microorganisms (Saiz-Jimenez, 2004). The visualisation of rDNA fragments by DGGE using DNA extracted from a cultural sample represents a specific fingerprint, which will display common and unique bands in comparison to similar samples (Gonzalez and Saiz-Jimenez, 2004). However, different sequences can migrate to the same location in a gel, so sequencing of the band must be performed to ascertain identity.

DGGE related studies on cultural heritage have been performed on a variety of samples to identify and monitor changes in microbial communities colonising frescoes, medieval wall paintings and building materials (Rölleke *at al*., 1996; 1998; Pinar *et al*., 2001a; 2001b; 2010; Ripka *et al*., 2006; Cappitelli *et al*., 2009), pictorial layers (Santos *et al*., 2009), glass samples (Schabereiter-Gurtner *et al*. 2001), paleolithic caves (Schabereiter-Gurtner *et al*., 2002a; 2002b; Portillo *et al*., 2008; Portillo and Gonzalez, 2009) and salt attacked monuments (Pinar *et al*., 2009; Ettenauer *et al*., 2010).

Other fingerprinting techniques that hold potential for the application in

cultural heritage science include terminal restriction fragment length polymorphism (t-RFLP) that can be easily automatised for a high-throughput scale, or the electrophoretic technique single-strand-conformation-polymorphism (SSCP) that separates DNA fragments under non-denaturing conditions due to differences in their conformation (Gonzalez, 2003).

4.4.2. Fluorescence in situ hybridisation for single cell assessment

Another technique used to visualise microbial cells in an environmental sample is fluorescence in situ hybridisation or FISH. It detects genes using specific oligonucleotide probes matching the target sequence, which are generally labelled with fluorescent dyes. Specific microbial cells can be visualised on a single cell level, enumerated in complex communities *in situ* and studied under the microscope (Amann *et al*., 1997). Labelled cells can be counted in a flow cytometer for quantitative analysis and interactions between known species can be studied.

One of the rare applications of FISH in the field of cultural heritage was performed by Pinar *et al*. (2001), who used FISH to detect and monitor *Halobacillus* populations from ancient wall paintings. In another study, La Cono and Urzi (2003) proved that FISH could be performed directly on adhesive tape used to capture bacteria from a mortar surface and from biofilms in Roman catacombs. The limited application of FISH in the field of cultural heritage might be due to the high-end equipment needed for analysis and the requirement for specific probes mentioned above. FISH can only detect a defined target sequence and is of limited use for the identification of whole unknown communities. Another limit to the method is related to the ribosomal content of cells, which is variable between species as well as within cells of one strain according to their metabolic state (De Long *et al*., 1999), and as a consequence, dormant cells contain less rRNA and are more difficult to detect. Conditions on items of cultural heritage like paper are mostly considered unfavourable for microorganisms and hence most of them can be in a dormant state, removing the advantage of the high sensitivity of FISH. Another problem especially for the identification of fungi using FISH is that fungal cell walls are often rigid and hamper the use of DNA probes (Teertstra *et al*., 2004).

5. Biodeterioration control and sterilisation treatments for paper

Treatments and products against biodeterioration of paper are aiming to either control or eradicate the causative organisms. Several factors make the control of biodeteriogens on objects of cultural heritage a complex task. According to Nugari and Salvadori (2003), an in-depth analysis of the biological growth is required to evaluate the effectiveness of the treatment towards the target organism and simultaneously its harmlessness on the substrate backbone of the object of cultural value. The protocols applied should also define standardised procedures for testing and evaluating methods and products. Long-term effects of the treatments have to be considered for future restoration and storage, and alternative or non-toxic methods should be proposed. There is also the need for prevention to halt re-colonisation of the treated material. Paper is considered to be a complicated substrate, as it is composite and fragile in nature so that most physical methods of removal of biodeteriogens like scalpels, scrapers or vacuum cleaners are restricted in their application.

When treating fungal infections on paper, the treatment should be directed towards the spores, as they represent the reproductive units or propagules and threat of new fungal colonies (Nitterus, 2000). Spores can also impose a risk or health hazard to humans as they can cause mycoses, mycoallergies and mycotoxicoses when they invade skin, lungs, eyes or brains.

5.1. Freeze-drying

The application of sub-zero temperatures to objects in combination with water removal is called freeze-drying. In this process, water is removed by sublimation, which means it bypasses the liquid phase and transforms from solid to vapour directly. A vacuum is involved to take advantage of the properties of water under low pressure, causing the water to vaporise at a lower temperature to avoid dimensional changes in the paper caused by evaporative effects of water (Carrlee, 2003). A cold condenser and a gentle heating element in the freeze-drying chamber is kept at a temperature slightly above the temperature of the frozen object to allow water molecules on the surface of the object to break free and gather on the condenser as frost (Schmidt, 1985).

Freeze-drying however is only useful when objects are wet, and most

paper objects generally lack sufficient moisture for freeze-thaw or dehydration mechanisms to occur (Carrlee, 2003). The average moisture content for organic artefacts in museum environments is 8-12% (Florian, 1986), a level that limits ice formation as the water is physically absorbed or chemically combined and therefore not available, a process known to cause freeze-thaw damage from the 9% volume expansion that takes place when water changes from the liquid to the solid phase (Franks, 1985). To avoid possible condensation of water on the paper or its swelling during the cooling or warming periods of the treatment, the object can be sealed into plastic bags with most of the air removed.

Other forms of potential damage like staining, embrittlement and shrinkage have been mentioned by conservators, but experiments suggest that no significant structural damage occurs in paper with repeated freeze-drying for pest control (Björdal, 1998). Other authors however observed that freeze-drying influenced paper characteristics such as moisture content, folding endurance and tear strength, particularly in paper with low initial strength (Carlsen, 1999).

As a consequence, freeze-drying of valuable books or documents is largely performed to save material that has been soaked with water in events like floodings or to control insect pests. It is not considered a form of treatment for mould. Freeze-drying in combination with dehydration can kill the vegetative parts of fungi like hydrated and germinating conidia and it stops the growth of the mycelium, but fungal spores can remain viable (Sussman *et al.*, 1966; Mazur and Schmidt, 1968; Mazur, 1970; Florian *et al*., 2002). Moisture content in treated paper is important as resting dry conidia can be activated through elevated moisture. Freeze-drying is still the most effective method for the physical, chemical and mechanical stabilisation of moisture damaged archival and library materials that need to be saved fast and in large quantities (Schmidt, 1985; Florian, 1990, 2002).

5.2. Electromagnetic radiation

Gamma rays are a form of high-energy electromagnetic radiation that causes direct damage to cell DNA through ionisation inducing mutation and killing of the cell (Da Silva *et al*., 2006). Gamma rays can deeply penetrate the treated material and show powerful biocidal action against insects and

microorganisms including spores (Brokerhof, 1989; Nitterus, 2000). Ionising radiation is quick, simple and leaves no hazardous residues, an advantage for the people involved in the process compared to other disinfection treatments, as the handling of treated paper is not hazardous. The drawbacks include lowered folding endurance and tear resistance of paper, its increased yellowing and embrittlement and depolymerisation of cellulose proportional to the absorbed radiation (Adamo *et al.*, 1998; Nitterus, 2000).

The use of gamma rays in conservation of cultural heritage is surrounded by controversy, as negative effects were observed in some studies but rendered unimportant in others (Adamo *et al*., 2001). Magaudda (2004) delivered a good summary of this controversy, admitting that radiation deteriorates cellulose and thus paper, but he made it clear that the observed effects on mechanical and physical properties of cellulose are true only to very high doses of radiation that are not needed for a recovery treatment of paper displaying biodeterioration. By keeping the duration of irradiation short, the possibilities of oxidative degradation induced by radiation are limited (Gonzalez *et al*., 2002).

Adamo *et al*. (2001) observed a synergetic effect of water during the irradiation process of wet paper. This indirect effect can be explained as a fortification of the treatment, because when water is subjected to radiation it forms free short-lasting radicals that have germicidal properties themselves, hence adding to the effect of the radiation itself. The same is reported for the radiolysis of cellular water with the formation of reactive oxygen species, free radicals and peroxides that cause single and double strand DNA breakages (McNamara *et al*., 2003).

5.3. Fumigation with ethylene oxide

Ethylene oxide (EtO) is a product of the oxidation of ethylene with air and its usage started as an insect and fungi fumigant in 1928 with wide application between 1970 and 1990 (Nugari and Salvadori, 2003). EtO was first applied to fumigate objects of cultural heritage in response to virulent outbreaks around 1933, and subsequently became a standard gaseous fumigant for libraries and museums (Ballard and Baer, 1986).

Vacuum fumigation of objects of cultural heritage is used to eradicate

bacteria, fungi and insects in a large numbers of objects at one time by exposing them to EtO in airtight chambers or sealed spaces (Nugari and Salvadori, 2003). Pressure increases the permeability of the treated material by the active gaseous compound, which is already higher than in liquid compounds. EtO does not require activation energy, expresses high reactivity and works by adding alkyl groups to DNA, RNA or proteins, which prevents normal cellular metabolism and the ability of the cell to reproduce (Rutula and Weber, 1999).

Unfortunately, EtO and some of its residues display chronic toxicity. As a directly alkylating and genotoxic agent, EtO was proven to have carcinogenic potential and this has consequently led to restricted use or a complete ban of its use as a sterilisation agent in many countries (Bolt, 1996; Angerer e*t al*., 1998). Dependent on the material treated, the amount of EtO used and the duration of the treatment, EtO residues evaporate over time, representing a risk to people involved in the handling of the material.

Furthermore, it was observed that EtO fumigated paper material was more susceptible to microbial attack during further storage (Valentin, 1986). As an explanation, Florian (1993) indicated the potential for ethylene glycol, a by-product of EtO fumigation, to activate fungal spores that are in metabolic arrest. Fungal spores form upon dehydration to survive adverse environmental conditions, but this is a reversible process, which needs water and an additional physical or chemical activator like ethylene glycol to reverse. Once activated, a spore can germinate immediately when water is available.

EtO fumigation also has long-term visual, chemical and physical effects that are known to conservators, archivists, librarians and scientists. It can react with sulphydryl groups in proteins and other polymers, can induce polymerisation and can oxidise copper and brass. Fumigation with EtO can also lead to a loss of strength in paper and cotton (Nugari and Salvadori, 2003) and is involved in changing the colours of pigments.

Other methods are regularly used for the treatment of biodeteriorated paper but do not form part of this thesis. Amongst them, local or overall aqueous washing and bleaching of paper can slow down the acidic hydrolysis and oxidation of the cellulose in foxing affected paper and solubilise fungal bodies and metabolites (Neevel, 1995; Florian, 1996)**.** In addition, lasers of different

wavelengths are used to sterilise paper by burning, cutting or disrupting molecular bonds in the surface of microorganisms. Growing fungi can be removed from a paper surface with laser cleaning without damaging the underlying artwork (Friberg *et al.,* 1997a, b). Unfortunately, laser cleaning sends out little shockwaves that can cause fungi to be dislodged from their original place on the paper surface and infect a new place and can consequently impose health hazards to people involved. Some authors discourage from laser cleaning of foxing affected paper as it appeared to damage the paper (Asmus, 1986), whereas others reported no damage to paper fibres (Szczepanowska and Moomaw, 1986).

6. Aims of the thesis

Foxing spots occur on paper-based objects independent of age, paper composition or manufacture and the involvement of fungi in the formation of foxing has long been postulated. Nevertheless, results have been inconclusive as most fungi identified are regarded as ubiquitous and no cause and effect relationship has yet been determined. As most studies on foxing are based on classical culturing or optical methods, it is expected that many fungi are undetected due to the well-known limitations of both methods. One main objective of this study was to apply molecular techniques that are widely used in environmental microbiology to identify fungal communities from foxing spots to close the gap between culturable and unculturable fungi and to find a link to the formation of the spots.

As foxing leads to aesthetic and possible structural damage of paper, means to control the biodeterioration with conservation methods are needed. The effect of such conservation treatments on fungi is therefore crucial for the maintenance of our cultural heritage. To achieve insight into fungal communities of foxing spots and their behaviour post treatment, the aims of the thesis were:

- Optimisation of molecular protocols for working with paper made objects focussing on non-destructive sampling and fungal spores (presented in Chapter II)

- Characterisation of the fungal flora associated with foxing spots from paper of different age and composition (as shown in Chapter III)

- Implementation of the developed protocol in the conservation strategy of real-case objects with visible biodeterioration (presented in Chapters IV and V)

- Monitoring of conservation treatments for paper with focus on fungal residues and viability over time (shown in Chapter VI)

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Chapter II

Application of molecular techniques for the identification of fungal communities colonising paper material

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Application of molecular techniques for identification of fungal communities colonising paper material

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Abstract

Archives and libraries all over the world suffer from biodeterioration of writings caused by microorganisms, especially fungi. With traditionally used culture-dependent methods, only a small amount of effectively colonising organisms is detected. Restoration and maintenance of written cultural heritage is therefore problematic due to incomplete knowledge of the deterioration agents.

In the present study, culture-independent molecular methods were applied to identify fungal communities colonising paper samples of different composition and age. Nucleic-acid-based strategies targeting the internally transcribed spacer (ITS) regions, which are nested in the nuclear rDNA repeats, were selected to investigate the fungal diversity on paper. The ITS regions possess a high variation among taxonomically distinct fungal species and even within the species.

With this aim, several molecular biological methods were optimised for working with paper materials. Here, we introduce a DNA extraction protocol, which allowed the direct extraction of PCR-amplifiable DNA from samples derived from different kinds of paper. The DNA extracts were used to amplify either the ITS1 or ITS2 region by using different fungi-specific primer sets. The ITS-amplified regions were subsequently analysed by denaturing gradient gel electrophoresis (DGGE). Conditions for DGGE analysis, gradient, voltage, and running time, were established to accurately discriminate different fungal species in complex communities. Pure fungal strains were used to constitute a marker for further comparative investigations of historic papers.

Keywords: Objects of art; DGGE; Internal spacer regions; PCR; Fungal communities

1. Introduction

In addition to presenting environmental concerns, microorganisms are responsible for the biodeterioration of cultural objects in the areas of art and writing. They often cause degradation of objects such as paintings, stone, wood, paper, masonry, leather, parchment, glass and metal, and cinematographic films (Cappitelli and Sorlini, 2005; Abrusci et al., 2005). The chemical and structural nature of the substratum, as well as environmental conditions such as moisture, temperature, pH, and light, are the significant parameters affecting quantity and quality of microbial colonisation on works of art (Saiz-Jimenez, 1993).

One type of deterioration found on cultural properties made of paper, and on books, is called foxing, the brown spots that appear to be caused by airborne fungi (Arai, 2000). It has also been suggested that contamination can occur during paper-making or book preparation, in addition to being caused by airborne fungi. Archives and libraries from all over the world suffer from biodeterioration phenomena, but with the traditionally used culture-dependent methods, only a small amount of effectively colonising microorganisms is detected and a relatively large amount of sample is needed. Restoration and maintenance of written cultural heritage is therefore problematic because of the incomplete knowledge of deteriorative organisms or agents. For correct conservation, it is important to identify the complete microbial community colonising art objects, using non-destructive sampling, or sampling that needs only small amounts of material.

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There are several works describing bacterial communities involved in the degradation of art objects (Schabereiter-Gurtner et al., 2001). However, the fungal flora responsible for the biodeterioration of such objects is not yet well described. There are very few works focusing on the investigation of the fungal flora responsible for the biodeterioration of paper materials by applying molecular techniques (Di Bonaventura et al., 2003a, b).

It has been estimated that only about 5% of fungal species involved have been accurately described owing to culture limitations, misidentifications in culture collections, and unexplored habitats (Hawksworth and Rossman, 1997). With classical cultivation or microscopic methods, species such as the cellulolytic *Chaetomium* spp., *Penicillium* spp., *Aspergillus* spp., *Eurotium* spp. or *Trichoderma* spp. (Szczepanowska and Cavaliere, 2000; Corte et al., 2003), could be detected on paper material. Other strains frequently found as contaminants in archives and libraries are *Paecilomyces variotii, Myrothecium verrucaria*, *Stachybotrys atra,* and Fungi Imperfecti (Deuteromycetes) (Florian and Manning, 2000).

Molecular approaches have been developed for the assessment of microbial diversity in complex communities (Gonzalez and Saiz-Jimenez, 2005). Methods based on DNA analysis can reveal fungal diversity in ecosystems, and offer the potential benefits of highly sensitive and rapid detection (Saad et al., 2004). The molecular identification of fungi to species level has been based mostly on the use of variable ribosomal-DNA (rDNA) internally transcribed spacer (ITS) regions. The non-coding ITS region consisting of ITS1, the 5.8S rDNA and ITS2, should produce a highly sensitive assay as the target sequence for amplification, because of its high copy number in the fungal genome as part of tandemly repeated nuclear rDNA. These regions benefit from a fast rate of evolution, which results in higher variation in sequence between closely related species, in comparison with the more conserved coding regions of the rRNA genes. As a consequence, the DNA sequences in the ITS region generally provide greater taxonomic resolution than those from coding regions (Anderson et al., 2003; Lord et al., 2002). Additionally, the DNA sequences in the ITS region are highly variable and might serve as markers for taxonomically more distant groups.

In this study, culture-independent molecular methods were applied to investigate fungal communities colonising paper material of different age and quality for the first time. Pure fungal strains were used to artificially infect different kinds of paper, which were subsequently used to compare and optimise established molecular biological methods for working with this kind of material.

Here we introduce a DNA extraction protocol, which allowed the direct extraction of PCR-amplifiable DNA from samples derived from different kinds of paper. The DNA extracts were used to amplify either the ITS1 or ITS2 regions, which were subsequently analysed by denaturing gradient gel electrophoresis (DGGE). Conditions for PCR, such as the use of different fungi-specific primer sets, as well as for DGGE analysis, such as gradient, voltage, and running time, were established to accurately discriminate different fungal species in complex communities. In addition, pure fungal strains were used to constitute a marker for further comparative investigations of historic papers.

2. Materials and methods

2.1. Preparation of samples

The optimisation of DNA extraction protocols to obtain PCR-amplifiable DNA from paper materials was performed with the following set of materials: (a) pure fungal colonies obtained from strains known to be involved in paper, wood, and cellulose biodeterioration; (b) samples of model-paper inoculated with suspensions of spores from single fungal strains or mixtures in known proportions; (c) paper samples with naturally occurring fungal infections; (d) paper samples with 20-year-old inocula and naturally occurring infections, selected in order to verify the chemical and physical changes in fungal nucleic acids on paper during time, and the effects on molecular diagnosis.

2.1.1. Fungal strains and growth conditions

The following four fungal strains, provided by the Institut für Holzforschung, Vienna, Austria, were used as marker strains: *Alternaria alternata* (BAM Berlin DSM 62010-VdL-RL06), *Aspergillus versicolor* (EMPA St.Gallen Nr.517), *Chaetomium globosum* (Messehalle Hannover) and *Cladosporium cladosporioides* (BAM Berlin DSM 62121-VdL-RL06). In addition to the aforementioned strains, fungal strains considered to be frequently associated with library material deterioration had been utilised 20years ago to inoculate paper strips, and this was repeated more recently. *Aspergillus terreus* Thom (strain no. 3) was obtained from the ATCC culture collection, *Aspergillus hollandicus* (Anam.: of *Eurotium amstelodami*, (Mangin) Thom and Church) and *Eurotium chevalieri* L. Mangin from the culture collection of the Istituto Centrale per la Patologia del Libro (ICPL), Rome, Italy, and were isolated from deteriorated paper materials. Additionally, *Penicillium rubrum*, *Byssoclamys fulva,* and *Aspergillus niger,* from the same collection, were used for inoculation 20 years ago.

For DNA extraction, the strains were grown on malt-peptone agar at 27 °C until vigorous growth was observed, prior to their harvest. The strains utilised for the inoculation of paper samples were grown on MEA (malt extract agar) for 7 days at 25 °C. The spore suspensions were obtained by gently scraping the surface of the 7-day-old cultures with a swab, washing it with 30 mL of H_2O containing 0.02% Tween 80 (Merck-Schuchardt, Hohenbrunn, Germany) and filtering through a sterile cotton cloth to remove impurities.

2.1.2. Artificial inoculation of paper material

Preparation and inoculation of paper materials was performed at the ICPL. Three types of paper with different composition, depending on the origin of the fibres and chemical treatments undergone during the manufacturing process, were used in this study (Table 1). Whatman paper is considered as a model because of its standard composition and manufacturing and its high level of availability in laboratories all over the world, although it is not used as writing material.

Paper strips of 2 × 6 cm of the three different paper types were exposed to UV light for 45 min on both sides to eliminate airborne fungal and bacterial cells from the surface prior to inoculation. The spore density used for inoculation of paper strips was defined for each fungal strain by optical microscopy in a Thoma Chamber. A defined volume of each spore suspension was diluted with Sabouraud Broth (DIFCO, Becton Dickinson, Heidelberg, Germany) in order to perform the inoculation with a comparable number of spores. Paper samples with single-strain inocula contained 3500 spores/100 µL. Mixtures with different proportions of spores were used to inoculate paper strips. The strips were inoculated with

Table 1
Paper types with fungal infections

Paper type	Composition	pH	Inoculation	Natural alterations
Whatman 1CHR paper for chromatography Cat. No. 3001 917	Pure cellulose	5.4	Single: *Aspergillus hollandicus* *Aspergillus terreus* *Eurotium chevalieri* *Mixed: 1:1:1* 3:1:1 3:3:1 20 y ago: *Chaetomium globosum* *Aspergillus niger* *Penicillium rubrum* *Byssoclamys fulva*	Recent 20 years ago
"Freelife vellum" from Fedrigoni Mills (Italy), long-life type (according to ISO CD 9706)	80% recycled fibres, 15% cellulose chlorine free, 5% cotton fibres	7.2	Single (as above)	Recent
"Old mill" ivory from Fedrigoni Mills (Italy), uncoated long-life type	100% cellulose fibres	6.9	Single (as above)	Recent

100 μL of broth with fungal spores distributed in 8 dots, and were incubated for 10 days at 27 °C and 75% relative humidity (saturated NaCl solution) each in 50 mL polystyrene vials. The incubation was performed in a thermostatic cell, and a sensor was used to register the relative humidity (Hygrolog-D Rotronic AY-Swiss).

2.2. DNA extraction from paper material

Two different methods were tested for the extraction of fungal DNA from paper materials of different age and quality: (A) DNA was extracted directly from paper by using the FastDNA Spin Kit for Soil (Qbiogene, Illkirch, France). The protocol of the manufacturer was slightly modified. About 10–20 mg (0.5–1 cm^2) paper was placed in the Multimix2 tissue tube with the appropriate buffer and then processed on a bead-beater twice for 1 min, with 1 min intervals on ice. The PPS reagent and the binding matrix solution were applied to the supernatant; the suspension was transferred to the provided spin filter and centrifuged at 13,000 rpm for 1 min following the instructions of the manufacturer. DNA was washed twice with 500 μL of the SEWS-M solution and eluted from the binding matrix with 100–150 μL DNase/Pyrogene Free Water. (B) DNA extraction was done following the protocol previously described by Schabereiter-Gurtner et al. (2001). Briefly, this method combines enzymatic (lysozyme and proteinase K) and mechanical steps (freeze and thaw cycles) in the presence of cetyltrimethylammonium bromide (CTAB).

DNA crude extracts were used directly for PCR amplification analysis or were further purified prior to PCR amplification with the QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer. The final elution step was repeated three times with 80 μL of 80 °C preheated ddH_2O (Sigma Aldrich, St. Louis, USA).

2.3. DNA extraction from pure fungal cultures

DNA was isolated from fresh mycelia taken from the surface of plate cultures. The method is based on that of Jasalavich et al. (2000), using CTAB in the presence of ß-mercaptoethanol. The mycelia was further processed mechanically with glass beads (0.1–2 mm) in a bead-beater and with two cycles of freeze–thawing (2 min at 95 °C, 2 min at −20 °C, 2 min at 95 °C), followed by organic extractions and isopropanol precipitation of DNA. The DNA yield was visualised in a 1% agarose gel.

2.4. PCR amplification of extracted DNA

All PCR reactions were performed in a Robocycler (Stratagene, La Jolla, USA) using PCR Master Mix (Promega, Mannheim, Germany). Fragments of about 300 bp in size, corresponding to either the ITS1 or ITS2 region of the ribosomal-DNA ITS region, were first amplified with the primer pairs ITS1 forward and ITS2 reverse, or ITS3 and ITS4, according to White et al. (1990). Primer pair EF3RCN (Lord et al., 2002) with the reverse primer ITS4 (as above) was also tested. All reactions were carried out in a 25-μL volume containing 12.5 pmol of each primer and 1.5–2.5 μL DNA as template, following the manufacturer's instructions for PCR Master Mix. The thermocycling program was as follows: 5 min denaturation at 94 °C, followed by 30 cycles of 1 min denaturation at 94 °C, 1 min annealing at 60 °C, and 1 min extension at 72 °C. Five minutes at 72 °C were used as a final extension step.

For DGGE analysis, a nested PCR was performed in a total volume of 100 μL (2 × 50 μL reaction size), each with 3.5 μL of PCR product from the first amplification as template DNA. The same forward and reverse primers were used, with a 37-base GC-clamp attached to either 5′-end of the forward primer, to stabilise the melting behaviour of the DNA fragments (Muyzer et al., 1993). The cycling scheme was as follows: 5 min denaturation at 94 °C, followed by 35 cycles of 1 min denaturation at 94 °C, 1 min annealing at 58 °C for ITS1-GC primer or 62 °C for ITS2-GC primer, 1 min extension at 72 °C with a final extension step at 72 °C. PCR products were analysed by electrophoresis in a 2% (w/v) agarose gel.

2.5. Denaturing gradient gel electrophoresis (DGGE)

For the genetic fingerprint of the amplified ITS regions, 50–100 μL of PCR products containing the GC-clamp were precipitated with 96% ethanol at −20 °C overnight, resuspended in 15 μL double-distilled H_2O, and separated by DGGE. Gel electrophoresis was performed as previously described (Muyzer et al., 1993) in 0.5 × TAE (20 mM Tris, 10 mM acetate, 0.5 mM Na_2EDTA; pH 7.8) with 8% (w/v) acrylamide gels containing a gradient of denaturants in a D-GENE-System (Bio-Rad, Munich, Germany). The gradient of denaturants, as well as running conditions, was optimised in this study and is given in the results section. After completion of electrophoresis, gels were stained in an ethidium bromide solution and documented with a UVP documentation system (Bio-Rad Quantity-One, Munich, Germany).

2.6. Preparation of a reference marker for DGGE analysis

A marker containing ITS1-GC or ITS2-GC PCR products, derived with primers ITS1-GC/ITS2 and ITS3-GC/ITS4 from seven different fungal strains, was designed to allow comparative analyses of DGGE patterns from different gels, and as a quality control of the gel run. One hundred μL PCR product from each species was pooled, precipitated in 96% ethanol at −20 °C, and then resuspended in 50 μL ddH_2O. Fifteen μL were used as marker for DGGE analysis. The reference species used were: *A. alternata*, *C. globosum*, *C. cladosporioides*, *A. hollandicus*, *E. chevalieri*, *A. terreus,* and *A. versicolor*.

2.7. SEM microscopy

SEM pictures of paper material with and without fungal colonisation were taken by a LEO 1450 VP from the Carl-Zeiss Electron Microscopy Group (Jena, Germany).

3. Results

3.1. Optimisation of DNA extraction protocols from fungal-infected paper samples and evaluation of their efficiency by PCR amplification

From paper material, DNA was extracted following two different protocols—the DNA extraction protocol described by Schabereiter-Gurtner et al. (2001) and the commercial kit FastDNA Spin Kit for Soil with modifications, as described in the materials and methods section. A further purification step using the QIAamp Viral RNA mini kit was carried out with the crude DNA extracts obtained from both DNA extraction methods to compare the PCR-amplifiability after a second purification step.

Both protocols (with and without the extra purification step) were tested for all three different types of paper (see Table 1). Each batch of paper samples consisted of several inoculated paper strips containing different mixture proportions of the selected fungal spores. In addition, each batch of paper samples included an incubated, non-inoculated paper as negative control.

DNA extraction efficiency was tested via fungal-specific PCR amplification of the minimal content of fungal DNA on the inoculated paper. The fungal specific primer pairs EF3RCN/ITS4, ITS1/ITS2, and ITS3/ITS4 allowed DNA of pure fungal strains to be amplified using these specific primer sets, whereas bacterial DNA from different phylogenetic groups, used as control for fungal specificity of the primer sets, was not amplified. No amplification was observed in any of the non-inoculated paper strip controls, excluding contamination during extraction procedure, as well as the amplification of plant material.

By using the DNA extraction protocol described by Schabereiter-Gurtner et al. (2001), only paper C gave amplifiable material without a further purification step. PCR products from papers A and B could be obtained only when this DNA extraction protocol was applied in combination with the QIAamp Viral RNA mini kit (data not shown). In contrast, the bead-beating homogenisation of the FastDNA Spin Kit proved to be more efficient for fungal DNA extraction from infected paper material. It resulted in pure DNA for direct use in PCR analysis, without the need for a second purification step, for all three different kinds of paper materials tested, independently of the primer set used. When this protocol was used in combination with the QIAamp Viral RNA mini kit as a second purification step, no differences in PCR amplifiability were observed from the first eluent of purified and non-purified DNA. A decrease in the intensity of PCR products obtained from the consecutive second and third eluents, according to their presumed DNA content, was observed, as shown in Fig. 1. Consequently, the FastDNA Spin Kit for Soil was selected for further experiments without the need of an extra time-consuming purification step. For DGGE analysis, a nested PCR with primers containing a GC-clamp was carried out, as mentioned in the materials and methods section. Some differences could be observed, depending on the primer pair used. Primer pair ITS1-GC/ITS2, amplifying the ITS1 region, produced specific PCR products, whereas amplification using the primer pair ITS3-GC/ITS4, for the ITS2 region, resulted in unspecific PCR products, which could not be eliminated by changing PCR conditions, such as number of cycles, annealing temperature, or amount of template (data not shown).

3.2. Optimisation of conditions for DGGE analysis

To establish the optimal DGGE running conditions for the fungal DNA fragments, a "time travel" experiment was arranged, in which the marker mixture of PCR products derived from genomic DNA of seven different fungal species, i.e., *A. alternata*, *C. globosum*, *C. cladosporioides*, *E. chevalieri*, *A. terreus*, *A. hollandicus*, and *A. versicolor*, was applied to a denaturing gradient gel containing a gradient of 20–60% of denaturants and run with a voltage of 200 V. The marker was loaded every 30 min over a total time of 6.5 h to get a variety of time points in one gel. The optimal running time of the gel was determined as the time necessary to achieve the migration of the seven DNA fragments as independent single bands. As shown in Fig. 2, the fragments could not be separated as single bands for the first 60 min. After this time, they started to separate from each other due to the denaturing conditions within the gel; the molecules were partially melted and slowed down. After ~7 h the maximum resolution of the three fragments was achieved for both ITS1 and ITS2 region analysis. In parallel, a time travel experiment keeping a low voltage of 100 V for 14 h was tested (data not shown). Results showed a better resolution of bands as when using 200 V and 7 h; therefore we used these conditions for further experiments.

For optimisation of the denaturing gradient, the concentration of denaturants was varied in subsequent experiments to obtain more accurate fungal fingerprints. Gradients of 30–50% and 30–60% were set for DGGE analysis of the ITS1 and ITS2 region, respectively.

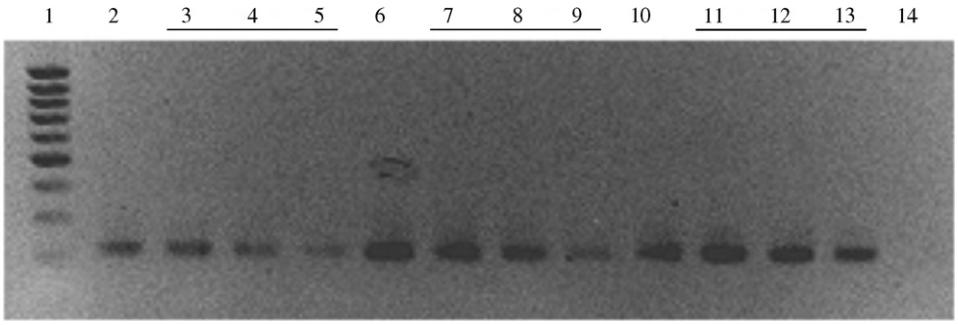

Fig. 1. PCR amplification of DNA extracted from infected papers by using the FastDNA Spin Kit for Soil with and without a further purification step with the Qiagen Viral Kit. PCR was performed with the primer pair ITS1/ITS2. Lane 1: 1kb marker; lane 2: paper A not purified; lanes 3–5: purified paper A eluents 1–3; lane 6: paper B not purified; lanes 7–9: purified paper B eluents 1–3; lane 10: paper C not purified; lanes 11–13: purified paper C eluents 1–3; lane 14: PCR negative control.

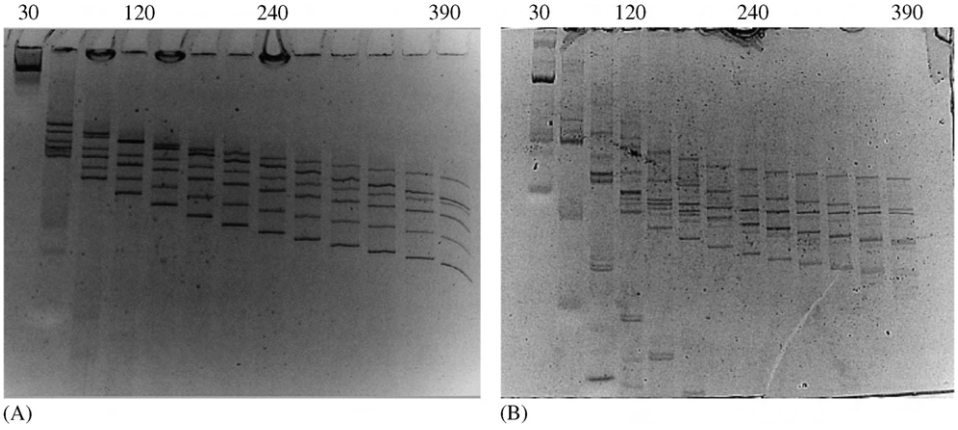

Fig. 2. Time travel experiment for the ITS1 and ITS2 regions. Ethidium bromide-stained DGGE separation pattern of fungal specific PCR fragments derived from the ITS1 (A) or the ITS2 (B) region. A mixture of PCR products obtained from *Alternaria alternata*, *Chaetomium globosum*, *Cladosporum cladosporioides*, *Eurotium chevalieri*, *Aspergillus terreus*, *Aspergillus hollandicus*, and *Aspergillus versicolor* was applied every 30 min over a total of 6.5 h. Both gels were run at 200 V with a gradient of 20–60% urea and formamide.

3.3. Creation of a fungal DNA reference marker for DGGE analysis

Seven different fungal strains (see Section 2.6) were selected for the creation of a fungal DNA reference marker for DGGE analysis. Three of them, namely *A. terreus*, *A. hollandicus*, and *E. chevalieri*, were the strains used to inoculate the paper strips. DNA extraction from pure fungal cultures was performed using the CTAB method, as described in the materials and methods section. PCR-amplifiable DNA could be extracted from all seven fungal strains without further need for purification or dilution (data not shown).

The extracted DNA was subsequently amplified with primers ITS1-GC/ITS2 and ITS3-GC/ITS4 and the resulting ITS-amplified fragments were used to create an internal reference marker for DGGE analysis of the ITS1 and ITS2 region, respectively (Fig. 3). DGGE of PCR fragments amplifying the ITS1 region resulted in reproducible clear single bands for each fungal strain (Fig. 3A). In contrast, DGGE analysis of PCR fragments amplifying the ITS2 region led to multiple bands for single strains and, therefore, to an overestimation of fungal strains (Fig. 3B). A discrimination between *A. hollandicus* and *E. chevalieri* strains was not possible in any case. Even by changing running conditions and/or denaturing gradients, no differences in the melting behaviour of these two fungal strains could be observed.

To correlate the consecutive order of the amplified fungal ITS regions in our marker with their phylogenetic identity, the marker and the DNA of the individual fungal strains were run together in one gel (Fig. 3). DGGE revealed the consecutive order of the fungal strains in our marker as being: *A. alternata*, *C. globosum*, *C. cladosporioides*, *A. hollandicus*, *E. chevalieri*, *A. terreus*, and *A. versicolor* for the ITS1 region DGGE marker, and *A. alternata*, *A. terreus*, *C. cladosporioides*, *A. hollandicus*, *E. chevalieri*, *C. globosum*, and *A. versicolor* for the ITS2 region marker. The creation of this marker allowed the identification of the inoculated strains in our samples, as well as the comparative analysis of DGGE patterns obtained from different gels.

3.4. DGGE analysis of naturally altered paper samples

Once the molecular strategy for working with fungal strains on paper materials was optimised, the next step was

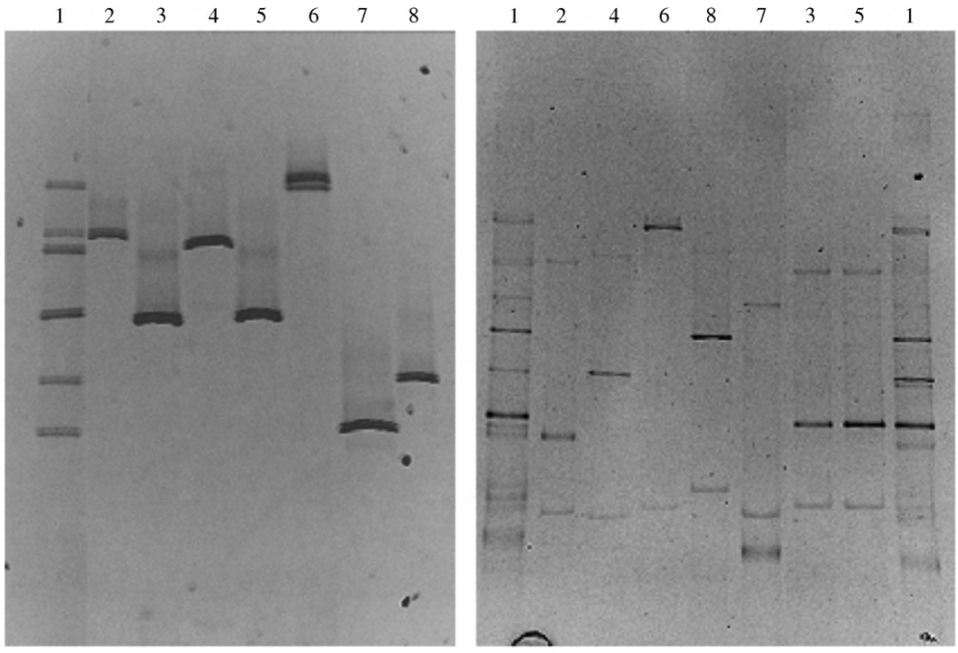

Fig. 3. Fungal DNA marker for DGGE analysis: (A) DNA amplified with primer pair ITS1-GC/ITS2. (B) DNA amplified with primer pair ITS3-GC/ITS4. Gels were run for 14 h at 100 V in gels containing a denaturing gradient of 30–50% for the ITS1 region or 30–60% for the ITS2 region. Nomenclature for both A and B. Lane 1: marker: mixture of all seven fungal strains; lane 2: *Chaetomium globo*sum; lane 3: *Aspergillus hollandic*us; lane 4: *Cladosporum cladosporioides*; lane 5: *Eurotium chevalieri*; lane 6: *Alternata alterna*ria; lane 7: *Aspergillus versicolor*; lane 8: *Aspergillus terreus*.

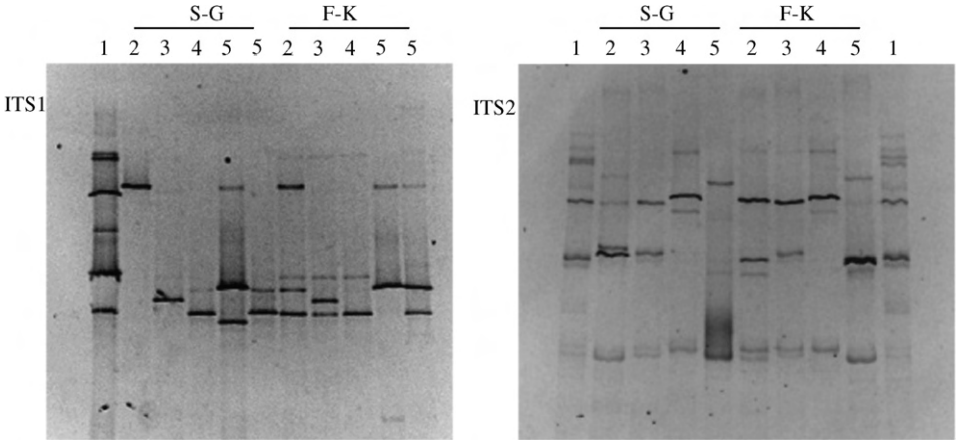

Fig. 4. DGGE analysis of different naturally infected paper types: a comparison between two types of DNA extraction methods and amplified-ITS target regions: (A) fingerprints of the amplified-ITS1 region on a gel with a denaturing gradient of 30–50% urea and formamide. (B) Fingerprints of the amplified-ITS2 region on a gel with a denaturing gradient of 30–60%. Both gels were run over 14 h with 100 V. Nomenclature counts for both. S–G indicates the extraction of DNA according to Schabereiter-Gurtner et al. (2001) whereas F–K stands for the use of the FastDNA Spin Kit for Soil DNA preparation. Lane 1: marker mixture; lane 2: paper A; lane 3: paper B; lane 4: paper C; lane 5: paper A naturally infected 20 years ago.

to test the established methodology for paper samples naturally infected or artificially inoculated 20 years ago (see Table 1). DNA extraction from infected paper samples was performed by using the two protocols tested in this study, as described in the materials and methods section.

To compare both methods, DNA extracts from all samples were used to amplify both the ITS1 and ITS2 regions, shown to be all PCR-amplifiable using either the ITS1 or ITS2 primer sets. Further DGGE analyses in 30–50% denaturing gels for ITS1-GC and 30–60% denaturing gels for ITS2-GC were carried out as mentioned above. Fig. 4 shows the DGGE fingerprints of paper samples naturally infected with fungal strains. DGGE profiles were different depending on the DNA extraction method. Using the DNA extraction protocol developed by Schabereiter-Gurtner et al. (2001), some dominant bands were completely absent, indicating that this lysis method was not suitable for fungal spores. In addition, as already observed for the fungal DNA fragments contained in our DGGE marker, only the fungal amplified ITS1 region fragments led to reproducible single bands on the DGGE fingerprints (Fig. 4A), whereas fingerprints obtained from

fungal amplified ITS2 region fragments produced no clear band patterns, with many weak bands (Fig. 4B). This let us to conclude that further experiments to investigate complex fungal communities on naturally infected paper samples will be based on the combination of the FastDNA Spin Kit for Soil DNA extraction protocol, followed by PCR amplification of the ITS1 region and the subsequent analysis of the resulting fragments by DGGE.

4. Discussion

Paper has been the main material for recording cultural achievements all over the world, and its microbial degradation is one of the most unappreciated and serious reasons for damage to library and archival materials. Fungal communities have been investigated with PCR-DGGE in different ecological systems such as grass, wheat, wood, soil (Smit et al., 1999; van Elsas et al., 2000; Vainio and Hantula, 2000), and historical church window glass (Schabereiter-Gurtner et al., 2001). Furthermore, the study of fungal communities colonising objects of art made of paper by using PCR amplification of the ITS of the nuclear ribosomal RNA genes has been already described (Di Bonaventura et al., 2003a, b). In this work, samples from fungal spots on Tiffany's drawings were removed and DNA was isolated from these fungal spots. ITS regions were amplified and the PCR products were cloned, sequenced and compared to existing ITS sequences of a large database of fungal species.

In this study, we focused on establishing a reliable and fast molecular strategy to investigate complex fungal communities on paper-based art objects. The major challenge of this study was to recover DNA directly extracted from paper materials suitable for PCR amplification of fungal-specific sequences. We compared two different DNA extraction protocols routinely used in our laboratory for direct DNA extraction from art objects, i.e., wall paintings, stone-works and glass, as well as from soil samples. The modified DNA extraction protocol described in the materials and methods section, based on the commercial FastDNA Spin Kit for soil, has the advantage that it is at least five times faster than previous methods described for DNA extraction from soil samples. The protocol includes an extra purification step for removing PCR inhibitors, which results in very pure, colourless DNA, suitable for cloning and PCR amplification. The protocol used for DNA extraction of art samples described by Schabereiter-Gurtner et al. (2001) has been developed on the basis of an existing protocol from Zhou et al. (1996). This modified extraction protocol, in combination with a method to purify the DNA, allowed successful DNA extraction from small sample amounts. In most extraction protocols, the DNA used for further DGGE analysis is recovered by phenol-chloroform extraction and precipitation by alcohol. These two steps raise the risk of contamination during the extraction procedure and impede extraction of DNA from tiny sample amounts. This problem could be minimised by passing the cell lysate through silica-gel membranes. We chose the QIAamp Viral RNA Mini Kit (Qiagen) because, according to the manufacturer, it removes PCR inhibitors. Furthermore, it contained carrier RNA, which facilitates the recovery of small amounts of DNA. From each sample, three DNA eluents were tested for PCR-amplifiable DNA using primers targeting the ITS1 and ITS2 regions.

Our results showed that, for pure fungal strains existing predominantly as mycelia, a combination of enzymatic and mechanical steps for DNA extraction, as performed in the protocol described by Schabereiter-Gurtner et al. (2001), is a useful tool. However, for paper materials containing mycelia as well as spores, it proved to be ineffective in spore lysis. As already observed by Saad et al. (2004) for the analysis of fungal communities in biofilms on paint coatings, DNA yield and quality varies greatly depending on the complexity of the microbial community and the DNA extraction protocols employed, particularly when dealing with fungal spores instead of mycelia. Spore characteristics may affect the procedure of cell wall disruption, which then directly affects the final quality of DNA released (Vainio and Hantula, 2000). In contrast, the optimised commercial FastDNA Spin Kit for Soil protocol showed the best results for the DNA extraction from fungal strains, even those containing thick cell walls, as well as from spores (Kuske et al., 1998; Hughes et al., 2001). Furthermore, this method had the advantage of being less time-consuming and needing no extra purification step, resulting in a convenient method to extract fungal DNA from communities colonising paper material. Also, a small amount of sample (about 5–10 mg) produced an amplifiable amount of DNA, which fulfils the requirement that objects of cultural heritage not be further destroyed.

For PCR analysis, the purity of the extracted DNA was the main factor. Cellulose from native wood, used for paper production from the beginning of the 19th century, exhibits a lower degree of polymerisation (up to 10,000), while the one from native cotton can reach up to 15,000. In addition to cellulose fibres, paper can contain hemicellulose (wood polyoses), lignin, and additives such as fillers, pigments, and metal ions (Fellers et al., 1991). All these components can act as inhibitors of PCR reactions, and therefore their efficient removal contributes to optimal results in DNA extraction from paper materials, as well as for the PCR amplification of the extracted DNA. Our results showed that, depending on the DNA extraction method used, a further purification step could be needed (as per the protocol described by Schabereiter-Gurtner et al. (2001)) or not (as per the FastDNA Spin Kit for Soil) to obtain reproducible PCR products from the crude DNA extracts. Furthermore, it was possible to observe differences in the amplification of the extracted DNA depending on the paper type. The three paper types tested in this study were of different qualities, depending on their composition, coating, and pH value. Our results showed that fungal DNA extraction was more effective from paper type C

(“Old mill” ivory from Fedrigoni Mills, Italy), an uncoated long-life type made of 100% cellulose fibres, than from paper type A (Whatman paper), or type B (Freelife vellum), which is also a long-life type but made of 80% recycled fibres, 15% cellulose chlorine free and 5% cotton fibres and of a more alkaline surface pH. It can be concluded that paper made from cellulose only leads to the best results in PCR amplifiable DNA due to its greater purity.

DGGE analysis carried out with amplified DNA extracted from pure fungal strains cultures, as well as from artificial and natural infected paper samples, revealed differences when targeting either the ITS1 or ITS2 region between the rRNA coding regions. When using the ITS2 region, multiple bands were visualised in the fingerprints derived from single fungal strains. This fact can lead to an overestimation of bands when working with unknown natural complex microbial communities. In contrast, the fingerprints obtained from the amplification of the ITS1 region showed unique bands for pure fungal strains displaying a one-fungi–one-band pattern in DGGE. Therefore, the amplification of this region was chosen for further DGGE analysis. Sequence variation within the ITS1 region is reported to be higher than in ITS2 for fungi such as *Aspergillus* and *Penicillium* (Gaskell et al., 1997).

Analysis of naturally infected paper samples corroborated the results obtained with paper samples artificially infected with pure fungal strains. Once more, the choice of DNA extraction had an influence on the resulting fungal community composition, as measured by DGGE analysis. Not only was the presence/absence of DNA bands affected, but their relative abundance in the total fingerprints was affected as well; this was also found by de Lipthay et al. (2004), Kozdroj and van Elsas, 2000, and Westergaard et al. (2001) for soil communities.

The molecular strategy described here also proved successful for the analysis of a 20-year-old paper. After this time, it is not expected to have any more viable material. This fact is also important because it allows the detection not only of viable fungi, but also of formerly active fungi, which could hence be responsible for the biodeterioration, as in the case of foxing spots. Florian and Manning (2000) proved that irregular fox spots in a 145-year-old book were caused by no longer viable fungal species, especially *E. amstelodami*, found randomly distributed on a page and throughout the book, and *Eurotium rubrum* and *Eurotium repens* in combination with ink, suggesting that irregular fox spots are an inherent feature of the book paper of the 19th century. Kirkpatrick et al. (1989) showed that modern paper can contain up to 10% of fungal hyphae because lignin digesting fungi are used in paper pulp for biological bleaching.

In conclusion, the bead-beating homogenisation of samples showed best results in fungal DNA extraction from paper materials of different compositions and ages. It rendered a pure DNA able to be directly amplified without the need for any extra purification step. By using this DNA extraction protocol, we expect to obtain the highest diversity of fungal sequences to be recovered from natural samples, as reported for bacteria (de Lipthay et al., 2004). However, the problem still remains that no single method of cell lysis is appropriate for all fungi potentially colonising paper-made art objects, and, therefore, the need for further modifications cannot be ruled out. The PCR amplification of the ITS1 region, followed by fingerprinting discrimination of fungal sequences in DGGE analysis is a reproducible and powerful technique for the visualisation not only of viable fungi, but also of formerly active fungi, which could have had an important role in paper biodeterioration processes. If the detection of only viable microorganisms is desirable, this could be overcome by using RNA assays. The molecular strategy presented here could be combined with the construction of clone libraries and sequencing of resulting clones to obtain a detailed phylogenetic identification of the individual members of the fungal communities present on paper.

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Chapter III

Molecular and SEM-EDS screening of foxing spots caused by fungi on paper of different age and composition

To be submitted to *FEMS Microbiology Ecology*.

Molecular and SEM-EDS screening of foxing spots caused by fungi on paper of different age and composition

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Abstract

Foxing is a deteriorative modification of paper occurring in the form of brown-reddish spots, which can be induced by fungi. Culturing identified mostly common fungi, but only a small number of foxing-related fungi have been identified by molecular techniques. In this study, nine different paper-made objects of different age and composition displaying typical foxing patterns were investigated to identify the fungal community present on foxing affected and unaffected areas. After initial denaturing gradient gel electrophoresis of DNA isolated from all samples, fungal clone libraries were constructed from the non-coding ribosomal-DNA internally transcribed spacer region and a total of 145 clones from foxing spots and unaffected areas were sequenced. Sequences were affiliated with 39 different species belonging to 15 *Ascomycetes* and 6 *Basidiomycetes* genera. *Aspergillus*, *Cladosporium* and *Penicillium* represented

the most commonly identified genera. Genera identified for the first time in connection with foxing were related to yeasts and *Basidiomycetes* in addition to interesting *Ascomycetes* candidates. Scanning electron microscopy analysis revealed fungal hyphae and spores present on all foxing samples and in some of the control samples, although in the foxing areas proliferation of mycelia was evident. Energy dispersion spectroscopy confirmed the presence of metal and other ions on the surface of the paper but no significant differences could be ascertained between foxing spots and control areas. This study identified for the first time fungi present also on visibly unaffected paper and adds a variety of species to the hitherto known fungi from foxing samples. It shows that the microbial community not only from inside but from around foxing affected areas should be considered for conservation and restoration attempts as they are possibly involved in ongoing biodeterioration.

Introduction

Our cultural and intellectual heritage is contained mainly in documents made of organic material like paper and thus susceptible to biological damage. The consequent loss of aesthetic composition, and often the irreversible degradation of important documents or works of art can have a significant impact on the economy of many nations (Rinaldi, 2006). A prevalent form of paper biodeterioration is foxing, an ambiguous term used for coloured spots that appear in libraries and archives worldwide and pose a threat to the conservation of our documentary heritage.

Foxing describes a large range of brown, brown-reddish or yellowish spots with sharp or irregular edges most of them showing fluorescence under UV light (Corte *et al*., 2003). Foxing spots are present on paper mainly dating from the sixteenth to the twentieth century. They can affect successive pages with a three dimensional migrating structure and appear to be increasing in number and size over time.

Foxing is considered either biotic or abiotic in origin with research pointing at fungi or chemical phenomena, as heavy metal deposits, cellulose oxidation and moisture condensation related to the non-homogeneity of paper, being responsible for its development (Arai, 2000; Bicchieri *et al*., 2001; Choi,

2007). However, there are some authors postulating that fungi are just opportunistic in occupying the ecological niche provided by chemical or mechanical weakening of the paper, as they observe no correlation between the intensity of the foxing spot's colour and the intensity of fungal growth (Daniels and Meeks 1988).

The microbial contamination of paper can be initiated by airborne fungi or even occur during the paper making process, book preparation and handling (Arai, 1990; Väisänen *et al.*, 1998, Florian and Manning 2000). In this regard, some studies have shown that foxing was initially caused by a group of spores deposited on the surface of the paper prior to the printing process, which germinated *in situ* during the slow drying of the paper (Florian and Manning 2000). Foxing caused by fungi shows fungal hyphae weaving around individual cellulose fibres without changing the constitution of the underlying paper (Meynell and Newsam, 1978). Moreover, recent work suggests a foxing mechanism where initial growth is suddenly aborted resulting in the subsequent auto-oxidation of the lipids (involving lipoxygenases), which may occur during the normal senescence of the biological tissues or the oxidization of fungal lipids by light and metals (Rakotonirainy *et al*., 2007).

Due to the multiple factors involved in the formation of foxing, it is very difficult to artificially reproduce the phenomenon under laboratory conditions. Despite that, Arai *et al*. (1990) could induce the formation of foxing spots by inoculating some fungi on paper, which lead to their theory that xerophilic fungi can be considered the main cause of foxing. Arai's biotic hypothesis is supported by Gallo's studies (1992), which found a relationship between the occurrence of 'biotic foxing' and environmental conditions, as a temperature range of 16–20°C and a relative humidity of 40 –60 % (Gallo and Pasquariello, 1992).

In the last fifteen years a variety of fungal species have been identified as possible causative agents of foxing spots by conventional culture-dependent and microscopic methods (Florian, 1996; Zyska, 1997; Florian and Manning, 1999, 2000; Arai, 2000; Szczepanowska and Cavaliere, 2000; Press, 2001; Corte *et al*, 2003, Rakotonirainy *et al.,* 2007; Zotti *et al*., 2008; Mesquita *et al.*, 2009; Pangallo *et al*., 2009).

With the classical cultivation or microscopic methods mentioned above, more than 84 fungal genera could be identified (Zyska, 1997). The most common genera in foxing spots are *Aspergillus*, *Alternaria*, *Cladosporium* and *Penicillium. Chaetomium*, *Eurotium* and *Trichoderma* are also frequently identified on paper material affected by foxing (Zyska, 1997; Florian an Manning, 2000; Szezepanowska and Cavaliere, 2000; Corte *et al*., 2003).

Unidentified yeasts were amongst some species found on foxing affected paper but they were not considered active or involved in the formation of foxing (Corte *et al*., 2003; Zotti *et al*., 2008). In addition, some bacterial species have been isolated from foxed papers, but they are mainly considered co-responsible in providing important nutritional contribution to fungal growth on paper or performing degradative activities in finished paper (De Paolis and Lippi, 2008).

There are very few studies based on molecular approaches targeting paper samples *in situ* to characterise the fungal species present in foxing stained areas. One of these studies concentrates on only one foxing affected page in a 19th century book without comparison to other samples (Rakotonirainy *et al*., 2007), and the other study focuses on the analyses of one drawing excluding unaffected areas (Di Bonaventura *et al.*, 2003).

We previously developed a culture-independent strategy to investigate fungal communities on paper material of different age and quality, which was successfully applied to investigate real paper samples with and without visible foxing spots (Michaelsen *et al*., 2006; 2009; 2010). In the present study, this molecular strategy was used to investigate and compare the fungal communities present in foxing spots, and in control areas without spots, of papers of different age and composition.

The molecular strategy combines fingerprinting by DGGE with the screening of fungal clone libraries by DGGE and sequencing. Clone libraries were constructed using the variable non-coding ribosomal-DNA internally transcribed spacer region (ITS), which is nested in the nuclear rDNA repeat of the eukaryotic genome. The ITS regions provide greater taxonomic resolution for fungi than coding regions like 18S rRNA. In addition, we performed SEM analyses to identify fungal material and other organic structures on the paper

surface, and to assign chemical characterisations of inorganic constituents by energy dispersion spectroscopy (EDS).

Material and Methods

Sample description

The nine paper samples analysed in this study listed in Table 1 belong to a wide collection of more than 100 foxing samples that was started in the 1960s by Dr. Fausta Gallo in the Laboratory of Biology at the ICPAL (Rome). Foxing spots (Figure 1) as well as corresponding control samples without visible deterioration were selected from a collection of paper that was inconclusively checked for statistically significant presence of living fungi and bacteria in the foxing spots by means of classic culturing methods in previous studies (unpublished). Fragments of paper (2-3 mm^2 width) each corresponding to a foxing spot (F), were collected using sterile tweezers. Areas without discolouration in the natural light and not presenting fluorescent signals under wood lamp excitation (Gallo and Pasquariello, 1989) were used as control samples (C). Sub-samples of foxing spots and control areas were used to obtain SEM images of cellulose fibres, sizing materials and fungal contaminants, while other spots were dedicated to molecular analysis.

DNA extraction from paper material

DNA was extracted directly from biodeteriorated paper using the FastDNA SPIN Kit for Soil (Qbiogene, Illkirch, France). The protocol of the manufacturer was slightly modified as described by Michaelsen *et al*. (2006). To ensure the lysis of all cells and spores on the paper surface, the samples were pre-treated with lysozyme and proteinase K as described by Schabereiter-Gurtner *et al.* (2001).

PCR amplification of extracted DNA

All PCRs were executed using PCR Master Mix (Promega, Mannheim, Germany) and were carried out as described by Michaelsen *et al*. (2006). Fragments of about 700 bp size corresponding to the ITS1, the ITS2 region and the interjacent 5.8S rRNA gene, were amplified with the primer pair ITS1 and

ITS4 (White *et al*., 1990). For DGGE analysis a nested PCR was performed in a total volume of 2 x 50 µl reaction size each with 2.0 µl of PCR product of the first round as template DNA using the GC-clamped primer ITS1GC and ITS2 (Muyzer *et al*., 1993; White *et al*., 1990). PCR products were run in a 2% (w/v) agarose gel, stained with ethidium bromide and visualized in an UVP documentation system (BioRad Transilluminator, Universal Hood).

Denaturing Gradient Gel Electrophoresis

For the genetic fingerprint of the amplified ITS region, 100 µl of PCR product containing the GC-clamp were precipitated with 96% ethanol at -20°C over-night, resuspended in 20 µl ddH_2O and separated by DGGE. Screening of clones was carried out with 20µl PCR. Gel electrophoresis was performed as previously described by Muyzer *et al*. (1993) in 0.5 x TAE (20 mM Tris, 10 mM acetate, 0.5 mM Na_2EDTA; pH 7.8) with 8% (w/v) acrylamide gels containing a gradient of 30-50 % denaturants in a D-GENE-System (Bio-Rad, Munich, Germany). Gels were run for 14 h at 100 V. Gels were stained in ethidium bromide and visualized as above.

A marker containing ITS1-GC PCR products from different fungal strains was used to allow comparative analyses of DGGE patterns from different gels, and as a quality control of the gel run. DNA of each pure fungal strain was extracted in the presence of CTAB following a modified protocol from Jasalavich *et al.* (2000), and amplified as described by Michaelsen *et al.* (2006). The used reference species were as follows: *Alternaria alternata* (Fr.) Keissler, *Chaetomium globosum* Kunze, *Cladosporum cladosporioides* (Fres.) de Vries, *Aspergillus hollandicus Samson* et W.Gams, *Aspergillus terreus* Thom and *Aspergillus versicolor* (Vuill.) Tiraboschi.

Construction of ITS rDNA clone libraries

Purified ITS PCR product (5 µl) generated with primers ITS1 and ITS4 was cloned using the pGEM-T Vector System (Promega, Mannheim, Germany), following the protocol of the manufacturer. *Escherichia coli* XLI-Blue was transformed and plated on LB medium containing ampicillin (100 µg/ml), tetracycline (10 µg/ml), X-Gal (0.1 mM) and IPTG (0.2 mM) (Sambrook *et al*.,

1989). To confirm the presence of inserts, 50 white colonies derived from each sample were picked, resuspended in 100 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0) and an aliquot of 2 µl was used as template for vector specific PCR with the primers SP6 and T7. Clones were screened in DGGE for different patterns (Schabereiter-Gurtner *et al*., 2001).

Phylogenetic analysis

For sequencing of clone inserts, 50 µl PCR product was generated with primers SP6 and T7 and purified with a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). Sequencing of cloned DNA fragments was done using the BigDye Terminator Cycle Sequencing Kit v3.1 and the ABI3130x1 Sequencer (Applied Biosystems Group, Foster City, CA, USA) following the manufacturers protocol. For a taxonomic affiliation of the obtained sequences they were compared against existing online databases using the BLAST algorithm (Altschul *et al.*, 1990).

SEM microscopy

SEM pictures of paper material displaying foxing were taken with an EVO 50 Scanning Electron Microscope instrument from the Carl-Zeiss Electron Microscopy Group (Oxford). The SEM used in this study is a Variable Pressure instrument that can be used with non-conductive specimens without the need for metallization (Goldstein *et al*., 2003). Paper samples of 2-5 mm in diameter presenting foxing, and control samples were mounted reversibly on a 12 mm metal stub using double–sided carbon adhesive tape. The sample areas were examined using a 20 kV electron beam, and both a variable pressure secondary electrons (VPSE) detector and an electron backscattered diffraction (BSD) detector were used to acquire SEM images of samples at various magnifications.

Chemical analysis

A chemical characterisation of the inorganic components of the paper samples was performed by means of electron dispersion spectroscopy (EDS), which allows for an X-ray area scanning of what is brought into focus in SEM images, thereby creating a compositional map of the sample's surface (Goldstein

et al., 2003). Reference elemental intensities acquired from pure compounds (standards) are commonly utilized for calibrating SEM-EDS systems. In this paper, conventional ZAF (atomic number Z, absorption A and fluorescence F) correction (Goldstein *et al.*, 2003) integrated into Oxford INCA 250 microanalysis package was applied (Oxford Instruments). EDS (EDS INCA Energy 250) analysis was performed at 20 kV accelerating voltage with a tungsten filament.

Statistical analysis

Analysis of Variance (ANOVA) was employed to compare the chemical composition of paper samples (Sneath and Sokal, 1973; Massart, 1988). Differences were considered significant if the probability was out of the confidence interval on the basis of the Fisher test (Fahmy, 2003). The test is a multiple comparison procedure generally used in conjunction with an ANOVA to find which means are significantly different from one another. The statistical package XLStat (Addinsoft 2007-Pro, Paris, France) was used (Fahmy, 2003).

Results and Discussion

DGGE and sequence analysis

In this study we performed direct DNA extraction and amplification from foxing areas as well as from control or unstained areas obtained from nine different paper-made objects of different age and composition. The presence of fungal DNA on all samples was confirmed through PCR and DGGE analysis. Crude DNA extracts did not result in visible bands in agarose gels but in positive PCR amplification of the ITS regions.

Figure 2 shows the DGGE fingerprints derived from all foxing samples (F) as well as from the corresponding control samples (C). In general, band-patterns of foxing areas had more bands than the control samples derived from areas without foxing, with exception of sample 5, which displayed higher diversity in the control samples. Some samples showed very similar fingerprints for foxing and control areas (as samples 8, 10 and 11) with the main bands being identical in both fingerprints. Apart from sample 3 and 5, the DGGE fingerprint of the foxing sample shared at least one band with the corresponding control.

We included unstained samples in our study to establish a connection between the existence of fungi and foxing spots, as Rakotonirainy *et al*. (2007) considered the complete absence of fungal material in unaffected areas as a support for the thesis that foxing is derived from fungi. The authors included non-foxing samples in their study but did not achieve any DNA extracts from their control samples. In contrast, all our DNA extracts from control samples were PCR positive for fungi.

To further identify fungal diversity in our samples and for more detailed comparisons of paper affected by foxing and unaffected paper, the PCR derived fungal ITS sequences were cloned, screened by DGGE and sequenced. The sequence analyse of 145 clones allowed the identification of a total of 39 different fungal species belonging to 15 *Ascomycetes* and 6 *Basidiomycetes* genera with sequence similarities from 91 to 100% to the reference species as shown in Table 2. The fungal diversity amongst samples ranged from one (sample 10F) to ten species (8F). Interestingly, some control samples displayed a higher diversity than foxing affected samples from the same source (samples 3C, 4C, 5C, 6C and 10C), which is not in compliance with the DGGE results with exclusion of sample 5C.

The best-represented genera amongst the 39 cultivated species were *Aspergillus*, *Cladosporium* and *Penicillium,* with eight, six or four different cultivated species respectively, as it was reported in other studies (Zyska, 1997; Corte *et al.*, 2003; Rakotonirainy *et al*., 2007; Zotti *et al.*, 2008; Mesquita *et al*., 2009). These species are ubiquitous and can produce numerous mitospores and conidia that are easily dispersed by air (Abrusci *et al*., 2005). The genus *Aspergillus* dominated also in the total number of 42 clones (28.9 %) found on 13 samples, followed by *Penicillium*, with a total number of 33 clones (22.7 %) on 9 samples, *Candida*, with a total number of 10 clones (6.9 %) on 8 samples, *Cladosporium,* with a total number of 8 clones (5.5 %) on 5 samples and *Eurotium,* with a total number of 3 clones (2 %) on 3 samples.

Furthermore, sequences affiliated with an uncultured endophyte accounted for a total of 11 clones (7.6 %) on 4 samples. Amongst the 39 species, six species were only found on control samples, seven species were detected exclusively on foxing samples and eight species were detected on both control and foxing spots (see Table 2).

The fungal communities detected were as different amongst each other as amongst different samples regarding foxing and control areas. The state of foxing on the paper or the paper composition could not be linked to a specific fungal species, genera or community. We assume that the microbiological properties of the probes are so different that one cannot have contaminated the other. The majority of the fungi we could identify on the samples are cosmopolitan species and the absence of prevalent fungi throughout the study makes solving the causes and effects of foxing a difficult issue. Additionally, a result from DNA extraction may originate from coincidental contamination by airborne spores, which may not be the actual cause of foxing.

Many of the species we identified have been reported as paper spoilers before and are xerophilic, osmophilic or osmotolerant, which makes them capable of surviving on paper with a low water activity (Florian, 1996). Interestingly *Chaetomium globosum*, a common airborne fungus, was not detected in our study but it was frequently described on foxing affected paper in previous studies (Pangallo *et al*., 2008; Mesquita *et al*., 2009). However, it is worth noting that these authors analysed isolated fungal strains and not DNA directly extracted from the samples. In contrast, Rakotonirainy *et al*. (2007) performed molecular analysis of fungi from paper and only found one single clone affiliated with *C. globosum*. This supports the theory of a low abundance of *C. globosum* (or its DNA) on the samples contrary to being a common airborne and easily cultivable strain that outgrows others when subjected to culturing in a mixed sample. Surprisingly, an uncultured endophyte proved to be more prominent on our samples (4F, 4C, 6F, 6C), matching sequences derived from the mycobiota in the roots of rare wild rice (*Oryza granulate*).

The species *Geomyces pannorum* (Link) Sigler & J.W was found on both a control and a foxing sample (3F, 3C) and has been emphasised by Zotti *et al*. (2008) before. It is ubiquitous but not widespread due to its low optimum temperature for growth, which correlates with the conditions most papers are stored in at 18-22 °C to prevent microbial growth (Florian, 1994). Another interesting candidate identified from a control sample (3C) is *Acremonium strictum* W. Gams, a filamentous, cosmopolitan fungus, which is primarily saprophytic in nature, living in soil and decaying material. It is known to be

fungicolous or mycophilic in nature, which means it thrives upon other fungi or plants and shows considerable activity in reducing the number of conidiophores, a trait that is trialled for use in biological control (Hijwegen, 1989). Interestingly *A. strictum* was identified amongst yeasts and a member of the *Basidiomycetes* as a 'real' fungus but no other of the common airborne *Ascomycetes* that produce conidiospores.

Davidiella tassiana (De Notaris) Crous & U. Braun has not previously been linked with deterioration of paper but was reported in dust collected by Charles Darwin during the nineteenth century as a very slow growing strain (Gorbushina *et al.*, 2007). The survival conditions in dust are even more stringent than on paper but do resemble them regarding low humidity and scarce food resources, as dust is also considered a food source on paper. Our finding of *D. tassiana* (8F, 8C) is in line with the niche described above. It has to be noted however, that *D. tassiana* represents the teleomorph, the sexual reproductive stage of *Cladosporium herbarum* (Pers.) Link, which is frequently associated with indoors air and paper spoilage.

The finding of *Eurotium halophilicum* Chr., Papav. & Benj. in our samples (4F, 7F, 6C) is worth pointing out, as *E. halophilicum* is a very rarely encountered obligate xerophile with a very slow growth rate (Hocking and Pitt, 1988). *E. halophilicum* was found in both foxing and control samples and is a candidate for further studies about its possible involvement in the formation of foxing spots, as it is considered an extreme xerophile and is able to outgrow other xerophilic species. In a previous study on a paper object from the thirteenth century by our group we also identified *E. halophilicum* and we concluded its impact may be underestimated because of the extremely slow growth in culture dependent studies (Michaelsen *et al*., 2010).

We found 6 sequences affiliated with the *Basidiomycetes* phylum that were not associated with paper before. Our sequencing results identified *Vuilleminia comedens* (Nees) Maire on a foxing sample (9F), which is a stemless fungus that grows in patches on dead wood. *Lycoperdon pyriforme* Schaeff. was identified from a control (3C) and is an edible puffball fungus that grows on rotting wood. The xylophagous fungus *Lenzites elegans* (Spreng.) Pat. was only found in foxing spots (7F, 8F) and belongs to the white rot fungi of wood and

bamboo where it can degrade both lignin and cellulose. Our findings of *Basidiomycetes* agree with those obtained in a recent study (Rakotonirainy *et al*., 2007) where two *Basidiomycetes* that usually grow on trees and cause white rot on wood were identified on paper samples.

Nothing is known about a possible role of *Basidiomycetes* in foxing and none of the species we found are known as contaminant fungi on historic paper documents, but they possess enzymes to metabolise cellulose, lignin and other organic compounds and can therefore contribute to the decay of paper. It is most likely that members of this phylum were introduced to the paper through the manufacturing process or as residues from wood pulp. Nevertheless, their frequent abundance also on paper made from cotton and linen might indicate further involvement and molecular studies tailored to identify *Basidiomycetes* could shed more light on this topic.

Furthermore, 3 out of 7 yeast species identified in our samples belonged to the *Basidiomycetes*. These results support the findings of Di Bonaventura *et al*. (2003), who isolated two *Basidiomycetes* related to the *Kockovaella* clade from brown spots in a Tiffany's drawing. They are yeasts closely related to *Bullera globispora* B.N. Johri & Bandoni found in a foxing spot (9F) in our study. *B. globispora* can perform aerobic cellobiose utilisation and can live on rotting wood. In our samples we found basidiomycotic yeasts that are all involved in decay of plant material. *Sporobolomyces roseus* Kluyver & C.B. Niel was found only in foxing spots (9F, 11F), a fungus that grows on the phylloplane or decaying plant material and is frequently found in air samples. It produces carotinoids whose synthesis can be strongly stimulated by enhanced aeration (Davoli and Weber, 2002) hinting at an involvement in the colour formation of foxing spots.

Cryptococcus saitoii A.Fonseca, Scorzetti & Fell was identified in a control (5C), a yeast species found in soil samples from Europe to Antarctica inhabiting arbuscular mycorrhizal roots or spores (Connell *et al*., 2008). The most abundant yeast in control samples was *Candida tropicalis* (Castellani) Berkhout, a pathogenic inhabitant of human skin, which has been found in the effluent of paper mills. It is mainly concentrated on control samples (3C, 5C, 6C, 7C, 8F, 8C, 10C, 11C) and it can either be it a possible contamination through the handling of

paper or invoved in the early stages of foxing development as its abundance on actual foxing samples is limited to one sample. *C. tropicalis* counts as an interesting candidate for further studies also because of its involvement in the decay of lignin and cellulose (Gonzalez et al., 1989).

The other yeasts observed are an uncultured form of *Cystofilobasidiales* found in a control sample (4C) that was previously identified as a carotinoid producer living on fouling concrete structures (Giannantonio *et al*., 2009), and *Dekkera bruxellensis* Van der Walt, which is commonly associated with wine spoilage but was also found on insects and in our case in a control sample (3C) (Woolfit *et al*., 2007). *Pichia jadinii* (Sartory, A. Weill, R. Weill & J. Mey.) Kurtzman was previously isolated from textile-dye polluted environments and displays high chromate resistance. We identified it in both a foxing sample and a control (3C, 8F).

The authors of another recent study cultured yeasts from different paper samples displaying typical foxing patterns, but did not identify them further (Corte *et al*., 2003). Interestingly, the abundance and frequency of yeasts amongst their samples is high and supports our findings.

The role of yeasts in foxing has not been emphasized in the literature so far and, to our knowledge, none of the yeasts we identified in this study had hitherto been identified in relation to foxing on paper. Yeasts like *Candida* and *Cryptococcus* were found to display fermentative activity with carbohydrates on frescoes and textile fibres, and were identified amongst our samples (Ranalli *et al*., 2009). Furthermore, as yeasts are considered active in other modifications of the paper (Gallo, 1992), and known to produce metabolites like carotenoids they are likely to play an active role in causing foxing stains. A succession of yeast populations was reported for wood decay, where initial ligninolytic *Basidiomycetes* enable the degradation of wood polysaccharides by other yeasts and fungi (Gonzalez *et al*., 1989).

The metabolism of fungi present on foxed paper material should be further investigated, as the foxing spots can be a result of enzymatic attacks not involving cellulose but nutrients that were deposited on the pages through human usage, insects, paints, coats or fillers used in the paper making process. The question also arises, whether fungi need to be alive to cause damage, or if they damage the

paper through residues like spores, segregated enzymes, metabolites or pigments long after the actual inoculation had taken place. The small size of the foxing stains and the relatively long time they need to develop might point to other sources of substrate than cellulose, which is highly abundant and does not seem to be digested. The paper substrate mostly stays structurally intact and foxing affected regions also do not appear mouldy. Fungal hyphae are reported to weave around the cellulose fibres without penetrating them (Meynell and Newsam, 1978, Corte *et al*., 2003).

The fact that not all sites of fungal growth show characteristic foxing stains may reflect a different physiological state of the fungus or a lack of maturity of fungal mycelium. This can explain the slow time-related flourishing of new spots on some documents: small centres of fungal growth with starving spores and old hyphae may release senescing chemicals, which can cause discolouration and cellulose degradation.

It is also known that fungi and yeasts follow successional pattern of growth on certain natural substrates (Dickinson and Underhay, 1977; Gonzalez *et al*., 1989), which could also be the case for foxing and could explain the variety of fungi identified.

SEM and EDS

Florian and Manning (2000) suggested that many of the fungal species recovered by culturing from foxing affected manuscripts were contaminants and used SEM to identify the fungal species in the spots *in situ* by analysing some fungal characters like the ornamentation of the conidia. In the present study, an attempt to compare the fungal species identified with molecular techniques and the SEM pictures of fungi grown on paper was performed. As SEM was performed in variable pressure mode, the paper samples were not stressed with metallization and high vacuum to the cost of details like fine conidia ornamentation, enabling the direct chemical analysis of particles co-occurring with fungal structures. The information obtained on fungal structures found on paper was relevant to dimensions, abundance, shape, presence or absence of ornamentation. Some fungal structures could be attributed tentatively to some peculiar systematic groups.

SEM observations of the paper samples are described in Table 3 on the basis of fungal structures and dimensions and shown in Figure 3. Fungal species that are consistently identified through both SEM and cloning in combination with sequence analysis are indicated in the table. Most of the fungi found by SEM and sequencing are common airborne species belonging to the *Aspergillus* and *Penicillium* genera. At least one fungal species or genera matching sequencing data could be identified per foxing sample included in this study, but only for half of the control samples.

Fungal structures were observed in half of the control samples, contradicting the assertion that no fungal material is present in areas unaffected by foxing (Rakotonirainy *et al*., 2007). We achieved positive DNA extracts from all samples that visually tested negative, underlining again the importance of embedding molecular methods in identification assays. It is interesting that *Geomyces pannorum* and *Eurotium halophilicum* were among the fungi observed with SEM. Both are very slow growing species and therefore likely to be outgrown by others and both are difficult to identify visually. They are not common paper spoilers but our data make them interesting candidates for further investigations including the artificial induction of the formation of foxing.

The fraction of spores that can be aerosolized to occupy a new environment from a contaminated material can vary sensitively according to the biology and ecology of the single fungal species and also to causal events. According to Górny *et al*. (2001) the release of fungal spores is driven by energy from external sources and may be significantly affected by environmental factors, such as air velocity. Spore detachment from mature mycelia of most *Aspergillus* and *Penicillium* species is caused principally by physical factors (Sivasubramani *et al*., 2004), and therefore mostly dependent on environmental and mechanical changes.

With the application of SEM-EDS to the foxing affected documents, it was possible to distinguish the white shining material spread along and on top of paper fibres as metallic impurity against darker vegetable fibres and fungal mycelium and spores (Fig. 3). The existence of inorganic materials enabled well-contrasted observation of filling materials and ion distribution on paper surfaces, in addition to the fungal structures growing on them. Fungal hyphae and conidia

are shown in Figure 3 growing on foxing affected areas on sample 7F made from linen and cotton.

The presence of iron and other chemical elements in both foxing and control areas in some samples was revealed, but almost all the combinations of the variables (fungi/iron/foxing, spot/blank) could be observed among the samples and no constant co-occurrence of iron and fungal structures in foxing spots was found. Average values of the elements found on the surface of the paper samples are reported in Table 4.

No overall statistically significant differences were found between foxing spots and control areas in terms of presence or absence of iron or other elements. The chemical elements detected in the whole set of samples and controls are sodium, magnesium, aluminium, sulphur, potassium, calcium and iron. Barium was only detected on sample 8. Particles with high iron content were found in control and foxed areas of paper samples 5, 6 and 7. A qualitative difference in particle distribution between control and foxed areas could be observed in samples 5 and 7. Figure 4 shows a histogram of weight percentage values of iron in control areas and foxed areas.

The EDS technique cannot be considered a quantitative analytical method since the volume actually analysed is unknown for materials with a variable density like paper. A deeper analysis by means of more penetrating and quantitative analytical techniques could shed more light on the role of iron and other elements in foxing formation. Our findings are in line with Press (2001), who found that iron concentrations did not show differences between foxing and unaffected control samples, he actually observed that foxing was associated with low rather than high iron content contradictory to most studies that link high iron content to foxing.

ANOVA analysis was used to group paper samples on the basis of their content of inorganic materials. Grouping of the samples according to foxing or control areas was not possible as the variance of the differences between the samples was too large. Given the high standard deviations between analysed particles, a higher number of particles should be taken into account. Results showed that the chemical composition was significant between samples as a consequence of differences in qualities of the paper.

Conclusions

The potential for fungal colonisation of paper appeared very high, with compact fungal communities identified using direct DNA extraction from both foxing spots and visibly unaffected paper. With respect to the variety of 39 different species from 21 genera, no direct link between fungal species and foxing could be established but species hitherto not linked to foxing were found, including a variety of yeasts and *Basidiomycetes* that should be investigated further. We proved for the first time that fungi do not only occur in foxing spots of damaged paper, but can be found in non stained areas. Due to the slow growth and long time development of fungi on paper, fungal material should be expected before formation of visible damage. Foxing spots may therefore be due to the fungi interacting with the paper, or by the presence of different fungi, some producing foxing stains and some not.

SEM analysis added visual data to the molecular approach and matches between both data sets included common airborne fungi well known in foxing samples. In most of the control areas on paper, no active growth of fungi could be observed by SEM, although amplification of fungal DNA could be obtained. Fungi were described by SEM as local spots, with production of mycelia or both hyphae and fruiting structures. The foxing spots can be considered as amplification sites for fungal strains. The presence of fungal DNA in samples where no growth was detectable could be interpreted as an early colonisation phase or as an old colonisation event. The progressive appearance of foxing alterations on paper can be related to the diffuse presence of fungal amplification sites or active propagules, and to micro environmental conditions favourable for fungal germination.

No significant differences were found between foxed and control areas of a given sample in terms of presence or absence of iron or other elements, but between different paper qualities. Some samples showed the presence of impurities containing elements that can induce paper degradation phenomena like iron or potassium (Waterhouse and Barrett, 1991). A high heterogeneity of the paper environment was observed in terms of mineral particle composition and distribution. This supports the hypothesis that the appearance of foxing stains are

multi-factorial events, due to the interaction of biotic and abiotic factors that can be found on paper with a high variability in space and time.

Our findings support the theory of intermediate processes in the formation of foxing, where filamentous fungi provoke paper discolouration that are intensified when metal oxides are present. In order to understand the formation of foxing, attention should be directed towards areas with early signs of foxing or even unaffected areas that show fungal communities with possible succession patterns. We propose that different consortia of fungi might be involved at different times in the formation of foxing and the visible spot is a late stage of development that started much earlier.

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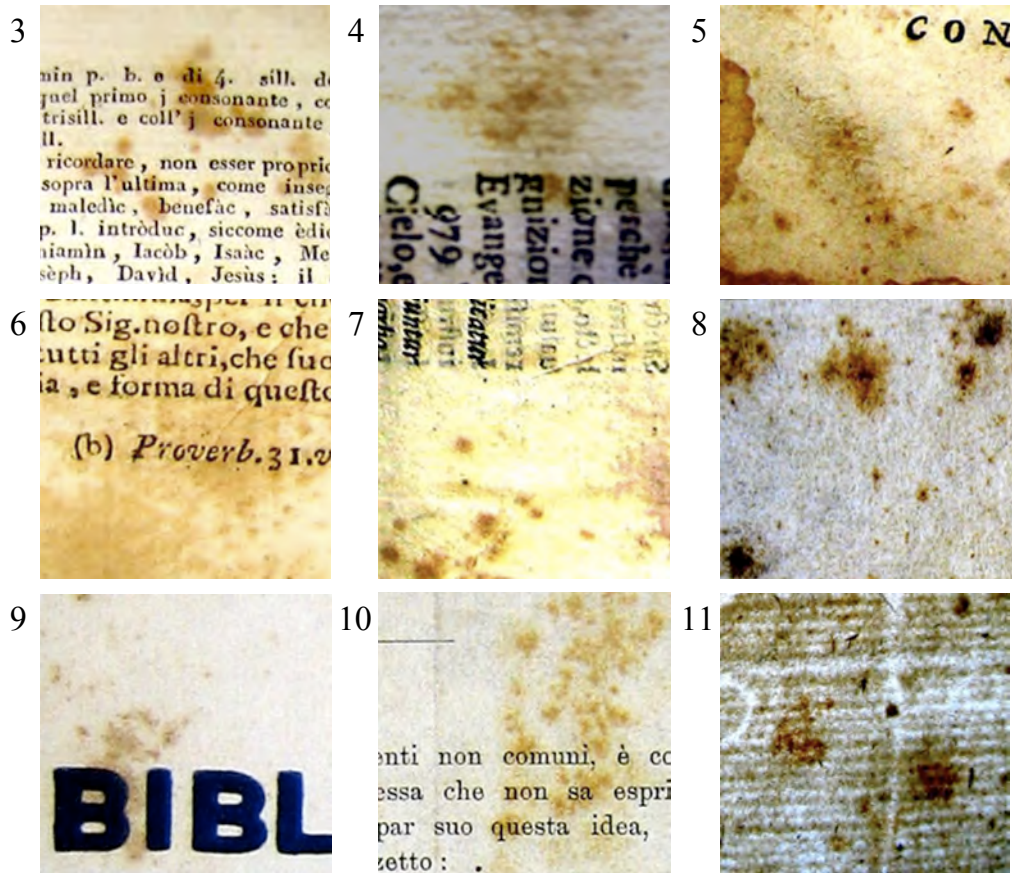

Figure 1. Foxing spots on paper samples. Samples were taken from nine different paper sources, as described in Table 1, displaying spots that are considered foxing. Various sizes, shapes and colours of foxing spots can be observed.

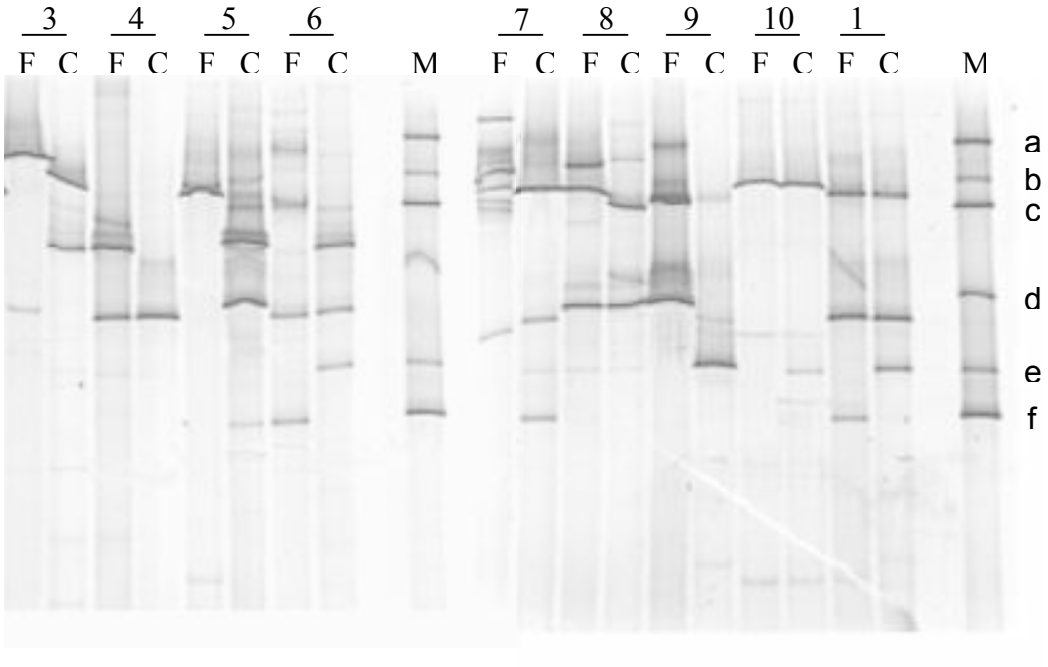

Figure 2. ITS-derived DGGE fingerprints from different historic paper samples (3-11) with (F) and without visible foxing spots (C). Gels contained a denaturing gradient of 30-50% and were run for 14h at 100V. Lane M constitutes of a marker containing fungal ITS amplificats. a: *Alternaria alternata*; b: *Chaetomium globosum*; c: *Cladosporium cladosporioides*; d: *Aspergillus hollandicus*; e: *A. terreus*; f: *A. versicolor*.

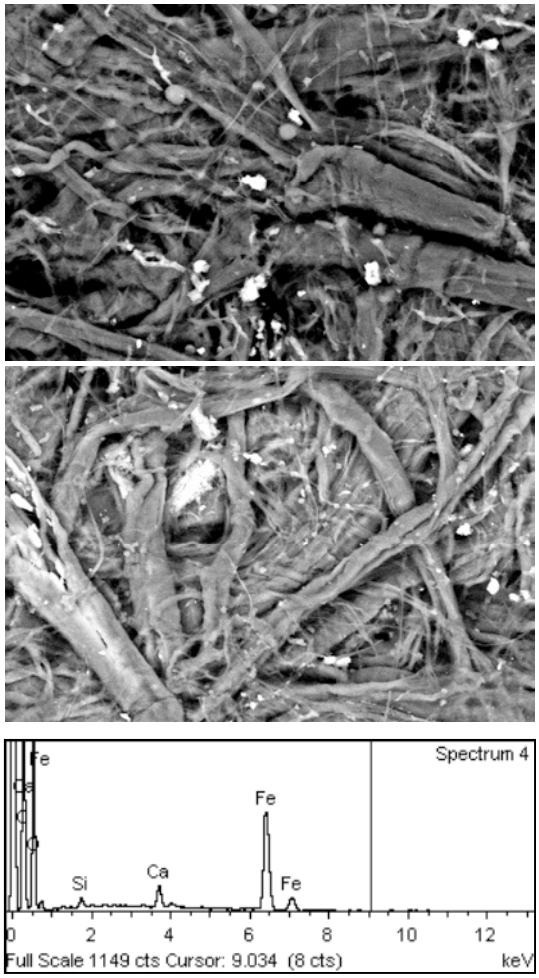

Figure 3. SEM pictures of metallic impurities and fungal structures (conidia and hyphae) in foxed areas on sample 7F. Due to the differences in chemical composition of organic and inorganic material, well-contrasted snapshots of white shining metal impurities against darker fibres or fungal mycelium were obtained. SEM combined with X-ray analysis of foxing infected areas revealed the presence of iron and other elements as shown for sample 7F in the spectrum below.

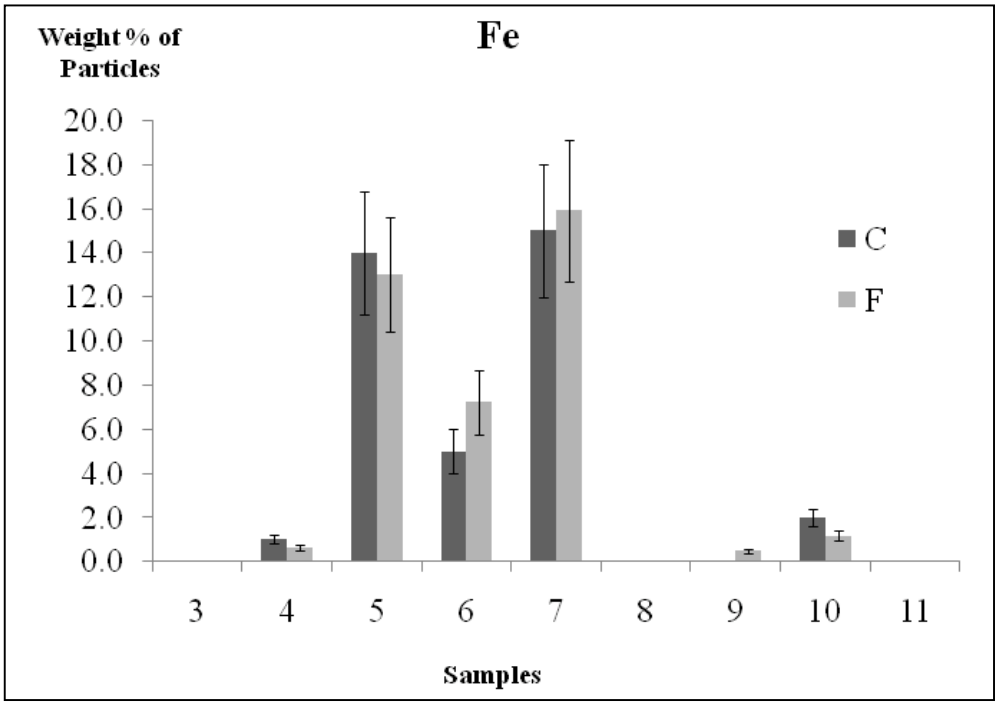

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Figure 4. Weight percentage values of iron (Fe) content in the foxing samples (F) and control areas (C). The iron content does not influence the appearance of foxing spots and iron is not present in all paper samples. Iron contents are comparable in sample and control whenever iron is present in a sample.

Sample	Material	Dated
3	cotton	1794
4	hemp, linen, cotton heavy sizing, covering fibres	1712
5	cotton	1784
6	cotton	1712
7	linen, cotton	1600
8	chemical pulp of wood	1960
9	conifer mechanical pulp, thick sizing, covering fibres	1930
10	manilla hemp, straw, rice straw	1880
11	cotton linter	1700

Table 1. Composition and age of the paper samples with foxing spots (F) used in this study. Control (C) samples were taken from the same objects from areas not displaying any visible discoloration or microbiological growth. All samples belong to the sample collection of the ICPAL, Rome, Italy.

Phylum	Genera	Closest species and accession number	3 F C	4 F C	5 F C	6 F C	7 F C	8 F C	9 F C	10 F C	11 F C	Clones/ Species	Clones/ Genera
Ascomycota	*Acremonium*	*Acremonium strictum* AY138846	- 5	- -	- -	- -	- -	- -	- -	- -	- -	5	5
	Alternaria	*Alternaria tenuissima* FJ949086	- -	- -	- -	- -	5 -	- -	- -	- -	- -	5	5
	Aspergillus	*Aspergillus penicillioides* AY373861	- -	1 -	- -	- -	- -	- -	- -	- -	- -	1	-
		Aspergillus restrictus AY373864	- -	- -	- -	5 -	- -	- -	- -	- -	- -	5	-
		Aspergillus sp. EF6502038, FJ471617, GQ169455	2 -	1 -	1 -	- -	- -	- -	- -	- -	- -	4	-
		Aspergillus terreus AB369899, AY373871, GQ461911	- -	- -	- -	- 2	- -	- 5	- 4	- -	- -	11	-
		Aspergillus versicolor AY373883, EF125026, FJ878627	- -	2 -	- 1	- -	- -	1 -	- -	- -	4 4	12	-
		Uncultured *Aspergillus* AJ582962, AM159626, FM165464, FN394520, FN394526	1 -	- -	3 -	- -	1 -	2 -	2 -	- -	- -	9	42
	Candida	*Candida tropicalis* AB467290, DQ680841, EU288196, EU924133, GQ376071	- 1	- -	- 1	- 1	- 2	1 1	- -	- 1	- 2	10	10
	Cladosporium	*Cladosporium cf. subtillissimum* EF679390	- -	- -	- -	- -	- 1	- -	- -	- -	- -	1	-
		Cladosporium cladosporioides FJ537128	- -	- -	- 1	- -	- 1	- -	- -	- -	- -	2	-
		Cladosporium ossifragi EF679382	- -	- -	- -	- -	- -	1 -	- -	- -	- -	1	-
		Cladosporium oxysporum EU759979	- -	- -	- -	- -	- 1	- -	- -	- -	- -	1	-
		Cladosporium sp. BC3 DQ317332	- -	- -	- -	- -	- -	- -	- -	- -	1 -	1	-
		Cladosporium sphaerospermum AM159631	- -	- -	- -	- -	1 -	- -	- -	- -	- -	1	-
		Uncultured *Cladosporium* EU516971	- -	- -	- -	- -	- 1	- -	- -	- -	- -	1	8
	Crysosporium	*Chrysosporium carmichaelii* AJ007842	- -	- -	- 1	- -	- -	- -	- -	- -	- -	1	1
	Davidiella	*Davidiella tassiana* EU343351, EU622926	- -	- -	- -	- -	- -	1 1	- -	- -	- -	2	2
	Dekkera	*Dekkera bruxellensis* AM491367, AM850055	- 2	- -	- -	- -	- -	- -	- -	- -	- -	2	2
	Epicoccum	*Epicoccum nigrum* FJ914708	- -	- -	- -	- -	- -	1 -	- -	- -	- -	1	1
	Eurotium	*Eurotium amstelodami* EU409808	- -	- -	- -	- -	1 -	- -	- -	- -	- -	1	-
		Eurotium halophilicum EF652088	- -	1 -	- -	- 1	- -	- -	- -	- -	- -	2	3
	Geomyces	*Geomyces pannorum* AY873967, DQ189224,	1 1	- -	- -	- -	- -	- -	- -	- -	- -	2	-
		Geomyces sp BC9 DQ317339	2 -	- -	- -	- -	- -	- -	- -	- -	- -	2	4
	Gymnoascus	*Gymnoascus ruber* AJ315839	1 -	- -	- -	- -	- -	- -	- -	- -	- -	1	1
	Penicillium	*Penicillium chrysogenum* AY373902, GQ121159	- -	- -	- -	- -	- -	- -	1 4	9 7	2 3	26	-
		Penicillium commune AY373902	- -	- -	- -	- -	- -	- -	- -	- -	1 -	1	-
		Penicillium pinophilum AB369480, AF176660	- -	- -	- 3	- -	- -	1 -	- -	- -	1 -	5	-
		Penicillium ramusculum EF433765	- -	- 1	- -	- -	- -	- -	- -	- -	- -	1	33
	Pichia	*Pichia jadinii* FJ865435	- 1	- -	- -	- -	- -	1 -	- -	- -	- -	2	2
	Thielavia	*Thielavia hyalocarpa* THY271583	- -	- -	- -	- -	- -	- -	- -	- -	- 1	1	1
	-	Unidentified fungi from glacial ice AY207370	- -	- -	1 -	- -	- -	- -	- -	- -	- -	1	1
		Uncultured *Cystofilobasidiales* EU409881	- -	- 1	- -	- -	- -	- -	- -	- -	- -	1	1
Basidiomycota	*Bullera*	*Bullera globispora* AF444407	- -	- -	- -	- -	- -	- -	2 -	- -	- -	2	2
	Cryptococcus	*Cryptococcus saitoi* EU149781	- -	- -	- 2	- -	- -	- -	- -	- -	- -	2	2
	Lenzites	*Lenzites elegans* FJ711054, FJ711055	- -	- -	- -	- -	1 -	1 -	- -	- -	- -	2	2
	Lycoperdon	*Lycoperdon pyriforme* AY854075	- 1	- -	- -	- -	- -	- -	- -	- -	- -	1	1
	Sporobolomyces	*Sporobolomyces roseus* AB030351, AY015438	- -	- -	- -	- -	- -	- -	1 -	- -	1 -	2	2
	Vuilleminia	*Vuilleminia comedens* FJ481021	- -	- -	- -	- -	- -	- -	1 -	- -	- -	1	1
Eukaryota		Uncultured endophyte fungi FJ524295	- -	4 2	- -	2 3	- -	- -	- -	- -	- -	11	11
		Uncultured fungus clone GQ513760	- -	- -	1 -	- -	- -	- -	- -	- -	- -	1	1
		Uncultured fungus singleton FJ760838	- -	- -	- -	- -	1 -	- -	- -	- -	- -	1	1
Total sequenced clones/sample			7 11	9 4	6 9	7 7	10 6	10 7	7 8	9 8	10 10		145

Table 2. Number and distribution of sequenced clones amongst the samples (F) and controls (C) according to the similarity of ITS1-5.8S-ITS2 sequences to their closest fungal reference sequence available from the NCBI database.

Sample	Fungal structures visible with SEM	Fungal species identified from ITS sequences consistent with the observed structure
3F	Cleistotecia of ascomycetes 15.5-16.5 µm, abundant web of aerial fungal hyphae, lemon-shaped ascospores 4.3x6.4 µm, several chains of arthroconidia 2-3 µm Geomyces-like	*Gymnoascus ruber* *Geomyces pannorum*
3C	Few, sparse lemon-shaped ascospores 4.3x6.4 µm, no visible mycelia. Chains of arthroconidia 2-3 µm Geomyces-like	*Geomyces pannorum*
4F	Abundant conidia 6,3-7,0 with ornamentation (echinated, like A. versicolor) and scars resembling that of some species of *Aspergillus* genera. Globose ascomata consistent with Eurotium species	*Eurotium halophilicum* *Aspergillus versicolor* *Aspergillus penicilloides*
4C	No fungal structures	
5F	Masses of 3,4-3,7 µm in diameter spherical conidia without evident ornamentation. Abundant hyphae covering cellulose fibres.	*Aspergillus* or *Penicillium*-like spores with no ornamentation
5C	Few, sparse round shaped conidia 1,5 µm, no visible mycelia.	*Aspergillus* or *Penicillium*-like
6F	Echinated ovoid spores.	*Aspergillus restrictus*
6C	Rare globose ascomata consistent with Eurotium species	*Eurotium halophilicum*
7F	Globose ascomata consistent with Eurotium species. Chains of arthroconidia 2,5-3,0 µm	*Eurotium amstelodami*
7C	No fungal structures	
8F	Rare masses of fungal conidia ranging from 1,0-3,3 µm in diameter. Collapsed hyphae.	*Cladosporium* or *Davidiella*
8C	No fungal structures	
9F	Abundant masses of small (1,5-1,7 µm in diameter) Penicillia-like spherical conidia. Hyphae.	*Penicillium chrysogenum*
9C	Rare sparse spherical conidia	*Penicillium chrysogenum* or *Aspergillus terreus*
10F	Spherical conidia of 1-1,5 µm in diameter	*Penicillium chrysogenum*
10C	No fungal structures	
11F	Yeast-like conidia in short chains ranging from small to large conidia (from 2-3 to 5 µm in diameter).	*Sporobolomyces roseus*
11C	No fungal structures	

Table 3. Consistency of fungi identified by structural observation using SEM and identified through sequence analysis of the ITS region.

Paper sample	Chemical element																							
	Na			Mg			Al			S			K			Ca			Fe			Ba		
3	0.0		B	0.0		B C	0.4		B C	8.9	A		0.0		B	10.4	A		0.0		B	0.0		B
4	1.4	A		0.8	A	B C	2.9	A	B C	2.1		B C	0.2		B	4.4	A	B C	0.6		B	0.0		B
5	0.2	A	B	0.2		B C	0.6		B C	0.0		C	0.3		B	4.4	A	B C	18.2	A		0.0		B
6	0.3	A	B	3.3	A	B C	2.8	A	B C	0.1		C	1.2	A	B	2.9		B C	7.2	A	B	0.0		B
7	0.0		B	0.1		C	0.0		C	0.0		C	0.2		B	2.9		B C	15.9	A		0.0		B
8	0.0		B	0.0	A	B	3.5	A	B	2.8		B C	0.0		B	0.6		C	0.0		B	12.0	A	
9	0.3	A	B	0.2	A	B C	2.3	A	B C	2.2		B C	0.1		B	2.7		B C	0.5		B	0.0		B
10	0.7	A	B	0.1	A		4.5	A		4.1		B	2.5	A		7.5	A	B	1.2		B	0.0		B
11	0.0		B	0.9		B C	0.3		B C	0.2		B C	0.5		B	3.7		B C	0.0		B	0.0		B

Table 4. EDS analysis of paper samples. The values in weight percentage present an average of a minimum of 10 inorganic particles analysed for each paper grade 3-11. ANOVA statistical analysis and Fisher test were used to group paper samples on the basis of their content of inorganic material. Values lower than 0.3% are under the detection limit of the instrument. The letters A, B and C refer to groups of samples with statistically significant differences according to Fisher test and the coexistence of two letters indicates that the sample belongs to more than one groups contemporaneously.

Chapter IV

Biodeterioration and restoration of a 16th-century book using combination of conventional and molecular techniques: A case study

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Biodeterioration and restoration of a 16th-century book using a combination of conventional and molecular techniques: A case study

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Restoration

ABSTRACT

In this paper we deliver a report on the study of microbiological damage found on the pages of a 16th-century book. Our aim is to describe the procedures needed to ensure a conservative approach to the restoration of valuable books and objects of art made from, or supported on, paper. The techniques employed to evaluate and describe the damage observed, as well as the organisms responsible for biodeterioration, are discussed. A range of sampling techniques and instruments were utilised, including swabs and adhesive tape. Conventional methods, such as classic culturing and the direct microscopic observation of sampled material, were coupled with DNA-fingerprinting and phylogenetic analysis. We postulated that the purple stains which migrate through the pages with a felted consistency (Fig. 2), based on all the information obtained using traditional and molecular means, were caused by a cellulolytic fungus producing purple essudates, characterised by echinated conidia and Hülle cells. These elements were consistent with the discovery of both *A. versicolor* and *A. nidulans* using molecular techniques.

1. Introduction

The physical and chemical procedures carried out on the materials and structure of objects of cultural or historical value are generally defined as "restoration". Prior to the application of any restorative intervention, books, like other objects of cultural heritage, usually undergo a thorough study in order to evaluate the damage they present with. The evaluation of the state of preservation of materials, from both the chemical and biological point of view, can be a highly complex and time consuming task, and has come to be referred to as "direct prevention" (Federici and Munafò, 1996). The modern approach to direct prevention and the restoration of precious books and objects of art made from paper involves the application of non-invasive techniques and protocols aimed, above all, at conservation.

Microbial, chemical, and physical deterioration occurs in paper of different ages and made using different manufacturing processes. This can affect a paper's fibres or its chemical components, depending on the raw materials used or work-up procedures (Kowalik, 1980; Gallo, 1985; Florian, 1999). The study of the causes of degradation is of great relevance if one is to acquire a better understanding of the mechanisms that leave objects of cultural heritage in a poor state of preservation.

Microbial degradation of paper causes different kinds of damage depending on the organisms responsible for the attack. Some filamentous fungi frequently associated with paper degradation are capable of dissolving cellulose fibres through the action of cellulolytic enzymes, or produce pigments or organic acids which discolour paper and cause serious damage to materials of cultural and historical importance made from paper (Nyuksha, 1983; Ciferri et al., 2000; Reese and Downing, 1951). Chemical damage is mainly the result of the oxidation of cellulose chains. Production of free carboxylic groups and redox or radical processes can involve all the constituents of paper, and leads to a general yellowing and weakening of a paper's structure. Inks, glues, impurities and other organic or inorganic materials can strongly influence, both negatively and positively, the ageing of paper (Nyuksha, 1983). Finally, physical damage, mainly due to adverse environmental conditions (light, temperature, humidity), often triggers subsequent chemical and microbial deteriorating processes. Aging of paper and chemical hydrolysis of cellulose chains can promote attacks by microbial and fungal saprophytic species. An advanced knowledge and thorough understanding of the materials from which a volume was made, together with the identification and characterisation of any original damage, are fundamental requisites prior to carrying out any

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restoration work. Nevertheless, the precise identification of the agent (or agents) responsible for any damage observed can be a highly complex task, and sometimes requires the application of technologies capable of disclosing a great deal of information.

One of the main problems encountered in the area of cultural heritage biological diagnostics arises when seeking to perform non-invasive sampling procedures. The study of objects of cultural heritage should be effected, if possible, without modifying the objects themselves, especially if these are of small dimensions.

The aim of this study was to describe the methodology required for a conservative approach to the restoration of precious books and works of art made from paper. In this report we present a case study of "direct prevention" involving a specific book. A copy of "Le Stanze del Bandello" by Matteo Bandello (Fig. 1), printed at the end of the 16th century in Italy and kept in the Braidense Library in Milan. The book was sent to the Istituto Centrale per la Patologia del Libro in Rome for restoration. We focused in particular on the study of microbiological lesions (Fig. 2) found on the pages of "Le Stanze del Bandello". A range of sampling techniques and instruments was employed, including swabs and adhesive tapes. Classic cultivation methods and the direct microscopic observation of sampled material were used in conjunction with molecular techniques to identify the organisms responsible for biological degradation. In addition, variable pressure SEM instrumentation was used in tandem with electronic dispersion spectroscopy (EDS) so as to obtain a chemical characterisation of inorganic constituents and a description of the biodeteriorated paper's surface, without the necessity of resorting to sample metallisation. Finally, an innovative and conservative *in situ* approach to the book's restoration is briefly described and discussed.

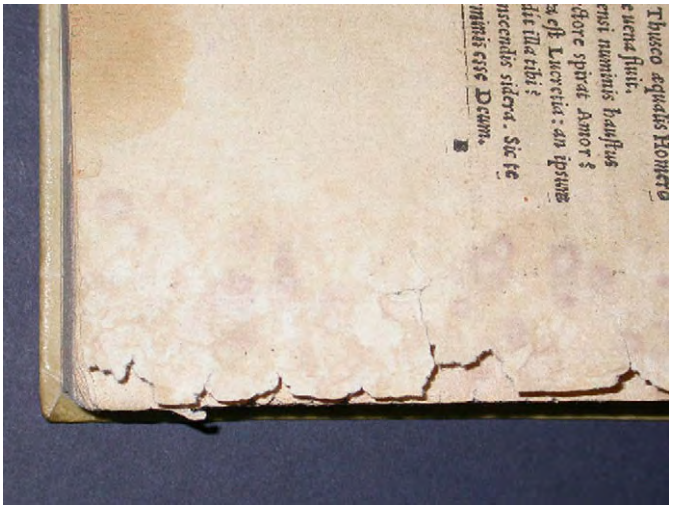

Fig. 2. A damaged page of the volume, with fungal stains. The felted consistence of paper resulted in a great fragility of the sheet's margins. Some fragments of paper (2–3 mm^2 width) were collected from this type of margin using watchmaker's tweezers.

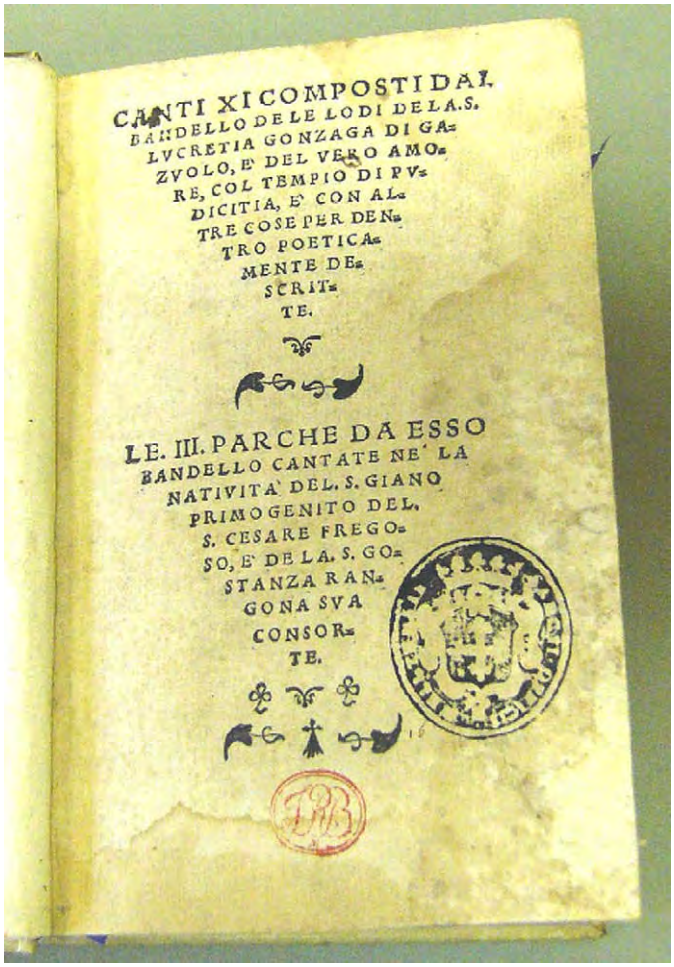

Fig. 1. The volume "Le Stanze del Bandello" by Matteo Bandello, printed at the end of the 16th century in Italy, conserved in the Biblioteca Braidense in Milan (Italy), and sent to the Istituto Centrale per il Restauro e la Conservazione del Patrimonio Archivistico e librario, in Rome, for restoration of damaged parts. The binding is made of parchment. In the picture, the frontispiece of the book with the following text "Canti XI composti dal Bandello de le lodi dela S. Lucrezia Gonzaga di Gazuolo e del vero Amore col Tempio di Pudicizia e con altre cose per dentro poeticamente descritte [sic]. Le III Parche da esso Bandello cantate ne la Natività del S. Giano, primo genito del Sr. Cesare Fregoso e della signora Costanza Rangona, sua consorte". S.n.t. [Agen, A. Reboglio, 1545].

2. Materials and methods

2.1. Sampling

Paper samples from "Le Stanze del Bandello" were obtained in different ways following techniques designed to minimise invasive action on objects made from paper. Before any sampling operation was carried out, the areas most suitable for this purpose were evaluated. Most of the pages composing "Le Stanze del Bandello" were observed in both natural light and under light rays emitted by a Wood lamp at 362 nm, so as to detect certain types of deterioration, such as mould damage, which can be invisible to the naked eye (Florian, 1999).

Sterile cotton swabs were used to obtain samples suitable for further fungal and bacterial culturing and identification. When collecting a sample using a cotton swab, the swab was wiped over the entire area of visibly damaged material, under normal light and UV light. The gentle application of dry swabs can legitimately be considered a non-invasive sample collection method.

Sterile needles were used to obtain samples of fibres and microbial structures suitable for microscopic examination and culturing. Some fragments of paper (2–3 mm^2 width) were also collected, mainly from the margins of the most degraded pages (Fig. 2), using watchmaker's tweezers.

Removable 3M™ adhesive tape was used to collect samples of fungal mycelia and sporulating structures from deteriorated paper found in "Le Stanze del Bandello". The choice of a tape that requires little effort to remove it from surfaces was influenced by the fragility of the paper and by the fact that a tape coated with a stronger adhesive would have picked up too many cellulose fibres. The tape was cut into 2 cm strips and gently applied to the discoloured surface of the leaves. The strips of tape were then transferred to a glass slide and examined under a transmitted light microscope. Cedar oil for immersion was used to render the tape translucent.

2.2. Agar/broth cultures

The viability of fungal mycelia observed on paper was tested using agar and broth cultures. Fungal structures sampled using cotton swabs were inoculated directly on to agar plates; the swabs were then immersed in sterile Czapek broth. Powdered paper samples obtained using needles were suspended in 2 ml of sterile water and inoculated on to agar plates using a glass pipette (1 ml of suspension for each Petri dish). Fragments of paper were used to perform a "25 points inoculum", consisting of multiple inocula on a Petri agar dish divided into a grid with 25 points; the sub-fragments of the sample paper were first washed several times with sterile

water. The aim of this procedure is to cultivate those micro-organisms that indeed affect cellulose fibres, and to avoid the development of airborne contaminants. When a statistically significant fraction of the 25 points develop the same fungal/bacterial species, it is reasonable to conclude that the developed strain represents the actual paper spoiler.

The media used to grow the inocula were malt extract agar (MEA , Samson et al., 2002), and Czapek yeast agar (CYA), prepared according to Pitt and Hocking (1985). Some of the powdery samples obtained by scratching purple stains on the paper with blades were inoculated onto M40 media (Samson et al., 2002), in order to look for the xerophilic species. Some ascomycetes presenting ascospores and globose asci, like *Eurotium* species are, in fact, moderately xerophilic and their growth can be favoured in a media that contains a high concentration of sucrose, and therefore low water activity. Czapek concentrate, yeast extract, agar, malt extract, peptone, glucose and sucrose were manufactured by DIFCO (Becton Dickinson, USA). The MEA, M40 and the CYA dishes were prepared by pouring 10 ml of sterilised substrata into ventilated Polystyrene 90 × 15 mm cell culture dishes (Corning Incorporated, New York, USA). The resulting depth of the medium was 4.4 ± 0.2 mm. All the inoculations and re-inoculations were performed within the confines of a laminar flow hood to assure sterility throughout the procedures.

2.3. Molecular analysis

2.3.1. DNA extraction from paper material

DNA was extracted directly from paper samples using the FastDNA SPIN Kit for Soil (Qbiogene, Illkirch, France). The manufacturer's protocol was slightly modified, as described by Michaelsen et al. (2006). DNA crude extracts were used directly for PCR amplification analysis of internal transcribed spacer regions (ITS regions).

2.3.2. PCR amplification of extracted DNA

PCR reactions were executed in a Robocycler (Stratagene, La Jolla, USA) using PCR Master Mix (Promega, Mannheim, Germany). Fragments of about 450–600 bp in size, corresponding to the ITS-1 and the ITS-2 regions, and the 5.8S rRNA gene situated between them, were amplified with the primer pairs ITS1 forward and ITS4 reverse (White et al., 1990). All reactions were carried out as described by Michaelsen et al. (2006). For DGGE analysis, a nested PCR was performed in a total volume of 100 μl (2 × 50 μl reactions), each with 3.5 μl of PCR product from the first round as template DNA. For the forward primer, the same primer as that used for the first round was employed, with a 37-base GC-clamp attached to each 5′-end, in order to stabilise the melting behaviour of the DNA fragments (Muyzer et al., 1993). For the reverse primer, the ITS2 was used (White et al., 1990). This primer pair amplifies the ITS-1 region. The cycling scheme was the same as that described by Michaelsen et al. (2006). PCR products were analysed by means of electrophoresis in a 2% (w/v) agarose gel.

2.3.3. Denaturing Gradient Gel Electrophoresis (DGGE)

To obtain the genetic fingerprint of the amplified ITS-1 region, 100 μl of PCR product containing the GC-clamp, were precipitated with 96% ethanol at −20 °C overnight, re-suspended in 20 μl double distilled H_2O and separated by DGGE. Gel electrophoresis was performed as previously described by Muyzer et al. (1993) in 0.5× TAE (20 mM Tris, 10 mM acetate, 0.5 mM Na_2EDTA; pH 7.8) with 8% (w/v) acrylamide gels containing a gradient of 30–50% denaturants in a D-GENE-System (BioRad, Munich, Germany). Gels were run for 14 h at 100 V. Following completion of electrophoresis, gels were stained in an ethidium bromide solution and documented using a UVP documentation system.

2.3.4. Creation of clone libraries and sequence analysis

To obtain detailed phylogenetic identification data on members of the fungal community, a clone library containing the ITS fungal regions was created. A 2 × 3.5 μl DNA template was amplified in 2 × 50 μl volumes using the primers ITS1/ITS4 (White et al., 1990), as mentioned above. The PCR products were pooled and purified using the QIAquick PCR Purification Kit Protocol (Qiagen, Hilden, Germany) and re-suspended in 30 μl ddH_2O water (Sigma). Purified PCR product (5.5 μl) was ligated into the pGEM-T Easy Vector system (Promega, Vienna, Austria), following the manufacturer's instructions. The ligation products were then transformed into *E. coli* XL Blue^{Tc}, which permits the identification of recombinants (white colonies) on an indicator LB medium containing ampicillin (100 μg/ml), tetracycline (10 μg/ml), X-Gal (5 bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 0.1 mM) and IPTG (isopropyl-β-D-thiogalactopyranoside, 0.2 mM) (Sambrook et al., 1989). Clones were screened on DGGE, as described by Schabereiter-Gurtner et al. (2001). The clones displaying different fingerprints were selected for sequencing, as described by Schabereiter-Gurtner et al. (2001).

Comparative sequence analyses were performed by comparing pair-wise inserts sequences with those available in the online databases (accessible to the public) provided by the National Centre for Biotechnology Information (NCBI) using the BLAST search program (Altschul et al., 1997).

2.4. Optical microscope observations

A stereoscopic microscope fitted with low temperature fibre optic lighting was used to examine the stained and deteriorated areas of the book. A Leica MZ16 dissecting microscope was used to perform a direct examination of the book prior to carrying out the sampling procedure. Illuminated microscopic examination of mounted slides carrying adhesive tape samples and fungal structures was performed using an Olympus AX60 microscope fitted with a phase contrast device and a digital camera.

2.5. SEM observations and EDS analysis

A variable pressure SEM instrument (LEO 1450 VP, Carl-Zeiss Electron Microscopy Group) was used together with elemental dispersive spectroscopy (EDS) for both the chemical characterisation of inorganic constituents and the description of biodeteriorated paper surfaces, thereby avoiding the need to metallise samples. The term "destructive analysis" implies that a sample (removed from an object) is destroyed whilst undergoing analysis, as opposed to when the sample (either still attached to the object or even if removed from the object) is not destroyed but remains viable for further testing. The variable pressure SEM/EDS instrument allowed for the non-destructive analysis of samples taken from "Le Stanze del Bandello".

The analysis was conducted by direct examination of very small (<4 mm) uncoated paper samples. The SEM technique coupled with electronic dispersion spectroscopy (EDS) allowed for both a deep characterisation of the paper grade (by means of a visual definition of the fibres' structure), and a chemical description of inorganic constituents, such as fillers and metallic impurities.

2.6. Restoration

The sheets of paper were freed of dust and spores with a soft, dry brush inside a laminar flow safety cabinet fitted with HEPA filters. Fungal stains were removed using a water–alcohol (v/v: 1:3) solution. The solvent was applied by blotting the stains with a suitably saturated sponge; the same method was employed on the paper's brown discoloration (caused by wet–dry interfaces). Following stain removal, sheets were partially glued with an alcoholic solution of Klucel G 2% (Botti and Scimia, 1996). Perforations, corroded areas, empty spaces and felted areas were restored using tissue paper for reinforcement (Udagami M2 No.63419 and Canson No.4891-130), and veil-like reinforcing sheets of Kami 7 E2 Tenguio (Japanese paper). These materials were applied to sheets using a thermocompression-type bonding process following their treatment with acrylic resins (Primal AC 33) (Botti and Scimia, 1996).

3. Results and discussion

3.1. Macroscopic appearance of stains

Upon first examination, the book appeared to display typical symptoms of biodeterioration. Most of the pages in proximity to the cover of the book showed great fragility and were of a felted consistency (Fig. 2). Pinkish to purple-coloured spots were distributed throughout the volume's leaves in a repetitive way, suggesting that these marks were caused by migration through the pages. Different areas affected by purple stains fluoresced differently when observed under UV light. Central areas of the stains did not fluoresce, while peripheral areas were conspicuously fluorescent (yellow–green fluorescence). The areas of the sheets that were free from purple stains emitted a blue-coloured fluorescence.

3.2. Microscopic observations

The samples obtained by scratching the surface of sheets and those collected using the adhesive tape technique, when examined using an optical microscope, all showed the presence of fungal structures, especially spores. The clearest evidence of fungal structures was observed on slides prepared with samples collected using adhesive tape: Fig. 3 shows ovate–echinulate conidia that can be attributed to an *Aspergillus* species, co-occurring with ascoma bearing smooth walled lemon-shaped spores. Fig. 4a shows a lenticular ascospore with a crest resembling, except for its pale colour, ascospores of *Emericella* (the teleomorph of *A. nidulans*) that are very distinctive, being red, lens-shaped, and featuring two equatorial crests (Domsch et al., 1993; Samson et al., 2000). Fig. 4b also shows further fungal structures which resemble Hülle cells. Hülle cells are characteristic structures that are normally produced by some species of the genus *Aspergillus* (Raper and Fennell, 1973).

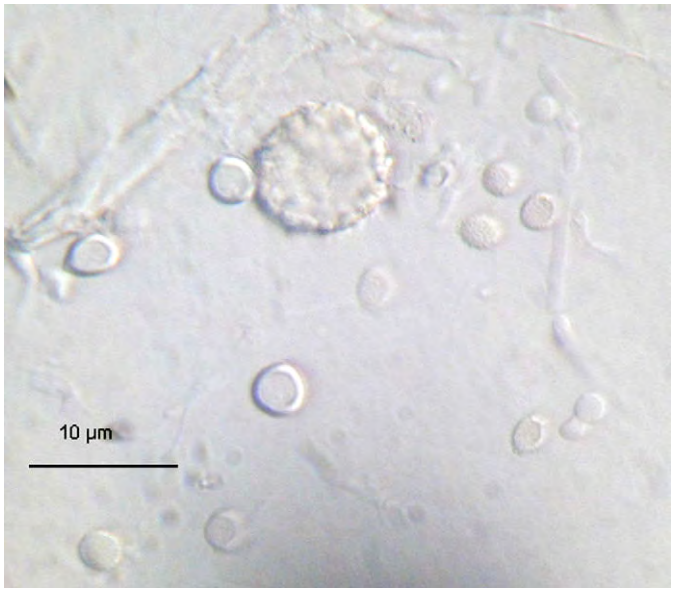

Fig. 3. Light microscopic examination of slides mounted with adhesive tape samples. Fungal conidia ovate to subglobose, echinulate, and a globose larger fungal fruit body. (Olympus AX60 microscope accessorised with phase contrast.)

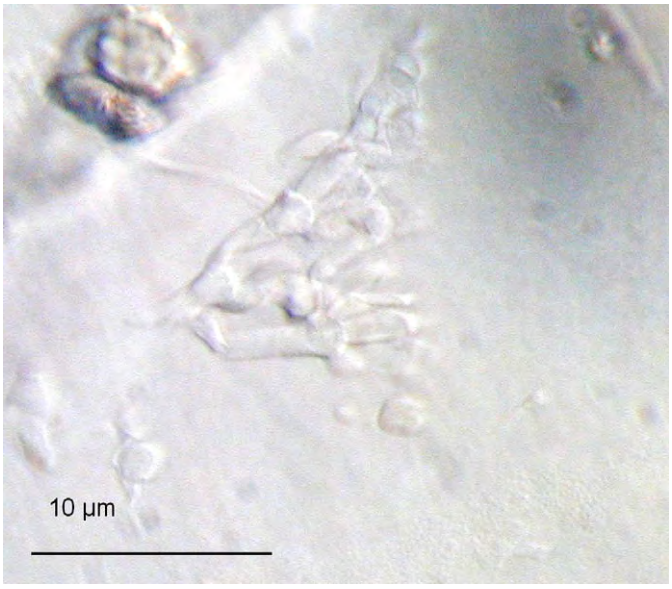

Fig. 5. Light microscopic examination of slides mounted with adhesive tape samples. *Penicillium sp.* conidiophores with conidia (Olympus AX60 microscope accessorised with phase contrast).

These cells are associated with the cleistothecia of all ascogenous species belonging to the *Aspergillus nidulans* group and occur in abundance in their vicinity. Globose and subglobose Hülle cells also appear in certain species of the *Aspergillus versicolor* group (Raper and Fennell, 1973). The association of Hülle cells with the cleistothecia that normally occur in certain Aspergilli groups suggests the participation of these cells in the formation of cleistothecia (Zonneveld, 1988).

Fig. 5 shows *Penicillium* sp. conidiophores with conidia. Several other fungal structures could be observed on the slides. The structures observed that were significant for diagnostics were asci with ascospores, and conidia. The ascospores appeared lenticular, 5–6 × 4–4.5 µm in diameter, and smooth walled; conidia were ovate to subglobose, echinulate, and 2.5–5 µm in diameter; asci were globose and 10–12 µm in diameter. Filamentous fungal isolates are usually identified by microscopic examination of characteristic morphologic structures after growth on appropriate media. Identification on this basis becomes extremely difficult if the isolate fails to form the diagnostically appropriate structures, or if these structures, as in our case, appear incomplete and collapsed. In the absence of any clear morphological feature of these fungi when cultured on different types of media, molecular identification becomes the sole criterion. PCR has become a preferred method used for the identification of fungal cultures deriving from environmental samples; paper originating from a book belongs to this category of sample.

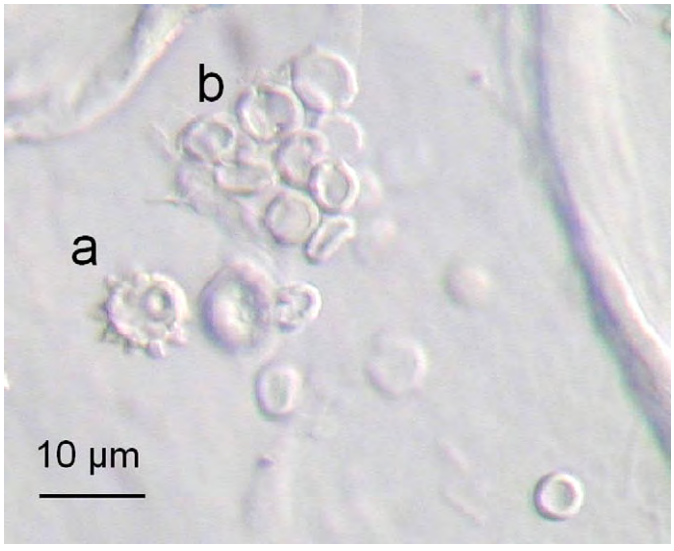

Fig. 4. Light microscopic examination of slides mounted with adhesive tape samples: (a) lens-shaped ascospore with denticular crest; (b) fungal structures resembling Hülle cells. (Olympus AX60 microscope accessorised with phase contrast.)

3.3. Cultivation

Cultures set up using fungal structures sampled from the book with the aid of cotton swabs and inoculated directly on to agar plates resulted in the development of a few colonies of fungi (eight colonies randomly distributed over 20 agar plates); distribution was not statistically significant. The eight colonies developed were identified as belonging to the species *Penicillium commune* Thom (1 isolate), *Penicillium chrysogenum* Thom (2), *Aspergillus niger* van Tieghem (1), *Cladosporium cladosporioides* (Fres.) de Vries (2), and *Cladosporium herbarum* (Pers.) Link (1). The same swabs, following immersion in sterile Czapek broth, developed three colonies of fungal strains which were different from those mentioned above (a species of *Mucor*, a *Trichoderma*, and an unidentified yeast).

Cultures prepared from powdered paper samples developed only a few fungal and bacterial colonies that did not resemble the genera regarded as typical paper biodegraders (Zyska, 1997). Also, the cultures prepared using M40 (15 plates, corresponding to as many sampling points in the book) yielded no results, thus suggesting that the fungal structures (both spores and propagules) observed through the microscope were no longer viable. The fragments of paper that were used to perform the "25 points inoculum" on agar also did not yield any positive results. None of the six plates set up with washed paper fragments displayed fungal or bacterial growth in a statistically significant number of points among the 25 points of the grid, thereby confirming the non-viability of the fungal mycelia observed on the paper under examination.

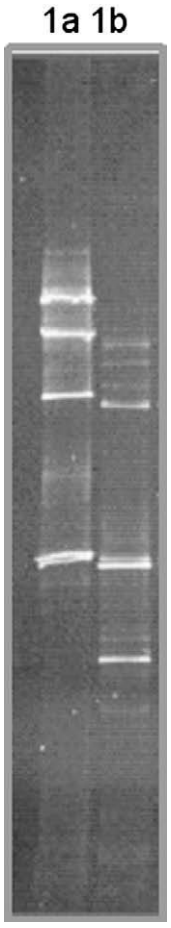

Fig. 6. ITS1-derived DGGE profiles obtained from two pieces of paper obtained from a copy of the book "Le Stanze del Bandello" by Matteo Bandello. For phylogenetic identification of members of the fungal community, a clone library was performed from sample 1a.

3.4. Molecular analysis

For molecular analyses, two fragments of paper, 1a and 1b of approximately 2–3 mm^2 obtained from the book (from the margins of the page shown in Fig. 2), were used for direct DNA extraction as described in Section 2. The DNA extracts were amplified by PCR with primers targeting the ITS regions. The duly obtained ITS-1-amplified fragments were further analysed using DGGE-fingerprints on 30–50% denaturing gels, as described in the Section 2. Fig. 6 shows the DGGE profiles obtained from the two fragments of paper, chiefly from the margins of the most degraded pages with the aid of watchmaker's tweezers. Both DGGE profiles revealed complex fungal communities consisting of four or five dominant DGGE bands as well as some other, faint, bands.

To accomplish phylogenetic identification of the individual members of the fungal community, a clone library containing the ITS fungal regions as well as the 5.8S rRNA gene was generated from sample 1a. The resulting clones were further screened on DGGE as mentioned in the Section 2. Clones displaying different fingerprints on DGGE were sequenced and compared with ITS sequences of known fungi listed in the EMLB database. Table 1 shows the phylogenetic affiliations of the fungal clones obtained in this study.

The comparative sequence analysis revealed great similarities – ranging from 98 and 100% – between different members of *Ascomycetes* and *Zygomycetes*.

Four sequences were affiliated with two genera of the order Eurotiales: *Penicillium*, namely *P. pinophilum* Thom (clones 1F1 and 1F33), and *Aspergillus versicolor* (Vuill.) Tirab. (clones 1F2 and 1F29). The sequences obtained for IF2 were also affiliated to *Aspergillus nidulans* (Eidam) G. Winter, the anamorph of *Emericella nidulans* (Eidam) Vuill. Two sequences were affiliated with a genus belonging to the order Capnodiales, namely *Cladosporium*: *C. cladosporioides* (clone 1F18) and *Cladosporium* sp. (clone 1F50). Clone 1F16 was phylogenetically affiliated to the order Pleosporales, as *Epicoccum nigrum* Link, and Clone 1F5 to the order Saccharomycetales, as *Debaryomyces hansenii* (Zopf) Lodder et Kreger-Van Rij, and clone 1F39 showed the greatest degree of similarity to a species belonging to the order Helotiales, namely *Botryotinia fuckeliana* (de Bary) Whetzel. Clone 1F17 was phylogenetically affiliated with *Rhizopus oryzae* Went & Prins. Geerl. (current name: *Rhizopus arrhizus* A. Fisch.), a member of the Zygomycetes.

Results obtained from the sequence analyses partially agreed with the results obtained from culturing experiments, and partially with results obtained through direct microscopic observations mentioned in Section 3.2.

C. cladosporioides is a cosmopolitan species and is abundant in air samples collected in many areas of the world (Marshall, 1997). This strain was detected using both culturing and molecular techniques. However, the number of colonies grown on agar media was not statistically significant enough to be blamed for the observed damage; this species can be considered an airborne contaminant of the paper samples.

P. pinophilum is also a cosmopolitan fungus (Samson and Pitt, 1985, 2000). Moreover, this species produces cellulases while growing on cellulose as a carbon source, and is able to induce hydrolysis of lignocellulosic materials, and presents high glucosidase activity (Krogh et al., 2004). This species was not cultivated from our paper samples, but some diagnostic fragments of *Penicillium* sp. consistent with the *P. pinophilum* species were found during the microscopic examination of the adhesive sampling medium employed. *P. pinophilum* belongs to the Biverticillium section and the Miniolutea series in the genus *Penicillium*. It was originally described in 1910, but afterwards confused or reported in synonymy with the rather similar species *P. funiculosum*; it was subsequently neotypified by Pitt (1979), and is now generally recognised as a different species (Van Reenen-Hoekstra et al., 1990).

Table 1
Phylogenetic identification of fungal sequences detected on a degraded paper of a 16th-century book

Phylum	Order	Clone	Length	Phylogenetic identification	Similarity (%)	Accession number
Ascomycota	Capnodiales	1F18	511	*Cladosporium cladosporioides* [EU497957]	100	FM165468
		1F50	510	*Cladosporium* sp. [EU497957; EF029809]	100	FM165472
	Eurotiales	1F1	538	*Penicillium pinophilum* [AB369480; AF176660]	98	FM165463
		1F33	537	*Penicillium pinophilum* [AB369480; AF176660]	100	FM165470
		1F2	529	*Aspergillus versicolor* [AY373883; AM883155; EU586040; EF125026; AJ137751]	99	FM165464
				Aspergillus nidulans [AF455505]	99	
		1F29	528	*Aspergillus versicolor* [AY373883; EU586040; AM883155]	99	FM165469
	Helotiales	1F39	499	*Botryotinia fuckeliana* [EF207415]	100	FM165471
	Pleosporales	1F16	505	*Epicoccum nigrum* [A EU232716; EF432273; AF455455]	100	FM165466
	Saccharomycetales	1F5	596	*Debaryomyces hansenii* [EF190234; EF197943]	99	FM165465
Zygomycota	Mucorales	1F17	582	*Rhizopus oryzae* [DQ990331; AY803928; DQ119030; AY213684]	99	FM165467

This species could be one of those responsible for the paper biodeterioration observed in Bandello's book.

A. versicolor is frequently found in stored cereals, hay, cotton, cheese, meat and other foods in a state of decomposition, as well as in various types of soil (Pitt and Hocking, 1985; Samson and Pitt, 1985, 2000). This species is a primary coloniser or storage mould, able to grow at a low water activity ($aw < 0.8$) and on very nutrient-poor materials, such as concrete and plaster (Fog, 2003). *A. versicolor* has highly variable culture morphology, but produces a consistent chemical profile on laboratory substrates, usually generating elevated quantities of the carcinogenic mycotoxin called sterigmatocystin and a pink–orange pigment known as versicolorine (Turner, 1971), whose colour is consistent with the stains we found on paper sheets. *A. versicolor* exhibits high amlylolytic and gelatinolytic activity, and a good level of cellulolytic activity (Reese and Downing, 1951). The conidia of *A. versicolor* are echinulate and 2–3.5 μm in diameter. *A. versicolor* can produce Hülle cells. This species is the second candidate whose appearance and metabolic characteristics fit with the deterioration observed on the paper from Bandello's book.

A. nidulans also produces Hülle cells, and its cultures can secrete a purple–brown pigment. *Emericella nidulans* and its anamorph produces a toxic metabolite, sterigmatocystin, and can be found on cotton and stored seeds; it can also be pathogenic to humans (Samson et al., 2000).

E. nigrum is a common secondary invader of plant materials, and is also found in soil and many other substrates (Domsch et al., 1993); it is a fungal species grouped among "common primary saprophytes", together with *Cladosporium* spp. *E. nigrum* is very sensitive to low water availability (minimum water potential for growth *aw* of 0.90–0.86). Epicocconone is a heterocyclic natural product generated by the fungus *E. nigrum* that fluoresces weakly in the green segment (520 nm) (Gopinath et al., 2005). No clear structures resembling those of *E. nigrum* were observed on the paper in question, although a green fluorescence was observed in stains when illuminated with a Wood's lamp.

B. fuckeliana is a filamentous, heterothallic, apothecial ascomycete that causes grey mould on many economically important crops worldwide, without any apparent host specificity (Coley-Smith et al., 1980). The fungus is the teleomorph of *Botrytis cinerea*, and it exhibits a high genetic variability expressed in the many phenotypical differences among isolates (Di Lenna et al., 1981). *D. hansenii* is a ubiquitous ascomycetous yeast which is commonly isolated from a variety of extreme environments such as animal products (especially cheese), high-sugar and high-salt foods, sea water or clinical swabs (Kurtzman and Fell, 1998). This species has the ability to grow in the presence of salt at low temperatures and to metabolise lactic and citric acids. It is phylogenetically very close to both *Candida albicans* and *Saccharomyces cerevisiae* (Nakase and Suzuki, 1985). Both *B. fuckeliana* and *D. hansenii* can reasonably be considered occasional contaminants of paper samples.

We postulate that the purple stains which migrate through the pages with a felted consistency (Fig. 2), based on all the information obtained using traditional and molecular means, were caused by a cellulolytic fungus producing purple essudates, characterised by echinated conidia and Hülle cells. These elements are consistent with the discovery of both *A. versicolor* and *A. nidulans* using molecular techniques. Moreover, an ascospore with diagnostic characters attributable to *E. nidulans* was observed through the optical microscope after being sampled directly from paper damage with the aid of adhesive tape (Fig. 4). It is worth noting that more than one fungal species probably contributed to the damage observed. *P. pinophilum* actively grew on paper, since it was detected on samples using molecular techniques; additionally, biverticillated penicillium-like structures were observed directly on the paper under investigation. Furthermore, a fungus species

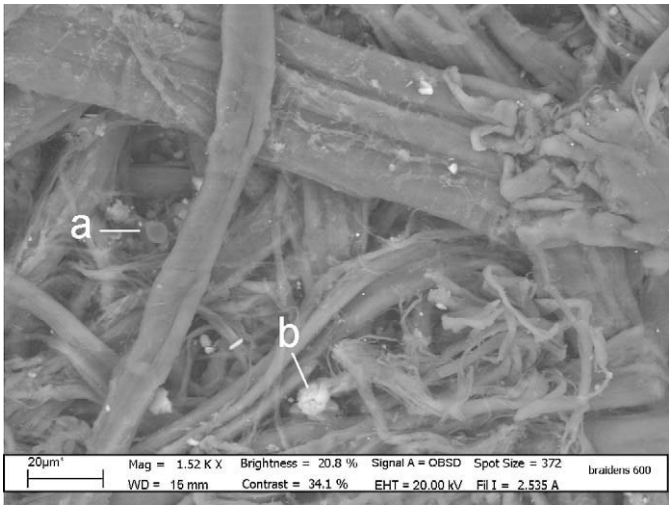

Fig. 7. Scanning electron micrograph of an uncoated sample. The image was obtained with a backscattered electron detector (QBSD). The differences in average atomic number of the organic and inorganic materials allowed for well-contrasted observation of filling materials and ion distribution on paper surfaces. Cellulose fibres of different sizes and with different degrees of integrity can be examined with detail in SEM pictures; fungal spore (a), crystals and aggregates of sizing materials and of impurities (b) are also visible.

producing lemon-shaped spores and globose aschi was also present, although none of the species found through molecular analysis could be coupled with these structures. The combination of traditional cultivation and molecular techniques demonstrated the advantage of validating results, as well as the additional information obtained through the application of molecular analysis, as sequences from fungi which could be not cultivated.

3.5. SEM/EDS observations

The samples taken from "Le Stanze del Bandello" and examined under SEM showed that the paper's surface was in an advanced state of deterioration, and that its constituent cellulose fibres were broken and projected from the material's surface (Fig. 7). The book contained a type of paper made from cotton linters. Cellulose fibres

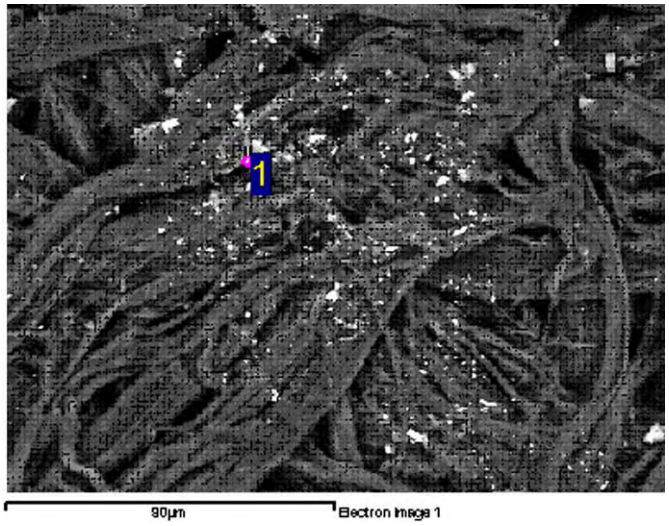

Fig. 8. Elemental dispersive spectroscopy (EDS) allowed a characterisation of impurities on paper. The presence of Hg (Table 1) in the form of a scattered powder observed on some samples is a result of the conservator's activity for protecting the paper against deterioration by micro-organisms.

Table 2
Qualitative chemical characterisation of the inorganic constituents of the samples

Element	Weight%	Atomic %
C	49.68	65.79
O	30.58	30.40
Na	0.35	0.24
Al	1.17	0.69
S	2.24	1.11
K	0.59	0.24
Ca	1.02	0.41
Hg	9.33	0.74
Pb	5.04	0.39
Totals	100.00	

The analysis was performed by means of electronic dispersion spectroscopy (EDS), which allows for an X-ray area scanning of what is brought into focus in SEM images, thereby creating a compositional map of the paper's surface. Processing option: all elements analysed (normalised). Number of iterations = 5.

of various dimensions and displaying different degrees of integrity can be observed in detail in SEM pictures; crystals and aggregates of sizing materials and impurities also become visible. In addition, because of the different conductivity of inorganic elements as compared to cellulose, mineral containing sizing materials and impurities stand out against the organic background of SEM pictures, since they are of a lighter tone of grey. Elemental dispersive spectroscopy (EDS) allowed for the characterisation of the paper's texture, sizing materials and impurities. The inorganic elements recorded in most of the EDS spectra performed on the paper samples were calcium, aluminium and silica, suggesting that both calcium carbonate or calcium hydroxide (lime) and silicates were used in the papermaking process as sizing and filling materials. The use of added lime during the beating process in early Italian papermaking and in the late 16th century is well documented (Hills, 1992).

Aluminium can be used in conjunction with gluing agents (gelatines) (Hunter, 1974). In Europe the use of alum for hardening gelatine sizing material was recorded during the 16th century (Barrett, 1989). The SEM-EDS technique allowed for the formulation of a hypothesis on the origin of some of the impurities and contaminants that are present in samples: according to their chemical nature, dimensions and relation to cellulose fibres, it can often be deduced whether or not they derive from the paper manufacturing process or not. This is the case with the high concentration of Hg and Pb in the form of a scattered powder observed in some samples, and shown in Fig. 8, Table 2. The Hg and Pb particles (Table 1, Hg = 9.33% and Pb = 5.04% in weight) appeared to overlap the fibres, suggesting that the contamination occurred after the papermaking process took place. The presence of mercury and lead compounds in paper is usually the result of a conservator's activities aimed at protecting the paper against deterioration caused by micro-organisms. The use of biostatic compounds and disinfecting interventions was common in the 18th century, and this suggests that the volume was subject to biological attack in the distant past.

3.6. Restoration

The restoration of the copy of "Le Stanze del Bandello" by Matteo Bandello was carried out according to the "*in situ* repair" method, which calls for limited operations to treat limited damage. Partial interventions are based on the theory of non-invasive restoration strategies aimed at safeguarding every original component forming a book. "Le Stanze del Bandello" was restored maintaining the integrity of the volume; the book's original binding, made from parchment, was kept intact. In this way all the historical and technical information contained in the original binding of the book was preserved. The paper leaves were cleaned

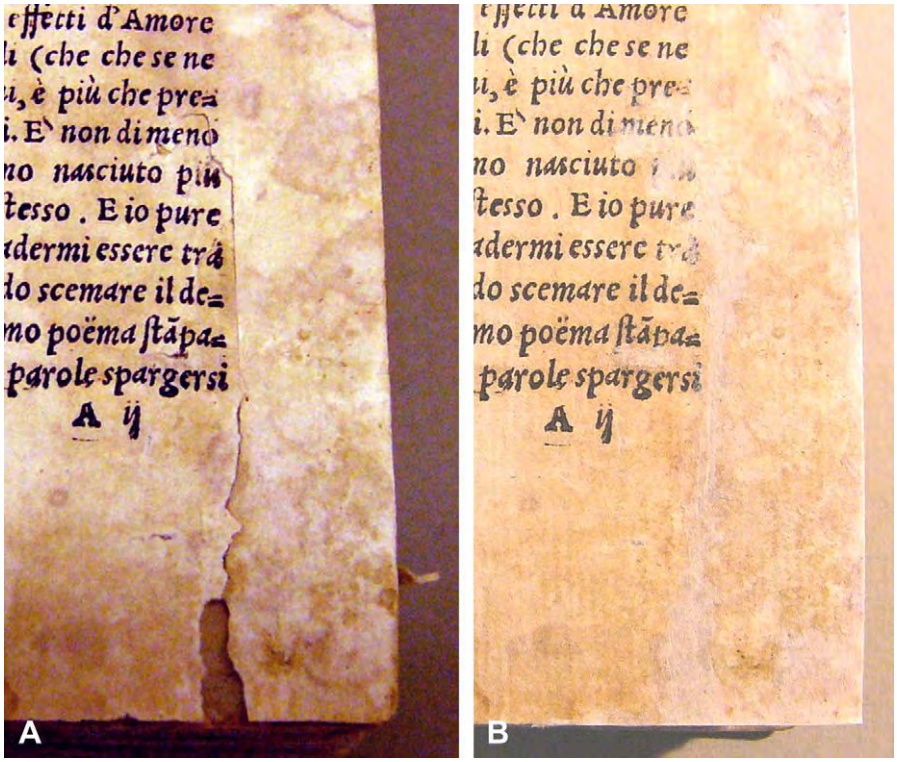

Fig. 9. The restoration of the copy of "Le Stanze del Bandello" by Matteo Bandello was performed according to the methodology of "*in situ* repair" which involves limited operations to treat limited damages. Paper sheets were locally reinforced with partial interventions in order to reintegrate empty spaces and strengthen felted areas and margins. A damaged area of a page before (A), and after (B) restoration. Empty spaces and felted areas were restored using tissue paper for reinforcement (Japanese paper) applied using a type of thermocompression bonding after treatment with acrylic resins.

and the stains produced by fungi were partially removed (Szczepanowska and Lovett, 1992). In addition, paper sheets were reinforced in places by means of localised interventions in order to fill empty spaces and strengthen felted areas and margins (Fig. 9a,b). A full disinfection procedure was not performed because agar cultures of samples obtained from the book developed a small number of fungal and bacterial colonies, which could be partly attributable to airborne contamination (Korpi et al., 1997) of the paper sheets. The stains caused by fungi were considered scarcely active and the treatment utilised, although not specific, was potent enough to kill those fungal colonies and airborne spores that showed activity upon culturing.

4. Conclusions

The copy of "Le Stanze del Bandello" by Matteo Bandello, printed at the end of the 16th century in Italy and kept in the Braidense Library in Milan is of great cultural heritage value. Despite the fact that the book presented some interesting damage for scientists studying ancient paper degradation, its study and the possibility for "direct prevention" presented some limitations, mainly due to restricted sampling opportunities. Nevertheless, the goal of defining the cause(s) and extent of damage was fulfilled. In this case study we demonstrated that the methodology required for a conservative approach to the restoration of precious books and objects of art made from paper encompasses a wide range of sampling techniques and instruments that cannot easily be standardised. Classic culturing, sampling methods and innovative techniques have to be specially adapted on a case-by-case basis so as to best suit the particular situations presented by materials encountered in unique objects of cultural heritage. The molecular techniques applied in this study have demonstrated the advantage gained from performing exhaustive tests on very small samples. We believe, therefore, that the use of molecular techniques in combination with conventional methodologies used in prevention should be adopted in the field of cultural heritage diagnostics.

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Chapter V

Molecular and microscopial investigation of the microflora inhabiting a deteriorated Italian manuscript from the thirteenth century

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ENVIRONMENTAL MICROBIOLOGY

Molecular and Microscopical Investigation of the Microflora Inhabiting a Deteriorated Italian Manuscript Dated from the Thirteenth Century

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Abstract This case study shows the application of non-traditional diagnostic methods to investigate the microbial consortia inhabiting an ancient manuscript. The manuscript was suspected to be biologically deteriorated and SEM observations showed the presence of fungal spores attached to fibers, but classic culturing methods did not succeed in isolating microbial contaminants. Therefore, molecular methods, including PCR, denaturing gradient gel electrophoresis (DGGE), and clone libraries, were used as a sensitive alternative to conventional cultivation techniques. DGGE fingerprints revealed a high biodiversity of both bacteria and fungi inhabiting the manuscript. DNA sequence analysis confirmed the existence of fungi and bacteria in manuscript samples. A number of fungal clones identified on the manuscript showed similarity to fungal species inhabiting dry or saline environments, suggesting that the manuscript environment selects for osmophilic or xerophilic fungal species. Most of the bacterial sequences retrieved from the manuscript belong to phylotypes with cellulolytic activities.

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Introduction

Biodeterioration of paper and parchment in ancient books and documents represents a cause of great concern for libraries and archives all over the world. The study of the mechanisms underlying the microbiological attack of historical materials has been widely practiced and still represents one of the main focuses of those institutions and laboratories that are involved in cultural heritage conservation. Microbial investigations based on cultivation strategies are not reliable because they yield only a limited fraction of the present microbial diversity [26]. The application of molecular biology techniques on cultural heritage environments has shown that new spoiling taxa and unsuspected microbial consortia are involved in the discoloration and biodeterioration of paintings and monuments [44]. Restricted sampling from art and documental objects results in additional problems for representative floristic analyses.

Filamentous fungi colonize different organic and inorganic materials and play an important role in biodeterioration processes [30, 35, 45, 57]. They can tolerate desiccation, high salt concentrations, and heavy metal compounds that are present in inks and pigments and are thus frequent inhabitants on paper-supported objects [14, 57, 58].

The fungal and bacterial communities that can develop on a book are similar to the communities of decomposers that, in natural environments, transform nutrients bound in dead organic matter into low molecular or inorganic forms,

making them available to plants. The development and maintenance of a fungal community on a shelf of a library or in a single book depends on the spores that reach the material's surface, on the microenvironment (temperature, relative humidity, light), on the water activity of the substrate, and on the casual events that help colonization of materials (insect dispersion, human contamination, external sources of fungal diversity) [14]. A library or a single book can be compared to a virgin land that can be reached by some colonizing organisms that behave like pioneer species on a nude soil. For Wardle [53] and Mikola et al. [29], species identity and composition of decomposers have far greater impacts on ecosystem processes than species richness per se. When considering paper stored in a closed environment, its colonization and biodegradation depends on species identity and composition since only cellulolytic organisms can exploit the bulk of the substrate. Like in natural environments, the diversity-functioning relationship is driven by the presence or absence of key species [52], by niche differentiation, and species interaction. Resource partitioning or facilitative (or negative) interactions between species [50] affect the substrate exploitation process in natural environments as well as in artificial ones.

In this case study, we present the application of non-traditional diagnostic methods to investigate the microbial consortia inhabiting an ancient manuscript. Molecular methods, including polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE), and the creation of clone libraries, were used as a sensitive alternative to conventional cultivation techniques.

Materials and Methods

History of the Object

A manuscript dated back to 1293 from Italy was sent to the Istituto Centrale per il Restauro e la Conservazione del Patrimonio Archivistico e Librario (ICPAL) in Rome for restoration [7]. The volume is composed of 222 sheets divided into six gatherings with a binding made of parchment. It was written on Arabic paper made of linen and was characterized by a singular and never described deterioration phenomenon that gave the paper a dramatically felted aspect, especially in the margins. The paper-supported manuscript belongs to La Spezia's Notaries Public Archive. It was edited by a Sarzanese notary called Parente Stupio between 1293 and 1294; it contains 464 deeds recording commodities, real estate, and land transactions. The volume, utterly transformed in appearance as a result of mechanical damage, damp, and microorganisms, was no longer in a consultable condition because the lower portions of its pages were shredded (Fig. 1a and b).

Figure 1 **a**, **b** Manuscript dated back to 1293 from Italy, which belongs to La Spezia's Notaries Public Archive

Sampling

Sterile cotton swabs were wiped along the most damaged margins of the outer and inner pages of the manuscript to obtain samples suitable for further fungal and bacterial culturing and identification. Loose fibers, dust, and powder were also collected from the manuscript by cleaning the pages with a soft brush; these materials were mainly used for microscopic observations. Some fragments of paper (2 to 3 mm^2) were also collected, mainly from the margins of the most degraded pages for microscopic examination and molecular analysis. Paper samples from the manuscript were taken by restorers after an evaluation of the areas most suitable for this purpose. Two samples of 4 mm in diameter containing ink were detached from their position: these could not be repositioned during restoration and were, therefore, used to SEM observations and specifically for ink elemental analysis.

Optical Microscope Observations

A stereoscopic microscope fitted with low temperature fiber optic lighting was used to examine the stained and deteriorated areas of the book. A Leica MZ16 dissecting microscope was used for the direct examination of the book prior to the sampling procedure. Illuminated microscopic examination of mounted slides carrying fibers and powdery

materials recovered from the manuscript was performed with an Olympus AX60 microscope fitted with phase contrast and a digital camera.

Agar/Broth Cultures

The viability of fungi and bacteria was tested using agar and broth cultures. Microbial structures sampled with cotton swabs were inoculated directly on to agar plates, and swabs were then immersed in sterile Czapek and Gelatine broth [42]. Some fragments of paper were used to perform a "25 points inoculum" [14], which consists of a multiple inoculum on agar divided into a grid with 25 points made up from sub-fragments of a paper sample previously washed several times with sterile water. The aim of this procedure is to cultivate those biodeteriogens that affect cellulose fibers and to avoid the development of air-borne contaminants. When a statistically significant fraction of the 25 points develop the same fungal/bacterial species, it is reasonable to consider that strain as being a paper spoiler.

The media used to grow the inocula were malt extract agar and dichloran 18% glycerol (DG18) agar prepared according to Samson et al. [42]. All the inoculations and re-inoculations were performed in a laminar flow hood to assure sterility throughout the procedures.

Molecular Analysis

DNA Extraction from Paper Material

DNA extraction was performed directly from the paper samples using the FastDNA SPIN Kit for Soil (Qbiogene, Illkirch, France) with modifications to the protocol as described by Michaelsen et al. [28]. To enhance DNA yields from all cells and spores on the paper surface, the samples were pretreated with lysozyme and Proteinase K as described by Schabereiter-Gurtner et al. [44]. DNA crude extracts were used directly for PCR amplification.

PCR Amplification of Extracted DNA

For the analysis of fungal sequences, fragments of about 700 bp in size corresponding to the ITS1, the ITS2 region, and the adjacent 5.8S rRNA gene were amplified with the primer pair ITS1 and ITS4 [5, 54]. For DGGE analysis, a nested PCR was performed with the PCR product of the first round as template DNA using the primers ITS1GC with a 37-base GC-clamp attached to the 5′ end [31, 32] and ITS2. All reactions were carried out as described in Michaelsen et al. [28].

For the identification of bacterial 16S rRNA sequences, DNA was amplified with the primer pair 341f/907r [31, 49]. For DGGE analysis, 200 bp fragments of the 16S rDNA were amplified with a nested PCR using the eubacterial specific primer 341f-GC with a 40-bp GC clamp added to its 5′ end [31] and the universal consensus primer 518r [33]. PCR conditions were as described by Schabereiter-Gurtner et al. [44]. All PCR products were analyzed by electrophoresis in a 2% (*w*/*v*) agarose gel.

DGGE

DGGE was performed as previously described [31] using a D-Code system (Bio-Rad) in ×0.5 TAE (20 mM Tris, 10 mM acetate, 0.5 mM Na_2 EDTA; pH7.8 with 8% (*w*/*v*) acrylamide).

For fungal fingerprints, gels contained a linear chemical gradient of 30–50% denaturants [100% denaturing solution contains 7 M urea and 40% (*v*/*v*) formamide] and were run at a constant temperature of 60°C for 14 h with a voltage of 100 V.

For bacterial fingerprints, gels contained a linear chemical gradient of 30% to 55% and were run at a constant temperature of 60°C during 3.5 h with a voltage of 200 V.

After completion of electrophoresis, gels were stained in an ethidium bromide solution and documented with a gel documentation system.

Creation of Clone Libraries and Sequence Analysis

To obtain a detailed phylogenetic identification of the microbial community members, clone libraries containing either ITS fungal regions (fungal community) or 16S rRNA fragments (bacterial community) were carried out. For fungal clone libraries, the DNA template was amplified using the primers ITS1/ITS4 [54] as mentioned above. For bacterial clone libraries, the primer pair 341f/907r was used as mentioned above. The PCR products were purified using the QIAquick PCR Purification Kit Protocol (Qiagen, Hilden, Germany) and resuspended in ddH_2O water. Purified PCR products were ligated into the pGEM-T easy Vector system (Promega, Vienna, Austria) following the instructions of the manufacturer. The ligation products were transformed into *Escherichia coli* XL Blue^{Tc} to allow the identification of recombinants on an indicator LB medium containing ampicillin, tetracyclin, X-Gal, and IPTG as described in Sambrook et al. [41]. Clones were screened in a DGGE gel and sequenced as described by Schabereiter-Gurtner et al. [44]. Comparative sequence analysis was performed by comparing pairwise insert sequences with those available in the public online database NCBI using the BLAST search program [2].

SEM Observations and EDS Analysis

The analysis of paper samples was conducted using a variable pressure SEM instrument (EVO50, Carl-Zeiss

Electron Microscopy Group) equipped with a detector for electron backscattered diffraction. In addition, a qualitative and quantitative chemical characterization of the inorganic constituents of the samples was performed by means of electronic dispersion spectroscopy (EDS), which allows for an X-ray area scanning of what is brought into focus in SEM images, thereby creating a compositional map of the paper's surface. Only after having observed samples with SEM in Variable Pressure mode, at 20 keV, some of the samples were covered with gold with a Baltec Sputter Coater for a further analysis with SEM in High Vacuum mode. The High Vacuum allowed for a higher magnification of samples' details.

Analysis of variance (ANOVA) was employed to investigate the differences in elemental composition of paper and ink with EDS data. In the ANOVA, each comparison is considered significant (the difference is significant) if the probability is out of the confidence interval on the basis of Tukey's honestly significant difference (HSD) test.

Results

Optical Microscope Observations and Cultures

The observation with the optical microscope of some fibers showed the presence of fungal spores and pigmented cellular structures (Fig. 2). Cultures set up with fibers and powder sampled from the book and those obtained from cotton swabs resulted in the development of a few colonies of fungi (five colonies randomly distributed over 10 agar plates); distribution was not statistically significant. The colonies developed were identified as belonging to *Penicillium commune* Thom (two isolates) *Cladosporium sphaerospermum* Penzig (two isolates), *Aspergillus niger* van Tieghem (one isolate). None of the plates with DG18 agar for xerophilic species supported the growth of fungal colonies. The fragments of paper that were used to perform the "25 points inoculum" on agar also did not yield any positive result.

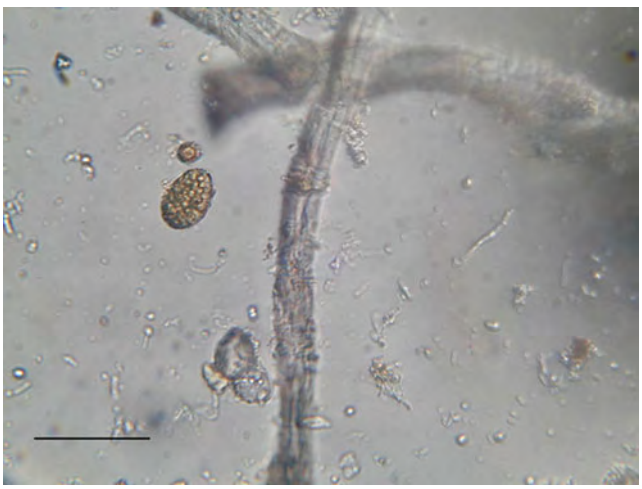

Figure 2 Pigmented fungal structures, fungal spores, hyphae and conidia (Olympus AX60 microscope, light field). The black bar indicates 100 μm

SEM-EDS

The microanalysis of the light-gray areas containing ink revealed the presence of statistically significant amounts of Fe, K, Al, Si, and Ca (Tables 1 and 2, Fig. 3a and b). Other elements present in paper but not statistically associated to inked areas were Na, Cl, Mg, S, and P. Na, Cl, and Mg relative percentages are consistent to the presence of sea salt, namely NaCl with traces of MgCl. The ink used for the writing of the manuscript can be considered an iron gall ink [7].

The SEM observation of the morphology of paper fibers allowed the definition of the plant origin of the cellulosic material. Some distinctive characters, like the dimension of the fibers and the presence of peculiar septa indicated that the fibers used in the manufacture of the volume's paper were from linen [7]. Moreover, starch granules were observed with SEM analysis, and its use as sizing material was confirmed by a colorimetric test [7].

SEM analysis at high magnification performed on samples both before and after coating with gold allowed the visualization of fungal spores and hyphae attached onto fibers (Figs. 4, 5, and 6). Fungal spores appeared globose and echinated, but any attribution to a genus would be misleading because of their desiccated state, which makes any systematic description useless.

Molecular Analysis

For molecular analyses, pieces of paper (approximately 2 to 3 mm^2) obtained from the book were used for direct DNA extraction.

The DNA extracts were amplified by PCR with primers targeting the ITS regions of fungi, as well as the 16S rRNA gene of bacteria. The fungal and bacterial amplified fragments were further analyzed by DGGE fingerprints. DGGE analysis revealed fingerprints for both bacterial and fungal communities.

Figure 7 shows the DGGE profile derived from the fungal community colonizing paper material; DGGE profile showed a complex fungal community consisting of seven to eight dominant DGGE bands as well as some other faint bands. DGGE derived from the bacterial community showed less bands, indicating a lower biodiversity of bacteria on the paper sample (data not shown).

Table 1 EDS microanalysis

	C	O	Na	Mg	Al	Si	P	S	Cl	K	Ca	Fe	Total
Ink	0.0	74.0	3.2	1.9	0.9	2.5	0.0	1.5	3.4	0.9	9.4	2.4	100.0
Ink	0.0	74.5	3.1	1.9	1.0	1.7	0.7	0.7	3.6	0.9	10.2	1.9	100.0
Ink	43.2	47.9	0.8	0.4	0.4	0.6	0.0	0.3	1.3	0.4	3.9	0.8	100.0
Ink	45.6	45.9	0.9	0.5	0.6	1.3	0.1	0.2	1.1	0.4	2.5	0.8	100.0
Paper	52.1	44.9	0.7	0.3	0.0	0.1	0.0	0.2	0.7	0.2	0.7	0.0	100.0
Paper	51.4	46.4	0.5	0.2	0.0	0.0	0.0	0.2	0.7	0.2	0.4	0.0	100.0
Paper	51.1	46.8	0.5	0.1	0.0	0.1	0.0	0.1	0.7	0.1	0.5	0.0	100.0
Paper	48.8	48.4	0.6	0.2	0.1	0.2	0.0	0.1	0.7	0.2	0.6	0.2	100.0

Quantitative chemical characterization of the inorganic constituents of the samples performed by means of EDS, which allows for an X-ray area scanning of what is brought into focus in SEM images, thereby creating a compositional map of the paper's surface. The results showed were obtained analyzing surfaces with and without the graphic sign (ink/paper) as was visualized with the Backscattered detector. All elements normalized. All results in weight%

EDS electronic dispersion spectroscopy

For phylogenetic identification of the individual members of the fungal and bacterial communities, two clone libraries containing either the fungal ITS regions and the 5.8S rRNA gene or the bacterial 16S rRNA gene were carried out. The resulting fungal and bacterial clones were further screened by DGGE. The obtained sequences were compared with ITS regions and 16S rRNA gene sequences of known fungi and bacteria, respectively, listed in the EMBL database. Figure 8 shows an example of the profiles derived from bacterial clones screened on DGGE. Tables 3 and 4 show the phylogenetic affiliations of the fungal and bacterial clones obtained in this study, respectively.

Table 2 Summary of all pairwise comparisons of the samples based on the average values obtained for the EDS analysis of paper and ink

Category	Paper	Ink
C	50.930 A	22.190 A
O	46.600 A	60.560 A
Na	0.549 A	2.003 A
Mg	0.202 A	1.163 A
Al	0.024 B	0.731 A
Si	0.099 B	1.536 A
P	0.000 A	0.211 A
S	0.137 A	0.660 A
Cl	0.713 A	2.331 A
K	0.159 B	0.649 A
Ca	0.570 B	6.494 A
Fe	0.038 B	1.464 A

α=0.05; test used: Tukey's, HSD; analysis of the differences between the categories with a confidence interval of 95%. Samples signed with different letters (A or B) are significantly different

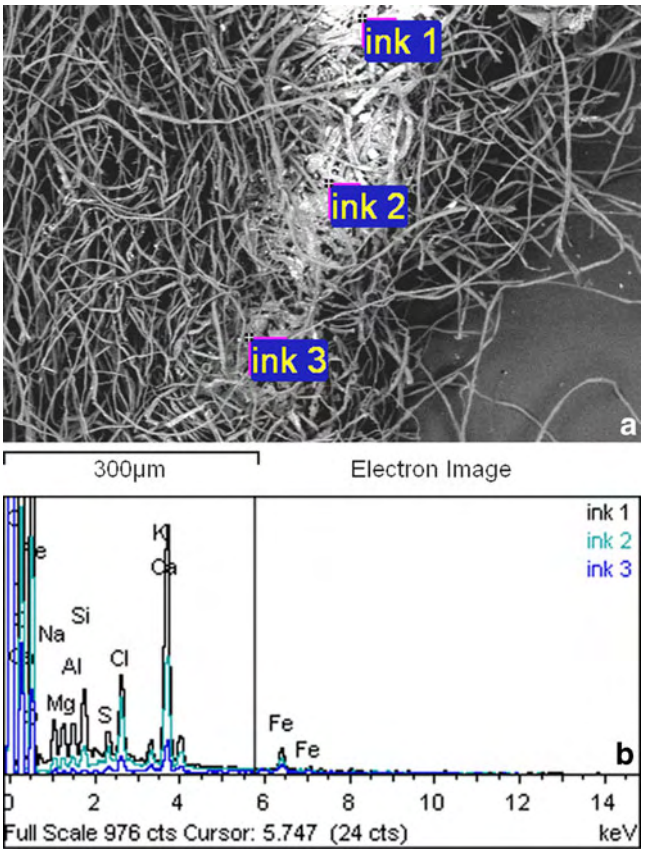

Figure 3 **a**, **b** Microanalysis of the light-gray areas containing ink revealed the presence of statistically significant amounts of Fe, K, Al, Si, and Ca

Figure 4 Scanning electron micrograph obtained wih a backscattered electron detector (QBSD) in Variable Pressure mode

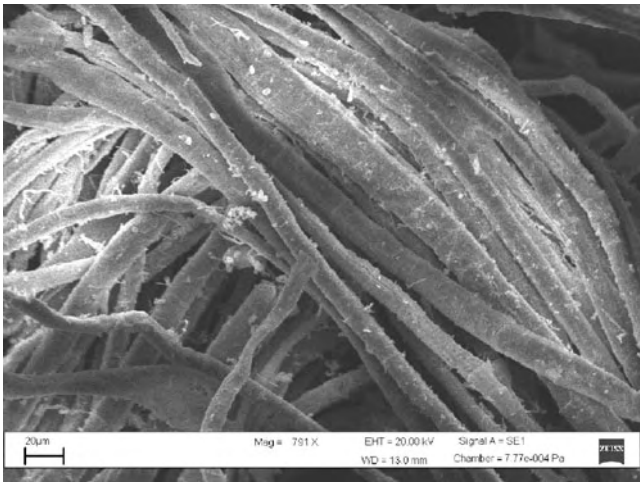

Figure 6 Scanning electron micrograph performed on samples after coating with gold. High Vacuum mode

Microflora Detected on Paper Material

Bacteria

Table 3 shows the phylogenetic affiliations of bacterial clones obtained in this study. Five bacterial sequences (clones K2, K14, K18, K20, and K31) showed high score similarities, between 97% and 99% similarity, with *Bacillus*-related sp. as well as with some uncultured bacterial clones. Clone K4 showed a high score similarity with *Acinetobacter* sp. and clone K21 showed a high score similarity with *Kocuria* sp. as well as with some uncultured bacterium inhabiting indoor environment and house dust.

Clone K27 showed high score similarity with *Stenotrophomonas maltophilia* and clone K29 showed high score similarity with *Clostridium colinum.*

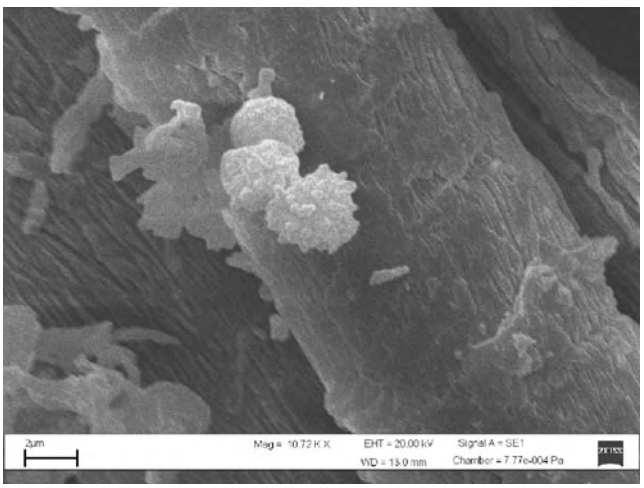

Figure 5 Scanning electron micrograph at high magnification (magnification 10.000×) performed on samples after coating with gold. High Vacuum mode

Fungi

Table 4 shows the phylogenetic affiliations of fungal clones obtained from the manuscript. Clone nF28 showed high similarity (98%) with *Aspergillus terreus* Thom. One sequence (clone F8) showed maximum score similarity (100%) with *Aureobasidium pullulans* (de Bary) G. Arnaud. Two sequences (clones K23F, nF20) showed a high score similarity with *Penicillium pinophilum* Thom.

Six fungal sequences (K5F, nF2, nF7, nF13, nF19, nF24) showed high score similarities, between 95% and 99%, with *Aspergillus versicolor* (Vuill.) Tirab.

Four fungal sequences (clones K31F, nF6, nF14, nF21) showed high score similarity with *Eurotium halophilicum* Chr., Papav. & Benj. Among the fungal DNA sequences obtained from the manuscript, clone F3 presented a high similarity with *A. penicilloides*, and clone F12 totally corresponded to *Wallemia sebi* (Fr.) Arx (100% similarity). Clone F28 gave a 100% similarity with a xerophilic fungal species: *Rhodotorula aurantiaca* (Saito) Lodder is an anamorphic basidiomycetous yeast species [12].

Four clones (F42, nF12, F25, F37) showed high similarity to sequences addressed to defined fungal genera but could not be identified at the species level [55]. These are all fungi that can be both considered dust inhabitants and paper spoilers, namely *Cladosporium* sp., *Alternaria* sp., *Penicillium* sp., and *Aspergillus* sp.

Discussion

The poor conservation condition of the manuscript presented in this case study did appear to be the result of biological activity, with SEM observations proofing several fungal spores attached to cellulose fibers.

Agar-based cultivation methods showed the presence of living fungal spores of few species that can be considered surface contaminants and dust inhabitants but not in a statistically significant number to be considered responsible for paper biodegradation. The conditions leading to a transformation from a surface contaminant into a paper spoiler are the micro-environmental conditions, the characteristics of the substrate, and the physiological attitudes of the organism itself. From the EDS and chemical characterization of the manuscript, we knew that its paper did not only consist of cellulose but also a complex mixture of starch, cellulose fibers, iron-containing ink, and other inorganic elements that could have represented, as a whole, a substrate for microbes. DNA sequence analysis confirmed the existence of fungi and bacteria on paper material that could not be cultivated with traditional methods.

Potential Deteriorative Actions by the Detected Microflora

Bacteria

Several bacteria have been already isolated from paper materials showing foxing deterioration [15, 25, 30], but to our knowledge, only few studies concerning their taxonomical identification or paper degrading activity has been published [11]. From the ecological point of view, the requirements of prokaryotes are very similar to the environmental needs of fungi and yeasts. One essential condition for bacterial life is a high level of humidity in the environment; thus, bacteria have to withstand drying to exist, as the sporogenic and osmophilic species discovered on this Italian manuscript do.

Facultative anaerobic or microaerophilic bacteria, as *Bacillus* and *Clostridium* strains have been detected in the book investigated in this study. They are cellulolytic bacteria and seem to play an active role in the deterioration processes. *Clostridium* sp. is forming a dominant group of cellulolytic bacteria in municipal solid waste [6, 10]. *Bacillus* sp. has been already isolated from paper affected by foxing [11] as well as from wooden art objects in museum environments [37]. Furthermore, *Bacillus* and related species have been shown to be the most commonly detected bacteria (up to about 20%) among the variety of microbial species isolated from the pulp and paper mill environment [8, 36, 46, 47, 51]. In addition, *Bacilli* have been found as the predominant cellulolytic group of bacteria in landfill, where cellulose accounts for 40% to 50% of the municipal solid waste [38], and they form a significant proportion of the intestinal microbial community of soil invertebrates, especially among cellulose degraders [23].

Table 3 Phylogenetic affinities of partial 16S rRNA coding sequences derived from bacterial clones libraries performed with paper samples obtained from the book written by Parente Stupio between 1293 and 1294

Clones	Length	Closest identified phylogenetic relatives [EMBL accession numbers]	Similarity (%)	Accession no.
K2	[589]	*Bacillus* sp. [DQ993298]	98.0	FN394538
		Caryophanon latum [X70319]	98.0	
		Uncultured bacterium clones [EU771735; EU466988]	98.0	
K4	[589]	*Acinetobacter* sp. [FJ544340] 16S rDNA of the microorganism resources for herbaceous fibers extracting.	99.0	FN394539
		Acinetobacter sp. [FM164629; FJ587508; FJ608713]	99.0	
K14	[589]	Uncultured bacterium clone zd3-48 [EU527183]	99.0	FN394540
		Bacillus sp. MB-7 and MB-1 [AF326364, AF326359] manganese(II) oxidative species.	97.0	
		Virgibacillus picturae [AJ276808] isolated from biodeteriorated wall paintings.	97.0	
K18	[588]	Uncultured Bacilli bacterium clones [EF664900; EF075265]	99.0	FN394541
		Sulfobacillus disulfidooxidans [AJ871255]	97.0	
K20	[589]	*Bacillus* sp. [AF548878; AF548879] Strains able of Specific Ureolytic Microbial Calcium Carbonate Precipitation	99.0	FN394542
		Sporosarcina sp. [EU182901, DQ073393; DQ993301; AB245381]	99.0	
K21	[571]	*Kocuria* sp. I_GA_W_11_16 [FJ267551] an airborne bacteria in indoor air	99.0	FN394543
		Kocuria sp. [FJ357623; FJ237398; EU073079]	99.0	
		Uncultured bacterium isolate [AM697331; AM696378] from a bacterial community in indoor environment	99.0	
		Uncultured bacterium clones [FM873202; FM873503; FM872761; FM873229] occupant as a source of house dust bacteria	99.0	
K27	[589]	*Stenotrophomonas maltophilia* [FJ405363; AY837728; AY841799; EU034540; EU294137]	99.0	FN394544
K29	[563]	*Clostridium colinum* type strain DSM 6011T [X76748]	94.0	FN394545
K31	[571]	Uncultured bacterium clones [EU466486;EU778711] gut microbes	99.0	FN394546
		Bacillus sp. [AB112729; EU249555; AJ717382; EF422411]	97.0	

Table 4 Phylogenetic identification of fungal sequences derived from fungal clones libraries performed with paper samples obtained from the book written by Parente Stupio between 1293 and 1294

Phylum	Order	Clone	Length (bp)	Phylogenetic identification	Similarity (%)	Accession number
Ascomycota	*Capnodiales*	F25	203	*Cladosporium* sp. [DQ780355; DQ780357; DQ092512; DQ299303]	98	FN394518
	Eurotiales	F3	250	*Aspergillus penicillioides* strain ATCC 34946 [AY373861]	98	FN394514
		F37	205	Aspergillus sp. [FJ196620; EU862194; DQ865103]	99	FN394520
		K5F	603	uncultured *Aspergillus* [FM165464] detected in a 16th-century book	96	FN394522
				Aspergillus versicolor [EF652480]	96	
		nF2	598	uncultured *Aspergillus* [FM165464] detected in a 16th-century book	99	FN394526
				Aspergillus versicolor [EF652480]	99	
		nF7	622	uncultured *Aspergillus* [FM165464] detected in a 16th-century book	99	FN394528
				Aspergillus versicolor [EF652480]	99	
		nF13	603	uncultured *Aspergillus* [FM165464] detected in a 16th-century book	99	FN394530
				Aspergillus versicolor [EF652480]	99	
		nF19	602	uncultured *Aspergillus* [FM165464] detected in a 16th-century book	99	FN394533
				Aspergillus versicolor [EF652480]	99	
		nF24	613	uncultured *Aspergillus* [FM165464] detected in a 16th-century book	95	FN394536
				Aspergillus versicolor [EF652480]	95	
		nF28	523	*Aspergillus terreus* [FJ011536; EU515150; AY360402]	98	FN394537
		K31F	627	*Eurotium halophilicum* isolate NRRL 2739 [EF652088]	95	FN394525
		nF6	711	*Eurotium halophilicum* isolate NRRL 2739 [EF652088]	99	FN394527
		nF14	692	*Eurotium halophilicum* isolate NRRL 2739 [EF652088]	99	FN394531
		nF21	701	*Eurotium halophilicum* isolate NRRL 2739 [EF652088]	99	FN394535
		nF12	604	*Penicillium* sp. OY12007 [FJ571473]	100	FN394529
		nF18	626	*Penicillium chrysogenum* [EF200090; AY373902; AF033465]	99	FN394532
		K23F	645	Uncultured *Penicillium* clone 1F33 [FM165470] detected in a 16th-century book	98	FN394524
				Penicillium pinophilum [AB369480; AF176660; AB194281]	98	
	Dothideales	nF20	634	Uncultured *Penicillium* clone 1F33 [FM165470] detected in a 16th-century book	99	FN394534
				Penicillium pinophilum [AB369480; AF176660; AB194281]	99	
		F8	227	*Aureobasidium pullulans* [FJ515165]	100	FN394516
	Pleosporales	F42	209	*Alternaria* sp. [FJ467349; FJ545250; FJ618522]	99	FN394521
Basidiomycota	*Wallemiales*	F12	160	*Wallemia sebi* strain UAMH 7897 [AY625073; AY302517]	100	FN394517
	Erythrobasidiales	F28	192	*Rhodotorula aurantiaca* [AB093528]	100	FN394519
	Non-clasified	K14F	562	Fungal sp. [AY843071] melanized fungi from rock formations in the central mountain system of Spain	91	FN394523
		F4	210	Uncultured basidiomycete isolate dfmo0688_100 [AY969394]	99	FN394515

Figure 7 DGGE profile derived from the fungal community colonizing paper material

Acinetobacter sp. and *Kocuria* sp. are osmophilic bacteria described as food spoilers [19, 20]. These species has also been detected as part of the microflora of the gut of termites and other invertebrates, and they are also involved in the degradation of polymeric material, as cellulose and hemicellulose, under oxygen limitation [22]. It can be hypothesized that these bacterial strains colonized cellulose fibers due to an occasional wetting event that raised water content in the manuscript to high values and that favored bacterial dispersal and growth.

S. maltophilia represents a rhizosphere bacterial species which has a potential agronomic importance due to its capability as biocontrol for plant diseases. Traits of *S. maltophilia* associated with biocontrol mechanisms include antibiotics production [18] and extracellular enzyme activities such as protease and chitinase [21, 56]. Many chitinolytic bacteria have found to produce more than one kind of chitinase. The efficient chitin degradation is assumed to be performed by the combination of these multiple chitinases. Synergistic effects on degradation of chitin or cellulose have been observed in the simultaneous action of different types of hydrolases [3, 16]. *S. maltophilia* could be described as a secondary colonizer of the manuscript that colonized fungal material actively growing on paper.

Fungi

Fungal cell walls are composed of chitin [12] which is a polymer containing Nitrogen. A high carbon-to-nitrogen ratio in a substrate represents a limiting factor for microbial colonization [53]. Following a fungal colonization, cellulosic material becomes more palatable for many microorganisms since it becomes enriched in nitrogen. The succession of biological "events" that could have occurred to the object is somehow recorded in the microbial and fungal dead or living material present in it. Among the fungal species that were found in the manuscript, some could be considered strongly cellulolytic and, therefore, capable of colonizing pure cellulose. It is the case of *A. terreus*, *A. pullulans*, and *P. pinophilum* which have all been already associated to the biodegradation of library and archival materials [39, 57, 58].

A. terreus is a fungal species used in biodegradation of lignocellulosic waste, thanks to its abilities in degrading both cellulose and phenolic compounds [13].

A. pullulans is a "black yeast" that grows on tree leaves and in salt water marshes [9]. The fungus contains multiple life forms (polymorphic) including blastospores, hyphae, chlamydospores, and swollen cells. The chlamydospores and swollen cells are considered resting forms. The fungus produces a green melanin which turns black over time.

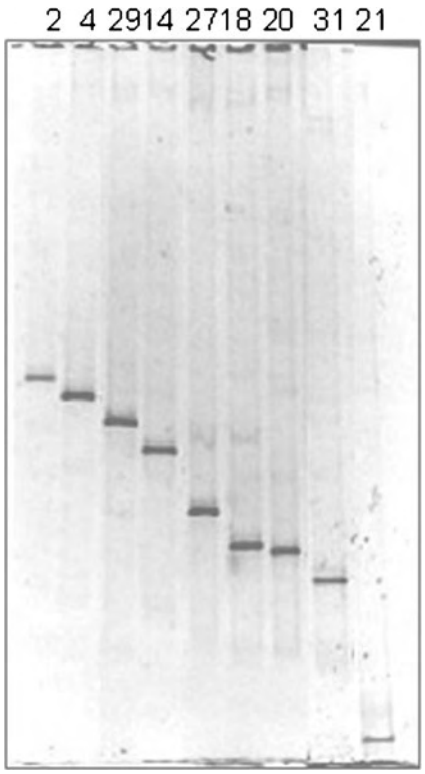

Figure 8 Example of the profiles derived from bacterial clones screened on DGGE

P. pinophilum is considered an efficient producer of cellulases and hemicellulases [4].

The colonization of the manuscript by *A. versicolor* is probably only secondary to the growth of strong cellulolytic species, or alternatively, its growth was supported mainly by starch and gelatine used in papermaking. *A. versicolor* has been isolated from both paper and parchment affected by discoloration and structural damage [14, 24, 58] and as a species exhibits a high amlylolytic and gelatinolytic activity, but it is only moderately cellulolytic [17]. It is a species that can deteriorate also polymeric materials [27], grow on building materials [34], and cinematographic films [1] indicating that it has a great plasticity and physiological versatility. *A. versicolor* is generally xerophilic, meaning that it can grow at low water activity (<0.80). The minimum and maximum growth temperatures for *A. versicolor* are 4°C and 40°C with an optimum at 30°C. Its optimal water activity is 0.95 with a minimum of 0.75 [42]. The manuscript presented some portions that were effectively reduced to shreds, suggesting that paper microbial degradation occurred with detriment of the glues and the sizing materials and not of mainly cellulose fibers, thus resulting in a loss of structure but not of substance. The high number of clones obtained for *A. versicolor* and the enzymatic abilities of this species for the materials constituting the manuscript suggests that it had a role in the biodeterioration of some parts of the book, although the fungal material recovered from the cellulose fibers was no longer viable or culturable.

E. halophilicum is a rare species, strictly xerophilic (tonophilic), but its occurrence in the environment and on materials in dry or salted habitats may be underestimated because it commonly fails to grow on agar media. *E. halophilicum* has been associated to *Aspergillus penicilloides* by Samson and Lustgraaf [43] as inhabitants of house dust [48]. *A. penicilloides* and *W. sebi* are tonophilic fungi that can grow on substrates with very low water activity [42]. These xerophilic species can be found as biodegrading agents of salted substrata, like salted meat or vegetables [42].

The volume was utterly transformed in appearance also as a result of a soaking event, as documented by some discolorations on the margins of paper sheets. The manuscript was used by a notary to record commodities real estate and land transactions in a sea town and was probably exposed to sea air and breeze, which correlates with the high contents of NaCl and MgCl that were shown by EDS elemental analysis (Tables 1 and 2). In fact Na, Cl, and Mg were not associated to inked areas and could not be considered paper or sizing constitutive elements [40].

The presence of 3% to 4% in weight of marine salt among paper fibers (Table 1) is consistent with the presence of a considerable number of osmophilic or tonophilic fungal species. Most of the fungal clones showed, in fact, similarity with species typical for dry or salted environments, suggesting that the manuscript is characterized by a microflora with a natural selection for osmophilic or xerophilic fungal species.

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Chapter VI

Monitoring of the effects of different conservation treatments on paper infecting fungi

To be submitted.

Monitoring of the effects of different conservation treatments on paper infecting fungi

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Abstract

Fungi are amongst the most degradative organisms that induce the biodeterioration of paper-based items of cultural heritage, and therefore effort is put into conservation and restoration treatments targeting them. Appropriate measures to deal with fungal infections include mechanical, chemical and biological methods, which also have to consider possible effects on the paper material itself and health hazards for humans. Three different conservation treatments, namely freeze-drying, gamma rays and ethylene oxide fumigation, were compared in their short and long term effectiveness to inhibit fungal growth on different paper samples. After inoculation with common paper spoilers *Chaetomium globosum*, *Trichoderma viride* and *Cladosporium cladosporioides* as single fungi or in a mixture of all, paper samples were treated and screened with DGGE for the recovery of fungal DNA. Freeze-drying displayed no effect on DNA recovery either short or long term when compared to non treated samples, whereas the application of gamma rays showed slight reduction of DNA recovery

and DNA fragmentation shortly after treatment but no difference could be singled out as long term effect. Additionally, RNA was used as an indicator for long-term fungal viability and minor differences in the mixed inocula were recorded for freeze-drying or gamma rays treated samples but overall RNA recovery for the single strains was good, contrary to most studies that proved gamma rays effective in fungal disinfection. Only treatment with ethylene oxide proved negative for both DNA and RNA recovery suggesting a possible bias in subsequent DNA-based methods that are used to analyse former biological infestations on EtO treated samples, as a molecular community analysis would render negative results in any case.

Introduction

A major part of our cultural heritage has been recorded in various forms on paper for centuries and is therefore vulnerable to biodeterioration of its organic components through fungi, imposing both structural and aesthetical damage (Nol *et al*., 2001; Corte *et al*., 2003; Cappitelli and Sorlini, 2005; Rakotonirainy *et al*., 2007; Zotti *et al.*, 2008; Mesquita *et al*., 2009). Fungal contamination is considered a major concern for libraries or archives full of paper-based books and documents. For the storage and maintenance of this often valuable material, not only the suppression and supervision of active fungal growth as hyphae, mycelium or mould is crucial, but the removal or reduction of reproductive bodies. The structurally resistant nature of spores and sclerotia allows the fungi to survive severe conditions and they are consequently harder to inactivate than the vegetative hyphae in their role as progenitors of future growth.

In unfavourable conditions, resting spores have low water content and their metabolism is inactivated but reversible (Florian, 1993; Deacon, 2005). Hence, any treatment to conserve objects of cultural value in order to perform their relevant aim should be directed towards the spores, as the vegetative hyphae are relatively easy to control by physical removal and through monitoring of the storage climate like temperature, relative humidity and activity of water (Nitterus, 2000). Fungal contamination is considered an increasing and dominant problem for archives and museums and thus the prevention of fungal growth and the

development of appropriate treatment measures for contaminated objects is a challenge for restorers, curators and scientist involved (Sterflinger, 2010).

A broad spectrum of chemical and non-chemical components has been utilised to sanitise microfungi attacking paper-made objects in an attempt to inhibit degradation (Magaudda, 2004). For our study we chose three different treatments for the paper samples to compare their effectiveness short and long term, namely gamma irradiation, freeze-drying and ethylene oxide fumigation.

The application of gamma rays in conservation science dates back to the 1960s when the radio-resistance of the most significant mould fungi from goods of cultural value was tested (Belyakova, 1960). Its high-energy electromagnetic radiation is deeply penetrating and biocidal through the denaturation and cleavage of nucleic acids, which leads to a simultaneous and indiscriminate devitalisation of all living organisms (Magaudda, 2004). As a secondary effect, free radicals and peroxides that originated from radiolysis of cellular water and a formation of active oxygen species can also cause DNA breakages (McNamara *et al*., 2003).

Gamma irradiation is successfully used to sterilize laboratory and hospital utensils and food, but can have unwanted side effects when applied in paper conservation, where high irradiation doses are often required in repeated doses, which can result in cumulative depolymerisation of cellulose in the paper. Severe ageing characteristics as lowered folding endurance and tear resistance, increased yellowing and general embrittlement were reported from paper treated with gamma rays (Butterfield, 1987; Adamo *et al*., 1998), whereas more recent studies suggested that the damage in mechanical-physical properties was not significant (Adamo *et al*, 2001; Gonzalez *et al*., 2002). The effect of gamma rays on fungi from paper confirmed that radiation treatment of books and documents was extremely effective as there was no fungal growth detectable (Jörg *et al*., 1992; Da Silva *et al*., 2006).

Ethylene oxide (EtO) has been widely used for sterilisation of objects of cultural heritage since 1933 (Ballard and Baer, 1986). EtO does not require activation energy, is used at room temperature and expresses high reactivity and diffusivity, all of which are of major importance for the inactivation of microorganisms (Mendes *et al*., 2007). By adding alkyl groups to DNA, RNA or proteins, EtO prevents normal cellular metabolism and the ability to reproduce

(Rutala and Weber, 1999). By mischance, this ability to act as an alkylating agent is widely accepted to indicate a carcinogenic potential, which has subsequently been proven for EtO and some of its residuals (Bolt, 1996; Angerer *et al*., 1998). Therefore, EtO has consequently been banned for paper conservation practise in many countries. Additionally, a study found paper material fumigated with EtO to be more susceptible to microbial attack after fumigation (Valentin, 1986). This phenomenon is not fully understood but it is claimed that ethylene glycol, formed as a by-product during fumigation, activates spores that contaminate the object in further storage (Florian, 1993).

Another strategy in paper conservation is freeze-drying, a method in which the water is frozen and then removed by sublimation, i.e. from the solid water to vapour bypassing the liquid phase. Sublimation allows the water to be removed without the effects of water evaporative forces, which can cause dimensional changes. Besides this, freeze-drying with dehydration can kill hydrated conidia and germinating conidia and it stops the growth of fungal mycelia and bacterial cells (Sussmann *et al*., 1966; Mazur *et al*., 1968; Florian *et al*., 2002). Yet if the moisture content in the thawed materials remains high, resting dry conidia may be activated. Freezing can also increase the porosity and thickness of organic materials and make them more hygroscopic. Although it cannot be considered a disinfecting treatment, freeze-drying is still the most effective method known for the physical, chemical and biological stabilization of water-damaged archival and library materials, especially when large quantities are involved and time is of the essence (Schmidt, 1985; McCleary, 1987; Parker, 1989; Florian, 1990; 2002).

In our study, effects of the three conservation treatments on fungal spores and mycelium viability and activity were compared. Two paper grades were used for the in vitro inoculation of spores from different fungal species to obtain infected paper samples. The physiological state of the fungal strains was evaluated by culturing at different time points after the treatments to determine time-dependant effects of treatments on fungal activity. These effects were also examined on a molecular level by generating DNA-DGGE profiles. As only metabolically active cells produce RNA, we used the ability to retrieve fungal specific rRNA directly from the paper samples as an indicator for the viability of

the respective fungi after the treated samples had been left for a year for eventual regrowth. The strategy is commonly used in bacterial ecology and is based on the fact that metabolically active species transcribe more rRNA for ribosome synthesis than inactive species (Prosser, 2002). As RNA is also highly unstable in the environment, the detection of RNA in an environmental sample has been used as strategy for detecting active microorganisms within a natural community (Gonzalez *et al*., 2006). To our knowledge this is the first study combining molecular methods including RNA detection of fungi with monitoring of conservation treatments for paper made objects.

Material and methods

Sample preparation and culturing

Fungal attack was induced in vitro by inoculating two types of paper with three fungal strains previously used in our group *Chaetomium globosum* Kunze, *Cladosporium cladosporioides* (Fres.) de Vries and *Trichoderma viride* Pers. (Michaelsen *et al*, 2006). Paper A was a Whatman paper type (Whatman 1 CHR category No. 3001917, not glued and not sized, 100% cotton linter, pH 6.5-7.0) consisting of pure cellulose with low ashes content, and paper D was a Mezzofino type paper with a high lignin content (naturally aged rag paper "Mezzofino", produced by the Istituto Poligrafico dello Stato in 1976 (Gallo *et al*.; 1999), No. 200953, bleached cellulose (45%), wood pulp treated with sulphites (25%), wood pulp from softwood (20%), glue (3%), Aluminium sulphate (5%), Kaolin (2%), pH 4.5).

Fungal strains were grown on Malt Extract Agar (2 %) to obtain colonies with mature spores. Spores were harvested with a sterile cotton swab and diluted in water with Tween 80 (0.01 %, Sigma, Italia) to obtain solutions with a standard concentration of about 5000 spores/ml. A further dilution was performed with a Czapek broth, to obtain 50 spores/µl. The water vapour sterilised paper samples were inoculated with each 50µl of broth to provide a physiological starter for germination. Each inoculum was applied to the sample in a single spot. About 100 % relative humidity (RH) value was maintained with distilled water during fungal growth in double-bottom glass containers. Samples were kept in a

thermostatic cell at 27 °C for 7 days (*Cladosporium* and *Trichoderma*), and 14 days (*Chaetomium* and mixed inocula) respectively.

The treated samples and the untreated control samples were tested for culturability of the fungal spores by means of the 25 points inoculum method: a set of three each paper sample was washed three times in sterile water and divided into 25 sub-samples that were inoculated directly on solid nutritive agar (Malt Extract Agar, 20 g/l) distributed according to a geometric scheme (Figure 1). The frame consists of a grid with 25 nodes spaced out equally. The ratio of development of the fungal mycelium in the nodes allows for a statistical comparison between different treatments and not treated samples (Dix and Webster, 1995). The inoculums on agar were performed directly after the treatments and 1 year later. Table 1 gives an overview of the samples and a description of visible fungal colonisation before conservation treatments.

Conservation treatments of paper samples

To avoid contamination from airborne fungi after the treatment, all samples were placed into sterile paper envelopes before being treated and left therein until further analysis. Gamma irradiation and EtO fumigation are hazardous and consequently conducted by specialists and approved companies.

Gamma rays

Irradiation treatment was performed at the Research Centre of ENEA Casaccia, Rome, Italy, by Dr Marianna Adamo. Inoculated paper samples underwent a gamma ray dose of 4756 Gy/h with an exposition time of 1 h and 3 min.

Ethylene Oxide

Fumigation treatment was performed by Spix Italia s.r.l. in a vacuum cell device with a mixture of Ethylene Oxide (10%) and CO_2 (90%) for 48 hours at a temperature between 20 and 22°C. After gas abatement, 20 washing cycles with air were performed. The treatment was verified with chemical and biological standard indicators.

Freeze-drying

The envelopes containing samples (a single envelope for each paper grade and each fungal inoculum) were positioned inside blocks of copying paper in

order to simulate the treatment of a thicker volume and to avoid a direct heating of the samples on the warming grid of the freeze-dryer. A set of 5 paper leaves was interposed between each envelope and 2 cm layer of paper leaves were used to isolate the envelopes from the heating plate. The treatment was conducted at the Centro di Legatoria e Restauro Frati e Livi s.r.l. (Castelmaggiore, Bologna, Italy) in a cycle of 5 hours with warming temperatures of 50 °C maximum.

Control samples

Sets of inoculated paper samples that developed fungal mycelium were air dried in sterile polystyrene ventilated Petri dishes at about 21 °C for one week. Following natural dehydration the samples were sterilely introduced into paper envelopes and kept for one year at an average air temperature of 22 °C and 50 % RH) to serve as untreated controls.

DNA extraction from paper material

Each paper sample existed as a set of small discs (1 cm diameter) in a paper envelope under sterile conditions. DNA was extracted directly from paper by using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, USA). The protocol of the manufacturer was slightly modified as described by Michaelsen *et al*. (2006). To ensure the lysis of all cells and spores on the paper surface, the samples were pretreated with lysozyme and proteinase K as described by Schabereiter-Gurtner *et al*. (2001) before the application of the Kit. DNA crude extracts were used directly for PCR amplification analysis. DNA extraction was performed one month after treatment and again one year later to test for effects of the different treatments on the DNA of the inoculated fungi.

DNA modifications

All PCR reactions were executed using PCR Master Mix (Promega, Australia). Fragments of about 500-700 bp size corresponding to the ITS1-ITS2 region of the ribosomal-DNA internal transcribed spacer region were amplified with the primer pair ITS1 and ITS4 (White *et al*., 1990). For DGGE analysis of the DNA fragments, a nested PCR was performed with the PCR product of the first round as template DNA using the primers ITS1GC with a 37-base GC-clamp attached to the 5`-end and ITS2 (Muyzer *et al*., 1993, White *et al*., 1990). All

reactions were carried out as described in Michaelsen *et al*. (2006) and PCR products were visualised by electrophoresis in a 2 % (w/v) agarose gel. Denaturing gradient gel electrophoresis (DGGE) was performed as previously described by Muyzer *et al*. (1993) in 0.5 x TAE (20 mM Tris, 10 mM acetate, 0.5 mM Na_2EDTA; pH 7.8) with 8 % (w/v) acrylamide gels containing a gradient of 30-50 % denaturants in a DGGE 2401 system (C.B.S. Scientific Co., USA). Gels were run at 60 ºC and 75 V for 14 h, stained with Ethidium bromide and visualised using a Bio-Rad Gel Doc imaging system (Bio-Rad Laboratories, Australia).

RNA extraction

RNA was extracted from the paper samples using a combination of the method developed by Griffiths *et al*. (2000) and the RNeasy Plant Mini Kit (Qiagen, Australia) with some changes to the manufacturers protocol. One disc per treatment was placed in a Lysing Matrix E tube (MP Biomedicals, Solon, USA) and 600µl of buffer RLC from the RNeasy Plant Mini Kit was added. The tubes were processed on the FastPrep beadbeater for 30 sec at 5.5, followed by a centrifugation for 2 min at 13.000 rpm. The supernatant was then used with the RNeasy kit following the protocol for fungi and plants from step 4 on according to the manufacturers instruction. An on-column DNAase treatment was performed using the Qiagen RNase-free DNase set (Qiagen, Australia). The final elution step was carried out two times with the provided water and stored at -80 ºC. RNA extraction was performed one year after the treatment of samples to allow conclusions on the viability and actual growth of the inoculated and treated strains to be compared with the DNA results.

RNA amplification

RT-PCR was carried out using 2 µl RNA template for the initial denaturing step with 1µl of reverse primer 18Sr (5'-GATCCTTCTGCAGGTTCACCTAC-3') for 5 min at 65 ºC. For DNA synthesis, 1 µl of AMV Reverse Transcriptase (Promega, Australia), 0.5 µl dNTPs (100 mM) and 2.5 µl buffer Omniscript were used in10 µl each reaction volume with molecular grade water (all Qiagen, Australia) for 60 min incubation at 37 ºC.

The cDNA was used to amplify approximately 250 bp products of 18S rRNA with primers EK555sr (5'-GCTGCTGGCACCAGACT-3') and 18S-300f GC which includes a 39-bp GC clamp on the 5'- end to be used in DGGE analysis (Moreno *et al.*, 2010). The PCR reaction contained 2 µl of cDNA with 2.5 µl AmpliTaqTM 10 x buffer, 1.5 µl $MgCl_2$ solution, 0.1 µl AmpliTaqTM DNA polymerase (all Applied Biosystems), 10 pmol of each primer, 0.5 µl dNTPs (10 mM) and 1.25 µl DMSO in a 25 µl volume. The PCR conditions used included an initial denaturation step at 95 ºC for 2 minutes, followed by 30 cycles of 95 ºC for 30 s, 61 ºC for 30 s and 72 ºC for 30 s, and a final extension cycle of 10 min at 72 ºC. The products were purified using the QIAGEN PCR purification kit. The GC-clamped PCR products were separated using DGGE on 10 % (wt/vol) polyacrylamide gels with a 30 to 50 % urea/formamide denaturing gradient using the DGGE 2401 system (C.B.S. Scientific Co., USA). Gels were run at 60 ºC and 55 V for 17 h, stained with Ethidium bromide and visualised using a Bio-Rad Gel Doc imaging system (Bio-Rad Laboratories, Australia).

Bands representing the expected fungal fragments were cut out from the gel and put into sterile water over night prior to amplification with 18SGC primers as above. After verifying the band in a subsequent DGGE (conditions as above), the PCR product was purified with the QIAquick PCR purification kit (Qiagen). Sequencing of DNA fragments was done using the BigDye Terminator Cycle Sequencing Kit v3.1 and the ABI3130x1 Sequencer (Applied Biosystems Group, Foster City, CA, USA) following the manufacturers protocol. For a taxonomic affiliation, the obtained sequences were compared against existing online databases using the search engines BLAST (Basic Local Alignment Search Tool) (Altschul *et al.*, 1990).

Results and discussion

Culturing of fungal spores from treated and untreated control samples

The inoculation of paper samples with fungal spores for both culturing and molecular analysis was followed by an incubation period of the paper in a thermostatic cell at 27 °C for 7 days (*Cladosporium* and *Trichoderma*), and 14 days (*Chaetomium* and mixed cultures) respectively. One part of the samples was used as control samples and was not subjected to any treatment, and the other

samples were treated with freeze-drying, gamma rays and EtO. Culturability of fungal spores was tested one month after treatment for all samples (T1) and one year later (T2) for the untreated control samples with the 25 points inoculum method.

The development of fungal mycelium in the 25 spot grid enabled statistical comparisons between the treatments and the control samples and amongst the control samples as presented in Figure 1 and 2 according to the analysis of variance (ANOVA) method and Fisher test. Non-treated control samples show good growth for all fungi with no statistical difference, but different inoculation results were obtained from paper samples subjected to freeze-drying dependent on the species. *Cladosporium cladosporioides* spores were no longer viable, and the recorded growth for *Trichoderma viride* was low and statistically different from both the control sample and *Chaetomium* (Fig. 2). No statistic difference was found for *Chaetomium* and mixed inocula samples in respect to the control samples. Both gamma irradiation and EtO fumigation did suppress fungal growth for all single species and mixed inocula in short term and showed a full efficacy as disinfecting agents when evaluated with culturing attempts.

Differences in the general long-term growth of the fungal strains without treatment (T2) were found for *Trichoderma* and *Cladosporium*, both of which were not able to grow after one year at RT. *Chaetomium* did not behave in a statistically different manner compared to the sample one year before.

Statistically significant differences were also noted between the two papers in a general favour of paper A, the Whatman paper, for general fungal growth after treatment. But as the same was found for the control samples of paper A, it indicates that cotton linter supported fungal growth generally better than rag paper D, the Mezzofino paper made from cellulose and wood-pulp, and the differences evaluated can be independent of the treatments.

DGGE fingerprints to compare the effect of the treatments

The internal transcribed spacer region 1 (ITS1) was amplified in PCR reactions with template DNA extracted directly from the paper samples after treatment and a non treated series as control, and separated in a DGGE gel against

an ITS generated marker containing pure fungal cultures (Michaelsen *et al*., 2006). DGGE is the technique most often used for the study of microbial communities colonising artworks to provide deeper insight into their structure (Portillo *et al*., 2008). We applied DGGE for the first time to paper samples that have been inoculated with fungal spores and subjected to conservation treatments to monitor structural changes on the DNA level due to treatment and to support predications of treatment efficiency.

Interestingly, there seem to be only minor effects on the recovery of fungal DNA after treatments with freeze-drying or gamma rays, as differences between the profiles of treated samples and non-treated controls are minor and mostly restricted to the mixed inocula samples and supposable a result of the competition between fungal strains during incubation.

DNA fingerprints derived from untreated control samples approximately one month after the treatment (T1) and one year later (T2) are nearly identical as shown in Figure 2. DNA from *Trichoderma viride* (lanes 5 and 6) was not detected in any sample when inoculated in a mixture with *Chaetomium globosum* and *Cladosporium cladosporioides* independent from the nature of treatment. However, it should be considered that *Trichoderma* was not competitive enough initially and outgrown by mycelium of the other two fungi. The amount of fungal spores and mycelium from *Trichoderma* on the samples to be inoculated could be considered beyond the detection limit of our PCR and DGGE conditions, which consequently implies proliferation and active growth of all other fungi that were detected.

Bands representing *Chaetomium* (lanes 3 and 4) in the non-treated samples are strong both in single as in mixed inoculum on both paper types at both T1 and T2, whereas *Trichoderma* only shows clear bands when inoculated as single culture at both time-points. Unexpectedly, no band could be obtained for *Cladosporium* inoculated as a single strain on paper D throughout the experiment, probably due to a mistake in the inoculation and therefore this sample group was excluded from further analysis, whilst it was detected for paper A and in the mixed cultures at both time-points.

Fingerprints derived from samples treated with freeze-drying show changes in band pattern compared to the untreated samples (see Figure 2b). One

month after treatment (T1) the fingerprints for the mixed inocula differ from the control and between paper A Whatman and paper D Mezzofino. *Cladosporium* is represented by a strong band on paper A (lane 1) but missing on D (lane 2). In the contrary, *Chaetomium* is strong on paper D but very faint on A, whereas *Trichoderma* is not detected in any mixed sample assimilable with the control. The bands representing the fungus inoculated in pure cultures do not show a difference due to treatment. Long-term effects of freeze-drying are only observed for *Chaetomium* with unspecific bands visible in the single inoculum samples (lane 3 and 4) and no bands at all when inoculated in a mixture with the other fungi (lane 1 and 2) at T2. The other fungal bands are in accordance with the control samples.

Samples treated with gamma irradiation show a pattern with numerous unspecific bands one month after treatment at T1. Bands seem to indicate the presence of more than one fungal strain, but faint bands are more likely to represent DNA artefacts as a consequence of the irradiation. Bands for individual fungi *Chaetomiun*, *Trichoderma* and *Cladosporium* are still observed but indecisive in some samples. After one year there is no more visible effect of the treatment on the DNA of pure strains compared to the untreated control samples and strong DGGE bands are observed, only the band for *Cladosporium* is weak in the mixed inocula on paper D.

In our study, only fumigation of paper with EtO caused the loss of amplifiable fungal DNA from all spore-inoculated samples tested in both single and mixed cultures. General EtO inhibition properties on PCR performance were ruled out with PCR controls with DNA extracts spiked with pure fungal DNA as template (data not shown).

RNA as indicator for metabolically active fungi

As we confirmed the presence of DNA on most samples but the ones treated with EtO, we were interested if the fungi inoculated on the samples were metabolically active. The existence of DNA alone does not lead to assumptions about viability because rRNA genes can persist in environmental DNA pools for species that are metabolically inactive and functionally less important (Ostle *et al*., 2003). Consequently, if responses of communities to environmental

perturbations are investigated, the rRNA gene approach seems problematic as rRNA genes may be detected in DNA pools for species whose growth or cellular activity has declined (Anderson and Parkin, 2007).

Instead of culturing to test for viable fungi, we attempted a molecular analysis of the RNA itself. Targeting the fungal rRNA directly from the environmental sample is meant to detect metabolically active and functionally important species as they transcribe more rRNA, a principle that is commonly exploited in bacterial ecology (Prosser, 2002). Determination of the viability of the causative agent is important in determining the active infection and in the evaluation of the efficacy of a particular treatment. Hence, to determine the viability of the fungi on the paper samples, additional RNA extractions were carried out one year after the treatment. The extracted RNA was transcribed into cDNA using 18S primers for the further use in DGGE. We successfully generated 18S rRNA profiles for DGGE for the untreated control samples and those subjected to freeze-drying and gamma irradiation as shown in Figure 3. No positive RNA extraction or PCR could be established for EtO treated samples. In all cases, PCR controls with the crude RNA extracts were performed to exclude DNA contamination in the extracts.

Surprisingly strong signals can be observed for all pure strains but *Trichoderma* on paper D in the control samples (Fig. 3a). The pattern of single bands obtained for the pure inocula indicates the presence of only one fungus on the paper respectively, a pattern that was correspondingly achieved for DNA extracts. The impact of either gamma ray or freeze-dry treatment on the viability of the fungal spores in pure inocula is minimal after one year of treatments. Only the mixed inocula used on paper D does give no RNA band at all or very faint signals (lane 2 Fig. 3b and 3c, respectively). All other samples deliver clear bands with a pattern that matches the expectations as discussed above and consistent for the different fungi.

Unfortunately, an amplification of the cDNA with ITS primers as used for the DGGE analysis of DNA failed. ITS sequences are the most popular choice for species identification of fungi in environmental DNA pools, but due to post-transcriptional processing of the main precursor rRNA molecules containing 18S,

ITS1, 5.8S, ITS2 and 28S rRNA, the ITS spacer regions are spliced out to leave the rRNA genes for ribosome synthesis (Anderson and Parkin, 2007).

There is evidence that the ITS regions can anyway be used for the detection of active fungi as performed by Anderson and Parkin (2007), but it is mainly considered impossible to detect ITS sequences in RNA pools (Hibbett, 1992). A successful amplification of ITS rRNA was performed by other groups from fungal isolate extracts and soil, and we assume there was more rRNA available in the samples itself as soils are active environments compared to paper environment that resembles starving conditions. Anderson and Parkin (2007) sequenced ITS RT-PCR derived clones and identified and confirmed all sequences as being ITS sequence of fungal origin. We observed a related phenomenon when we tried to verify the origin of the 18S rRNA derived sequences from the cut out bands. Sequences were aligned using the BLAST online tool and results are shown in Table 2. All sequences were affiliated to fungal 18S rRNA but to different species than the ones that were used for inoculation. Interestingly there is a consistent allocation of the same sequence to a resembling band, which was confirmed in repeated gels and sequence analysis. We argue that the bands indicate the fungal strains used but do not lead to proper phylogenetic identification due to the shortness of the amplified sequence and the nature of the fungal 18S for species detection in general, as the lack of taxonomic resolution between 18S rRNA genes to closely related taxa is well known for fungi and limits its usefulness for fungal species identification (Anderson and Cairney, 2004).

Effects of treatments on fungal growth and paper condition

Temperature has an effect on the growth and survival of fungi as it dictates the rate of the reactions catalysed by enzymes, but importantly, a temperature below no growth occurs should not be understood as a lethal temperature for fungi (Nitterus, 2000). Reducing the temperature to subzero temperatures as performed in freeze-drying cycles reduces viability of many fungi and can finally even exhaust dormant spores if maintained over long periods after initially activating them (Florian, 1993). As spores and hyphae in mature and active fungal colonies contain considerable amounts of water, the majority of

them is said be killed at temperatures below 0° C (Nitterus, 2000). Contrary, our results show that culturing and molecular techniques both produced positive results for fungal growth or activity on samples subjected to freeze-drying comparable with untreated samples. This explains also why freeze-drying is not considered a conservation treatment but an emergency mass rescue procedure for flooding and other water related incidents.

Different functions of the paper or its components like cellulose are affected by gamma irradiation, and its germicidal power on fungous microflora is reported to be dose dependent (Magaudda *et al*., 2000; Adamo *et al*., 2001). A dose of about 5 kGy like applied in our experiment was found to cause statistically significant variations on almost all paper properties like tensile strength, tearing resistance and folding endurance, but also reduced the fungal population down to the 'blank' samples level, even if no complete sterilisation was achieved (Adamo et al., 2001; Magaudda, 2004). Relatively low irradiation doses of 2-3 kGy delivered comparable decontaminating power without the increasingly depolymerising effects of gamma rays on cellulose.

According to our molecular results contradicting the culturing results, gamma rays seem not to be able to eliminate fungal colonisation of paper even in relatively high doses but could potentially reduce or slow down the growing process. DGGE profiles obtained from DNA extracted directly after the irradiation process display a certain amount of fragmentation and unspecific bands that could be related to the effect of gamma radiation on the tertiary or secondary structure of the DNA itself, but no long-term effect of gamma rays on DNA or RNA is obvious. Controversially, gamma rays have been reported to inactivate fungi from and related to books (Jörg *et al*., 1992; Da Silva *et al*., 2006). However, Da Silva determined the efficiency of the radiation treatment on fungal spores performing culturing attempts to define the microbial load and therefore described a higher degree of reduction than our DNA and RNA based studies. Our culturing results are in accordance to his observations, but are proven wrong when including molecular data.

Finally, the observation that EtO can significantly reduce or erase the amount of amplifiable DNA on small items has been reported before. EtO is successfully applied in forensics to remove DNA contamination on items used

both at crime scenes and in the forensic laboratory, without significantly affecting any needed downstream DNA analysis when used to sterilise equipment (Shaw *et al*., 2008). The gaseous EtO is considered a radiomimetic agent, which means it is similar to ionising radiation by virtue of its ability to induce the same biological end-points like gene mutations mainly by alkylating and reacting with nucleophile centers such as nitrogen and oxygen atoms in the DNA bases (Bolt, 1996; Rutala and Weber, 1999; Chovanec *et al*., 2001). Double-strand-breakages are induced leading to fragmentation of the DNA helix. Our results could be explained by such a fragmentation of fungal DNA through EtO fumigation with PCR not delivering any evidence of fungal DNA or RNA on all samples submitted to fumigation. Compliantly, a study on viability of fungal spores on paper after EtO treatment indicated that fungal spores became non-cultivable and non viable with EtO inhibiting spore activity (Rakotonirainy *et al*., 2003).

In accordance to Shaw and colleagues (2008) we observed that EtO fumigation was more effective than the application of gamma rays in reducing DNA from the surface of samples. The superiority of EtO as a sterilising agent compared to gamma radiation was explained by Chovanec (2001) as a combination of differences in the nature of damage caused and the distribution of target. Ionising radiation can react more local and focussed with free radicals following tracks along covalent bonds, whereas small molecules like EtO can diffuse quite freely in the cell nucleus causing damage on a broader scale. Although EtO application is banned for the use in conservation in many countries, it should still be highly recommended in certain cases where the careful restoration and conservation of valued material is assessed.

It is worth emphasising that no DNA or RNA could be recovered from paper samples fumigated with EtO, a fact that is of particular importance for studies that address a molecular analysis of old contaminations or biological materials in cultural heritage characterisation and diagnostics. EtO fumigation was used by museums and libraries in the recent past to treat insect or mould infestations, but is often not traceable or not clearly mentioned in the reports, which accompany the treated objects. Consequently, misleading diagnostics could be produced in subsequent molecular studies. This result calls for a better documentation of the conservation history of the objects of cultural value, in

order to account for treatments that can alter the future studies and analysis to avoid any impediments by past treatment based on EtO.

Conclusions

As a summary, large amounts of paper can be treated simultaneously and without subsequent chemical hazard with gamma rays, but this can only be considered a decontamination treatment, a method to remove biodeteriogenous microorganisms to a controllable or blank level. This target of reducing and holding the biodeteriogens under the danger threshold is crucial in paper conservation and can be achieved with gamma radiation, whereas freeze-drying can be applied to stop heavy mould before further treatment. Our results point at ethylene oxide fumigation as the only effective treatment for paper when the sterilisation of a fungal infected paper object is needed. Subsequent contamination is however possible, as ethylene oxide does not represent a chemical that remains as preventive biocide on materials.

Generally, any treatment that is applied to prevent the biodeterioration of paper documents or books has to be carefully assessed beforehand. The entity and typology of the biological attack and the urgency of intervention have to be considered when choosing amongst available treatment alternatives. Economic factors like cost of treatment, quantity of objects to be treated and their historical or commercial value have also an impact on the decision of conservators.

To preserve materials, minor negative effects of treatments that can incidentally degrade materials are acceptable if the alternative is the complete disintegration of the document. Especially freeze-drying, but to some extend gamma radiation also, are ideal to treat severely attacked historical paper or for disaster recovery after flooding or other catastrophic events, and to stop ongoing growth and deterioration. Both treatments can help to control microbiological outbreaks until further cleaning or sterilisation can take part. Both are also cost-effective and compatible with mass treatment, increasingly important factors as fungal biodeterioration is affecting books with growing frequency.

In our study, the discrepancy between results delivered via culturing or molecular techniques highlights the importance of molecular tools to complement

conventional conservation techniques, as they display higher sensitivity and bypass cultivation restrictions.

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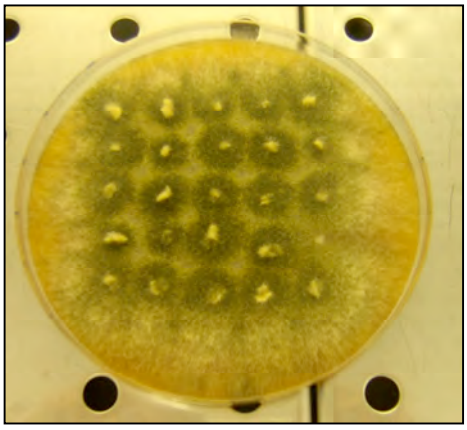

Figure 1. Example of a 25 points inoculum of *Chaetomium globosum* on paper A taken from untreated paper samples serving as control. The development of mycelium in the points allows for a statistical comparison between different treatments and untreated samples.

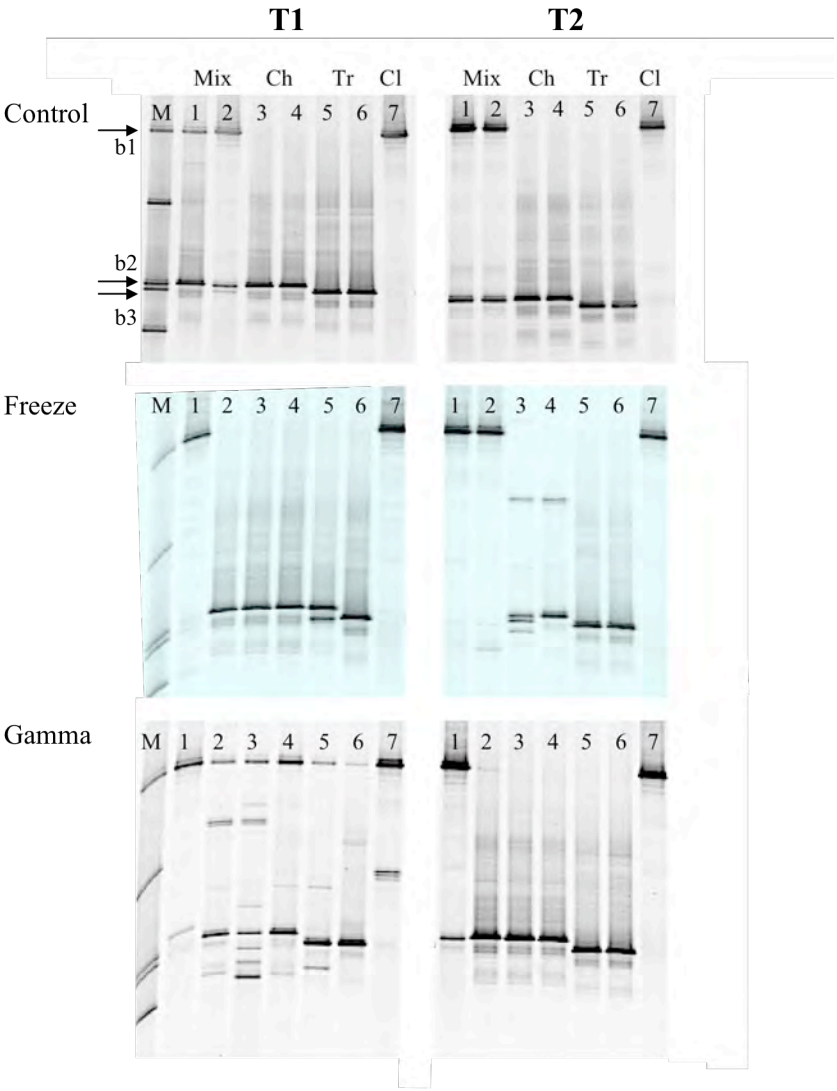

Figure 2. DGGE profile generated from ITS 1 derived DNA of untreated control, freeze-dried and gamma rays treated paper samples extracted after the individual treatment (T1) and one year later (T2). Lanes contain Marker (M), mixed inocula (Mix) on paper type A and D (lanes 1 and 2), *Chaetomium globosum* (Ch) on paper type A and D (lanes 3 and 4), *Trichoderma viride* (Tr) paper on type A and D (lanes 5 and 6) and *Cladosporium cladosporioides* (Cl) on paper type A (lanes 7). No result was obtained for *Cladosporium* for paper D as a consequence of a failed inoculation. No DNA was obtained for any sample treated with EtO. The marker contains fragments from *Cl. cladosporium* (b1), *Ch. globosum* (b2) and *T. viride* (b3).

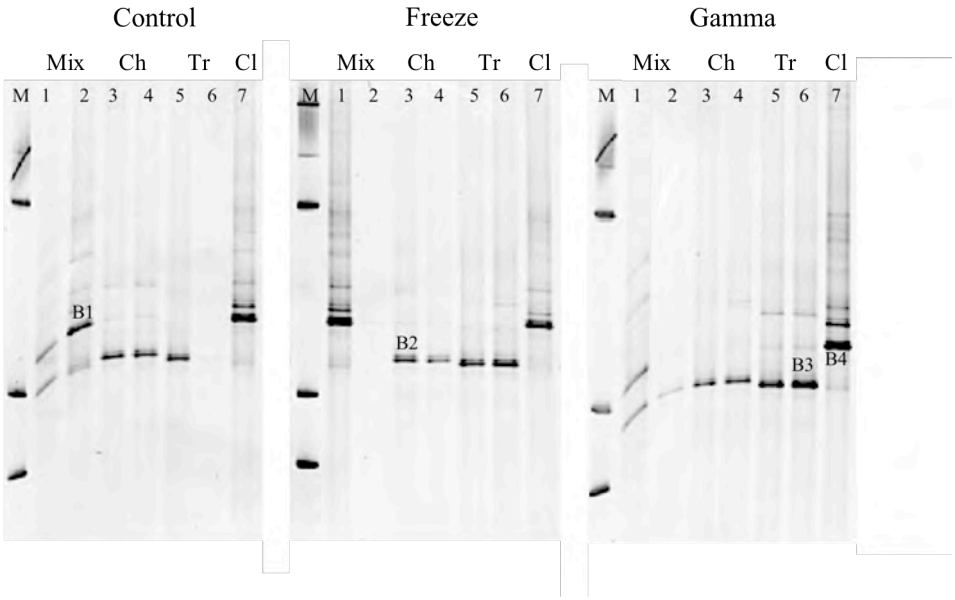

Figure 3. DGGE profile generated from 18S cDNA of untreated control, freeze-dried and gamma rays treated paper samples one year after treatment (T2. Bands B1-B4 were excised from the gel and sequenced (see Table 3). Lanes contain Marker (M), mixed inocula (Mix) on paper type A and D (lanes 1 and 2), *Chaetomium globosum* (Ch) on paper type A and D (lanes 3 and 4), *Trichoderma viride* (Tr) on paper type A and D (lanes 5 and 6) and *Cladosporium cladosporioides* (Cl) on paper type A (lanes 7).

Sample inoculated with	Paper A (Whatman)	Paper D (Mezzofino)
Control	white	darker, more yellowish
Chaetomium globosum	fungal colonies visible as dark spots; paper is grey/reddish with no stains where the colonies are; colonies around coloured spots (=area where inocula medium was applied)	almost no colonies/spots visible
Trichoderma viride	very small colonies; no stains	big colonies on border of stained area; dot in the middle where paper was inoculated
Cladosporium cladosporioides	very small colonies; slight stains	dark colonies; big colony in middle surrounded by smaller ones; reddish stain
Mixed inocula	small dark colonies all over area where medium was applied	big dark colony in the middle and small ones all over where medium was applied; paper coloured dark; ring of colonies on border of applied medium

Table 1. Paper samples and description of visible fungal colonisation before conservation treatments. The described colonisation pattern also represents untreated control appearance for T1 after treatment.

Treatment	Fungi inoculated			
	Chaetomium	*Trichoderma*	*Cladosporium*	Mixed inocula
Control (T1)	22.0 a	19.5 a	25.0 a	23.0 a
Control (T2)	16.3 a	0.0 b	0.0 b	13.3 a
Freeze-drying	18.7 a	1.2 c	0.0 b	8.5 a
Gamma rays	0.0 b	0.0 b	0.0 b	0.0 b
EtO	0.0 b	0.0 b	0.0 b	0.0 b

Table 2. Statistical analysis of the 25-inoculum growth test. The comparison between treatments is derived from average fungal growth values of both paper grades. ANOVA and Fisher test (95 % for $p < 0.05$) were used to group treatments and growth behaviour of control samples with a different letter (a, b, c) within each treatment/sample indicating a statistically significant difference.

Chapter VII

Summary/Zusammenfassung

Summary

Foxing spots appear on paper and its derivatives like books, documents, sketches, stamps and drawings, ranging between single stains to pages blotched with the typical irregularly shaped spots of small size with colours ranging from reddish to black-brown and yellowish. They are found on paper from the 16th century on, seemingly increasing on younger paper possibly linked to the poorer quality of paper through changes in composition and manufacture. A main concern for libraries and archives is both structural and aesthetical destruction of objects of cultural value through foxing. Unfortunately, the slow development of foxing spots complicates the identification of its origin, and very little is known about how foxing is initiated. Fungi, metal or cellulose oxidation are considered responsible for the formation of foxing, with fungi being regarded as the main trigger.

In the frame of this thesis we wanted to understand the initiation and forming of fungal induced foxing spots on paper. Hence, the fungal diversity present in foxing spots was assessed in detail. Following the field of general microbial ecology, molecular profiling techniques were sought in this thesis to elucidate the taxonomic and functional characteristics of fungal communities living on cultural assets beyond the gap between culturable and unculturable organisms. An introduction into the topic and background information is provided in Chapter I.

Chapter II of this thesis describes the application of molecular techniques to identify fungi on paper-made objects. When working with objects of cultural and financial value, sampling methods can be a first crucial step as a further intrusion on a sample is either not possible or has to be confined to a minimum. With the development of a DNA extraction protocol applicable for small samples and swabs we avoided extensive sampling strategies as needed for culturing and reduce the further damage on the valuable samples.

The efficiency of different DNA extraction protocols on paper samples inoculated with fungal spores was determined by their ability to retrieve PCR grade DNA of different fungi inoculated on paper and the avoidance of additional steps like purification. All fungal species subjected to the analysis were

distinguishable in a DGGE gel when amplified with primers ITS1/ITS2 targeting the internal transcribed spacer 1 region, which is described as the environmental barcoding marker for fungi. To ensure comparability of subsequent DGGE gels we designed an internal marker that consisted of ITS fragments of six fungal strains that were used for the inoculation of the paper material for the DNA extraction studies.

Chapter III of this thesis describes the implementation of the developed method to the analysis of naturally occurred foxing spots on samples of paper different in age and composition and thus representing different ecological niches. Surprisingly, DGGE fingerprints revealed a variety of fungal bands for all foxing and unaffected control samples submitted to the study, which was confirmed by sequencing of selected clones.

A total of 39 different fungal species belonging to 15 *Ascomycetes* and 6 *Basidiomycetes* genera were identified with diversity ranging between one to ten abundant species per sample. *Basidiomycetes* and yeasts were amongst the interesting candidates identified as their number and frequency reported in this thesis could be regarded too high to be coincidental. Both have hitherto been overlooked in the search for foxing related fungi, but are involved in the decay of wood and in fermentative activities with carbohydrates on frescoes and textile fibres. Their production of carotenoids as metabolites could contribute to the colour formation of foxing spots.

In contradiction to published studies, we identified fungi not only in foxing spots, but also in unaffected paper used as controls. This is an important finding, as the main focus so far was put on the spots themselves and it was postulated as actual evidence for the fungal origin of foxing that fungi were only detected in foxing spots. However, paper without visible foxing can be regarded as fungal propagules to foxing and should be the subject of further research into the early stages of foxing development. If we consider foxing to be a succession of fungal colonisation, research needs to be done to identify stages and their succession to enable early prevention. SEM and EDS analysis revealed fungal proliferation in foxing spots but in only one control sample. No significant difference in terms of quantitative presence or absence of iron or other elements could be established.

No specific type of fungal colonisation or chemical composition of the paper could be linked to foxing, and the fungal communities detected in this study were as different amongst each other as amongst different samples. This fungal diversity we identified would have gone unnoticed if molecular studies where not included in the study. This is an important fact that needs to be communicated to restorers and conservators involved.

Two case studies on biodeterioration observed on valuable paper objects were incorporated into this work. Here, a conservative approach to the restoration is dependent on a detailed description of the damage observed and its cause, but a standardisation of processes to achieve this goal is limited due to composition and value of the unique objects in cultural heritage.

The analysis and restoration of a book from the 16th century is described in Chapter IV of this thesis. The limitations of culturing were highlighted as no positive results could be achieved, whereas SEM observations could identify certain fungi but were also limited by the low abundance and incompleteness of diagnostically important structures of the fungi. Only the molecular strategy was able to supply knowledge of the fungal community in the book and confirmed fungi to be responsible for a purple staining of the paper, specifically two *Aspergillus* species.

Chapter V describes the second case study involving a manuscript from the 13th century with a rare biodeterioration of almost felted appearance that could not be linked to microbiological attacks with classical techniques. In response to observations of specific fungal and bacterial species that were identified by sequence analysis, we constructed a succession of events that could have lead to the observed biodeterioration. Mostly cellulolytic fungal species were identified that could provide palatable cellulose based material through enrichment in nitrogen as a food source for use by the identified bacteria. Glues and sizing material degradation could have provided a more varied microbial diet. Optical evidence for a soaking event of the manuscript could explain the initial ability of the detected bacterial species to settle on paper, and they are able to withstand temporary dry conditions.

The high salt content of the paper and the geographic location of the object selected for a microflora composed of osmophilic and xerophilic fungal

and bacterial species. The molecular data provided a possible deterioration record for conservators and restorators to improve treatments and future storage. Both real cases demonstrate how our innovative molecular approach for paper can be adopted in conventional studies on a case-by-case basis.

The impact and efficiency of common paper conservation treatments on diverse fungal strains was investigated in Chapter VI. Samples of two different types of paper were infected with fungal spores from *Cladosporium cladosporioides*, *Trichoderma viride* and *Chaetomium globosum* and subjected to freeze-drying, gamma irradiation and ethylene oxide fumigation. Culturing showed full immediate efficacy of both gamma rays and ethylene oxide as disinfecting agents whereas freeze-drying samples overall behaved like untreated controls.

ITS-fingerprints however represented a different picture directly after treatment, with a fragmented band pattern in DGGE for gamma ray treated samples and the complete absence of bands for EtO treated samples. Long-term effects of treatments on DNA level included negligible effects of freeze-drying on the spores of *Chaetomium*, whereas gamma ray treated paper showed a reduction of DNA fragmentation to the level of untreated control samples. Only EtO fumigation did lead to long-term loss of amplifiable DNA, implying no regrowth had occurred.

However, as rRNA genes can persist for metabolically active and inactive species in environmental DNA pools, we determined fungal viability by targeting rRNA itself. We concluded from the RNA recovery from samples treated with freeze-drying and gamma rays that both methods did not influence the long-term growth of either of the fungal species. They should only be used to control outbreaks or to keep a colonisation below a certain threshold as fungal spores can survive the conditions imposed by these methods. This result for gamma rays is in contrast to other studies that claim successful inactivation of fungi by radiation in doses comparable to our study but assessed by culturing.

No residual RNA could be recovered from EtO fumigated paper, an important finding clearly separating EtO fumigation from the other methods. Amongst the tested conservation methods, EtO proved superior in inactivating fungi on paper with long-term effects, if a subsequent colonisation can be

avoided. Unfortunately, due to its carcinogenic nature, EtO is banned for use in conservation in many countries, but in accordance to our results should be considered when complete sterilisation is needed. Molecular methods should be applied increasingly to determine appropriate treatments to avoid unnecessary exposure or damage of valuable items due to ineffective treatments.

The molecular method we developed in the frame of this thesis can help to further the conservation and preservation of paper-based objects beyond their natural lifetime. Objects of cultural value are not only conserved for display purposes or storage, they might be reanalysed or reinterpreted in the future. In imbedding our molecular strategy in real case conservation studies, we could supply restorers with possible culprits for biodeterioration phenomena observed on the paper, which was not possible with conventional techniques.

The comparison of conservation treatments on a molecular basis revealed big differences in sanitising efficiency and long-term effects as when performed by conventional methods. The results call for a closer relationship between researchers and conservators to ensure that molecular strategies evolve into future conservation practise or knowledge.

Zusammenfassung

"Foxing spots" oder Stockflecken erscheinen auf Papier oder papierhaltigen Produkten wie Büchern, Dokumenten, Skizzen, Briefmarken und Zeichnungen, als einzelne Flecken bis hin zu Seiten die mit den typischen unregelmäßigen Flecken von geringer Größe übersäht sind, deren Farbe von rötlich bis schwarz-braun oder gelblich reichen kann. Man findet sie auf Papier ab dem 16ten Jahrhundert und scheinbar vermehrt auf jüngerem Papier, was wahrscheinlich auf die minderwertigere Papierqualität zurückzuführen ist, abhängig von Änderungen in der Zusammensetzung und der Produktion des Papiers. Eine durch Stockflecken hervorgerufene strukturelle und ästhetische Zerstörung von Objekten mit kulturellem Wert ist eine der größten Sorgen für Büchereien und Archive. Leider behindert das schleichende Auftreten von Stockflecken eine Aufschlüsselung ihrer Herkunft und Ursachen, und so ist nur wenig über die Bildung von Stockflecken bekannt. Pilze, Metalle oder die Oxidation von Zellulose werden als Auslöser von Stockflecken betrachtet, wobei Pilze als Hauptverursacher gesehen werden.

Im Rahmen dieser Doktorarbeit wollten wir die Ursache und Entstehung von Stockflecken nachvollziehen, die von Pilzen hervorgerufen werden. Daher haben wir die Diversität von in Stockflecken vorhandenen Pilzen im Detail untersucht. Wie auch schon in der allgemeinen mikrobiellen Ökologie vorgemacht, haben wir molekulare Techniken eingesetzt um ein Profil der taxonomischen und funktionellen Pilzgemeinschaften von Stockflecken kulturell wertvoller Objekte zu erstellen, und die Diskrepanz zwischen kultivierbaren oder nicht kultivierbaren Organismen zu überwinden. Eine Einleitung in das Thema und Hintergrund Informationen werden im Kapitel I dargestellt.

Kapitel II dieser Doktorarbeit beschreibt die Anwendung von molekularen Techniken um Pilze auf papierhaltigen Objekten zu identifizieren. Wenn man mit Objekten von kulturellen oder finanziellen Wert arbeitet, können Methoden zur Probennahme einen ersten kritischen Schritt darstellen, da ein weiterer zerstörender Eingriff in eine Probe entweder nicht möglich ist oder auf ein Minimum beschränkt werden muss. Mit der Entwicklung eines Protokolls für eine DNA Extraktion von sehr kleinen Papierproben und Abstrichen konnten wir die

erheblichen Probennahmen die für Kultivierungen nötig sind vermeiden und damit die Beschädigung von wertvollen Objekten minimieren.

Die Effizienz von verschiedenen Protokollen zur DNA Extraktion von mit Pilzsporen beimpften Papierproben wurde hinsichtlich der Fähigkeit bestimmt, inwieweit die DNA der verschiedenen Pilze dem benötigten Reinheitsgrad für PCRs entsprach ohne zusätzliche Manipulationen wie Reinigungsschritte zu benötigen. Alle im Experiment behandelten Pilzarten konnten in einem DGGE separiert werden, wenn sie mit den Primern ITS1/ITS2 amplifiziert wurden, die auf die "internal transcribed spacer" ITS1 Region abzielen, welche auch als ökologischer Strichcode für Pilze beschrieben wird. Um eine Vergleichbarkeit von DGGE Gelen zu ermöglichen wurde ein ITS-Marker aus den in den Extraktionstudien benutzten sechs Pilzarten entwickelt.

Kapitel III dieser Arbeit beschreibt die Anwendung des entwickelten Protokolls für natürlich auftretende Stockflecken auf Papier verschiedener Zusammensetzung und Alters und folglich unterschiedlichen ökologischen Nischen. Überraschenderweise wurde eine Vielfalt von Pilzbanden für alle Proben mit und ohne Stockflecken erzeugt, was durch eine Sequenzierung ausgesuchter Klone bestätigt wurde.

Im Ganzen wurden 39 Pilzarten identifiziert die zu 15 Gattungen der *Ascomyceten* und zu 6 Gattungen der *Basidiomyceten* gehörten, mit einer Diversität zwischen einer und zehn vorhanden Arten je Probe. *Basidiomyceten* und Hefen waren unter den interessanteren Kandidaten, da ihre Anzahl und Häufigkeit im Rahmen dieser Arbeit zu hoch lag um zufällig zu sein. Beide wurden jedoch bisher in der Suche nach Pilzen die an der Entstehung von Stockflecken beteiligt sind übergangen, obwohl sie an der Holzzersetzung und Fermentierung von Kohlenhydraten in Fresken und Textilien beteiligt sind. Ihre Produktion von Karotinoiden als Stoffwechselprodukt könnte zur Farbbildung von Stockflecken beitragen.

Entgegen anderer veröffentlichter Studien haben wir Pilze nicht nur in Stockflecken identifiziert, sondern auch in nicht betroffenem Papier welches als Kontrolle betrachtet wurde. Das ist ein wichtiges Ergebnis, da bisher ein Hauptaugenmerk auf Stockflecken selbst gelegt wurde, und es als eigentlicher Beweis für die Beteiligung von Pilzen an Stockflecken gesehen wurde, dass Pilze

nur in den Flecken gefunden wurden. Papier ohne sichtbare Flecken kann allerdings als Vorläufer für pilzgenerierte Stockflecken gesehen werden und sollte daher in weiterführende Studien der frühen Entwicklung von Stockflecken einbezogen werden. Wenn wir Stockflecken als eine Abfolge von Besiedelungen durch Pilze betrachten, muss mehr Forschung betrieben werden um frühe Stadien und ihre Reihenfolge zu identifizieren damit Prävention geleistet werden kann.

SEM und EDS Analysen enthüllten eine Verbreitung von Pilzstrukturen in allen untersuchtuen Stockflecken aber nur in einer Kontrollprobe. Es konnte kein signifikanter Unterschied hinsichtlich Vorhandensein oder Abwesenheit von Eisen oder anderen Elementen hergestellt werden.

Es konnte kein Zusammenhang zwischen einer spezifischen Besiedelung mit Pilzen oder einer chemischer Zusammensetzung des Papiers und Stockflecken aufgezeigt werden, und die Pilzgemeinschaften die im Rahmen der Studie identifiziert wurden unterschieden sich in untereinander sowie in verschiedenen Proben. Die Diversität von Pilzen wäre unbemerkt geblieben wenn molekulare Methoden nicht in die Studie integriert worden wären. Das ist eine wichtige Tatsache die den beteiligten Restauratoren und Konservatoren vermittelt werden muss.

Zwei Fallstudien zum biologischen Zerfall und der Erhaltungsmaßnahmen von wertvollen Papierobjekten wurden in diese Arbeit einbezogen. Hierbei ist ein konservatives Herangehen an die Restaurierung abhängig von einer detailgetreuen Beschreibung der vorhandenen Zerstörung und ihrer Ursache, wobei eine Standardisierung der notwendigen Prozesse hierfür auf Grund der Zusammensetzung und des Wertes der einzigartigen kulturell bedeutenden Objekte beschränkt ist.

Die Analyse und Restaurierungsmaßnahmen eines Buchs des 16ten Jahrhunderts wird in Kapitel IV dieser Arbeit beschrieben. Beschränkungen der Kultivierungstechniken wurden deutlich da keine Ergebnisse erzeugt wurden, wobei mit Hilfe von SEM einige Pilze identifiziert wurden, welche jedoch auch durch den Mangel an diagnostisch notwendigen Pilzstrukturen und deren generellen Zustand eingeschränkt wurden. Nur mit Hilfe der molekularen Strategie konnten Daten über die Pilzgemeinschaft des Buches gewonnen werden

und Pilze als Verursacher der vorhandenen lila Flecken des Papiers bestimmt werden, genauer zwei *Aspergillus* Arten.

Kapitel V stellt die zweite Fallstudie eines Manuskripts aus dem 13ten Jahrhundert vor. Die einzigartige, filzartige biologische Verfallserscheinung konnte mit konventionellen Methoden nicht mit mikrobiologischem Befall in Zusammenhang gebracht werden. In Anlehnung an die Entdeckung besonderer Pilz- und Bakterienarten durch Sequenzanalysen konstruierten wir eine Abfolge von Ereignissen die zu dem vorliegenden Verfall geführt haben könnten. Vorwiegend cellulolytische Pilzarten wurden identifiziert, welche zellulosehaltiges Material durch die Anreicherung von Stickstoff in verwertbarer Form für die vorhandenen Bakterien liefern könnten. Die Zersetzung von Klebern und Füllmaterialien könnte zu dieser für Bakterien abwechslungsreicheren mikrobiologischen Diät beigetragen haben. Sichtbare Hinweise für eine Quellung des Manuskripts lassen außerdem die anfängliche Fähigkeit der gefundenen Bakterienarten sich auf dem Papier niederzulassen erklären, wobei diese auch kurzfristige Trockenheit überstehen können.

Der hohe Salzgehalt des Papiers and die geographische Ortsangabe des Objekts selektierten für eine Mikroflora aus osmophilen und xerophilen Pilzen und Bakterien. Ein denkbares Verfallsszenarios konnte anhand der molekularen Daten erstellt werden, um Konservatoren und Restauratoren bei der Verbesserung der Behandlungen und Lagerbedingungen in der Zukunft zu unterstützen. Beide Studien veranschaulichen, wie unsere für Papier innovative molekulare Herangehensweise in Einzelfällen in konventionelle Konservierungsstudien integriert werden kann.

Die Wirkung und Effizienz von gebräuchlichen Behandlungen in der Konservierung von Papier auf verschiedene Pilzstämme wurde in Kapitel VI untersucht. Proben von zwei verschiedenen Papiersorten wurden mit Pilzsporen von *Cladosporium cladosporioides*, *Trichoderma viride* und *Chaetomium globosum* inokuliert und mit Gefriertrocknung, Gammastrahlung und Ethylenoxid-Begasung behandelt. Eine komplette und unmittelbare Effizienz von Gammastrahlung und Ethylenoxid als Desinfizierungsmittel wurde mittels Kultivierung festgestellt, wohingegen gefriergetrocknete Proben sich in Ganzen wie die unbehandelten Proben verhielten.

ITS-Fingerabdrücke präsentierten jedoch ein anderes Bild im direkten Anschluss and die Behandlungen, mit einem fragmentierten Bandenmuster im DGGE Gel für die mit Gammastrahlung behandelten Proben, und einer kompletten Abwesenheit von Banden für die EtO begasten Proben. Langzeitwirkungen auf DNA-Ebene beinhalteten eine zu vernachlässigende Auswirkungen von Gefriertrocknung auf die Sporen von *Chaetomium*, wobei die Fragmentierung der DNA in dem mit Gammastrahlen behandelten Papier auf das Niveau der unbehandelten Kontrollen zurück ging. Nur eine Begasung mit EtO führte zu langfristigem Verlust von amplifizierbarer DNA, was darauf schließen lässt das kein Wachstum stattgefunden hat.

Da rRNA Gene allerdings in metabolisch aktiven sowie unaktiven Arten eines umweltbedingten DNA-Pools fortbestehen können, bestimmten wir die Viabilität der Pilze durch die rRNA selbst. Abhängig von der Ausbeute an RNA von Proben die gefriergetrocknet oder mit Gammastrahlen behandelt wurden konnte geschlossen werden dass beide Behandlungen das Langzeitwachstum von keinem der Pilze beeinflussen. Sie sollten daher nur eingesetzt werden um akute Massenvermehrungen zu kontrollieren oder um eine Kolonisation unter einem bestimmten Schwellenwert zu halten da Pilzsporen die extremen Konditionen der beiden Verfahren überleben können. Das dieses Resultat für Gammastrahlung erzielt wurde steht im Gegensatz zu anderen Studien, die von einer erfolgreichen Inaktivierung von Pilzsporen durch Gammastrahlung vergleichbarer Dosis berichten, festgestellt jedoch durch Kultivierung.

Keine Rückstände von RNA konnten auf EtO begastem Papier gefunden werden, ein wichtiges Ergebnis welches die Begasung mit EtO eindeutig von beiden anderen Verfahren abhebt. EtO erwies sich als überlegen gegenüber den anderen getesteten Behandlungsmethoden in seiner Fähigkeit Pilze langfristig zu inaktivieren, insofern eine nachfolgende Kolonisation vermieden werden kann. Leider ist EtO aufgrund seiner krebserregenden Wirkung in vielen Ländern auf der Verbotsliste für eine Anwendung in der Konservierung, sollte aber in Übereinstimmung mit unseren Ergebnissen in Betracht gezogen werden wenn eine komplette Sterilisation erforderlich ist. Molekulare Methoden sollten zunehmend herangezogen werden um angebrachte Behandlungen zu bestimmen

und eine unnötige Beanspruchung oder Zerstörung von wertvollen Gegenständen aufgrund unwirksamer Verfahren zu vermeiden.

Die im Rahmen dieser Doktorarbeit entwickelte molekulare Strategie kann dazu betragen, papierhaltige Objekte über ihre natürliche Lebenszeit hinaus zu bewahren. Objekte von kulturellem Wert werden nicht nur für Austellungszwecke oder Lagerung erhalten, sondern um eventuell neu ausgelegt oder interpretiert werden zu können. Indem wir unsere molekulare Strategie in echte Fallstudien der Papierkonservierung eingebunden haben konnten wir den Restauratoren mögliche Verursacher des beobachteten biologischem Zerfalls des Papiers nennen, was mit konventionellen Methoden hier nicht möglich war.

Ein Vergleich von Methoden der Papiererhaltung zeigte große Unterschiede zwischen einer molekularen und konventionellen Herangehensweise zur Bestimmung von Effizienz und Langzeitwirkung auf. Die Ergebnisse fordern eine engere Zusammenarbeit zwischen Wissenschaftlern und Konservatoren um sicher zu stellen, dass molekulare Strategien zukünftig in Methoden und Grundlagen der Konservierung eingebunden werden.

Appendix

Abbreviations

A	adenine
AD	anno Domini
bp	base pair
BLAST	basic local alignment search tool
C	cytosine
cDNA	complementary DNA
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DMSO	dimethyl sulfoxide
EDS/EDX	energy dispersive X-ray spectral analysis
et al.	*et alii*
EtO	ethylene oxide
Fig.	figure
FISH	fluorescence in situ hybridisation
FTIR	Fourier transform infrared spectrometry
G	guanine
ICPL	Istituto Centrale per la Patologia del Libro
ITS	internally transcribed spacer regions
LCR	ligase chain reaction
PCR	polymerase chain reaction
pH	potential of hydrogen
qPCR	quantitative polymerase chain reaction
rDNA	ribosomal deoxyribonucleic acid
RH	relative humidity
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
RT-PCR	reverse transcriptase polymerase chain reaction
SEM	scanning electron microscopy

sp.	species
s.r.l.	societa a responsabilita limita (Italy; Private limited company)
SSCP	single-strand-conformation-polymorphism
T	thymine
t-RFLP	terminal restriction fragment length polymorphism
UV	ultraviolet
w/v	weight per volume
X-ray	Roentgen radiation

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