



This article appeared in a journal published by Elsevier. The attached copy is furnished to the author for internal non-commercial research and education use, including for instruction at the authors institution and sharing with colleagues.

Other uses, including reproduction and distribution, or selling or licensing copies, or posting to personal, institutional or third party websites are prohibited.

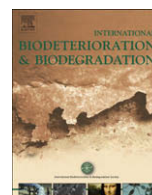
In most cases authors are permitted to post their version of the article (e.g. in Word or Tex form) to their personal website or institutional repository. Authors requiring further information regarding Elsevier's archiving and manuscript policies are encouraged to visit:

<http://www.elsevier.com/copyright>



Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

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

Astrid Michaelson^{a,1}, Guadalupe Piñar^b, Mariasanta Montanari^c, Flavia Pinzari^{c,*}^a Department of Microbial Ecology, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria^b Institute of Applied Microbiology, Department of Biotechnology, Universität für Bodenkultur, Muthgasse 18, 1190 Vienna, Austria^c ICRCPAL – Istituto Centrale per il Restauro e la Conservazione del Patrimonio Archivistico e Librario, Laboratorio di Biologia, Ministero per i Beni e le Attività Culturali, Via Milano, 76, 00184 Rome, Italy

ARTICLE INFO

Article history:

Received 3 July 2008

Received in revised form 7 August 2008

Accepted 14 August 2008

Available online 13 November 2008

Keywords:

Paper

Fungi

Biodeterioration

DNA-fingerprints

Phylogenetic identification

Cultural heritage

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.

© 2008 Elsevier Ltd. All rights reserved.

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

* Corresponding author. Tel.: +39 06 48291215; fax: +39 06 4814968.

E-mail address: flavia.pinzari@beniculturali.it (F. Pinzari).¹ Current address: University of New South Wales (UNSW), Centre for Marine Biofouling and Bio-Innovation, Sydney, Australia.

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

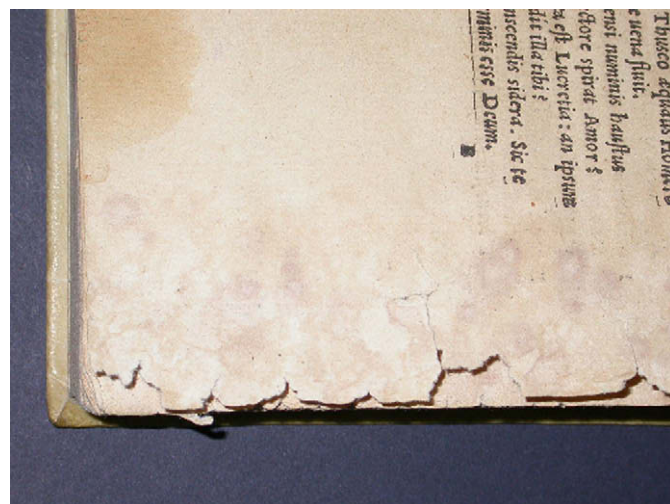


Fig. 2. A damaged page of the volume, with fungal stains. The felted consistence of paper resulted in a great fragility of the sheet's margins. Some fragments of paper (2–3 mm² width) were collected from this type of margin using watchmaker's tweezers.

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.

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 mm² width) were also collected, mainly from the margins of the most degraded pages (Fig. 2), using watchmaker's tweezers.

Removable 3MTM 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

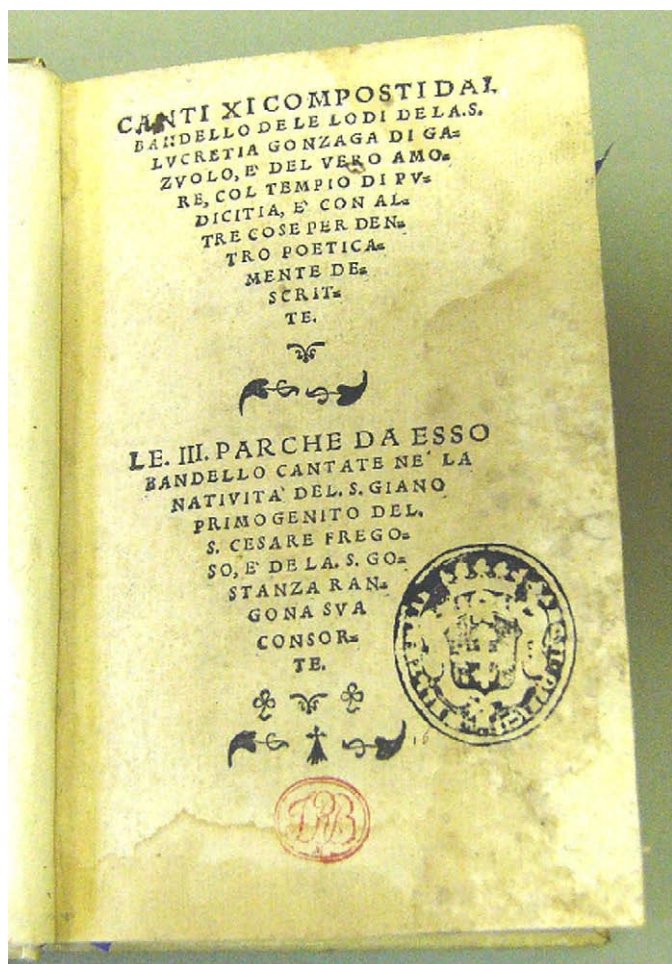


Fig. 1. The volume “Le Stanze del Bandello” by Matteo Bandello, printed at the end of the 16th century in Italy, conserved in the Biblioteca Braidense in Milan (Italy), and sent to the Istituto Centrale per il Restauro e la Conservazione del Patrimonio Archivistico e librario, in Rome, for restoration 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. Lucretia 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].

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 H₂O and separated by DGGE. Gel electrophoresis was performed as previously described by Muyzer et al. (1993) in $0.5 \times$ TAE (20 mM Tris, 10 mM acetate, 0.5 mM Na₂EDTA; 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 ddH₂O 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 (iso-propyl-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, $5\text{--}6 \times 4\text{--}4.5\text{ }\mu\text{m}$ in diameter, and smooth walled; conidia were ovate to subglobose, echinulate, and $2.5\text{--}5\text{ }\mu\text{m}$ in diameter; asci were globose and $10\text{--}12\text{ }\mu\text{m}$ 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.

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.

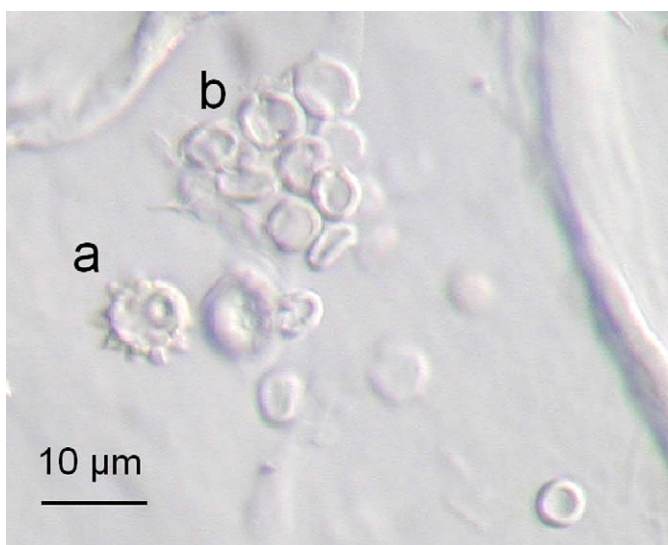


Fig. 4. Light microscopic examination of slides mounted with adhesive tape samples: (a) lens-shaped ascospore with denticular crest; (b) fungal structures resembling Hülle cells. (Olympus AX60 microscope accessorised with phase contrast.)

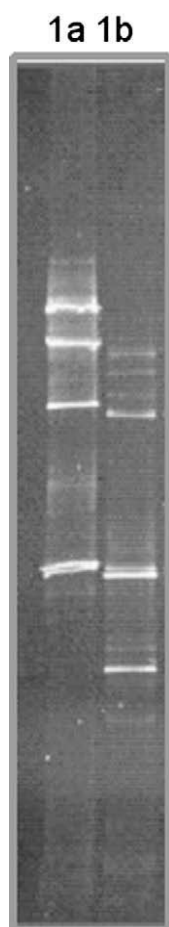


Fig. 6. ITS1-derived DGGE profiles obtained from two pieces of paper obtained from a copy of the book “Le Stanze del Bandello” by Matteo Bandello. For phylogenetic identification of members of the fungal community, a clone library was performed from sample 1a.

3.4. Molecular analysis

For molecular analyses, two fragments of paper, 1a and 1b of approximately 2–3 mm² 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 1F2 were also affiliated to *Aspergillus nidulans* (Eidam) G. Winter, the anamorph of *Emmericella 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	<i>Cladosporium cladosporioides</i> [EU497957]	100	FM165468
		1F50	510	<i>Cladosporium</i> sp. [EU497957; EF029809]	100	FM165472
	Eurotiales	1F1	538	<i>Penicillium pinophilum</i> [AB369480; AF176660]	98	FM165463
		1F33	537	<i>Penicillium pinophilum</i> [AB369480; AF176660]	100	FM165470
		1F2	529	<i>Aspergillus versicolor</i> [AY373883; AM883155; EU586040; EF125026; AJ137751]	99	FM165464
				<i>Aspergillus nidulans</i> [AF455505]	99	
		1F29	528	<i>Aspergillus versicolor</i> [AY373883; EU586040; AM883155]	99	FM165469
	Helotiales	1F39	499	<i>Botryotinia fuckeliana</i> [EF207415]	100	FM165471
	Pleosporales	1F16	505	<i>Epicoccum nigrum</i> [A EU232716; EF432273; AF455455]	100	FM165466
	Saccharomycetales	1F5	596	<i>Debaryomyces hansenii</i> [EF190234; EF197943]	99	FM165465
	Mucorales					
		1F17	582	<i>Rhizopus oryzae</i> [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 amylolytic 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). Epicoconone 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 phenotypic 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

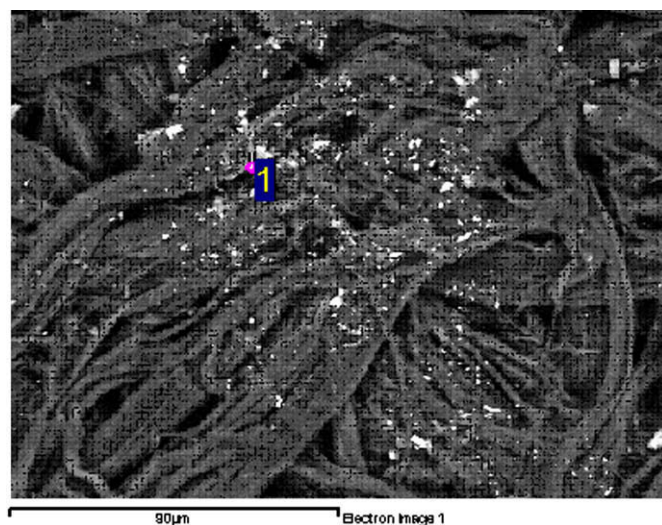


Fig. 8. Elemental dispersive spectroscopy (EDS) allowed a characterisation of impurities on paper. The presence of Hg (Table 1) in the form of a scattered powder observed on some samples is a result of the conservator's activity for protecting the paper against deterioration by micro-organisms.

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

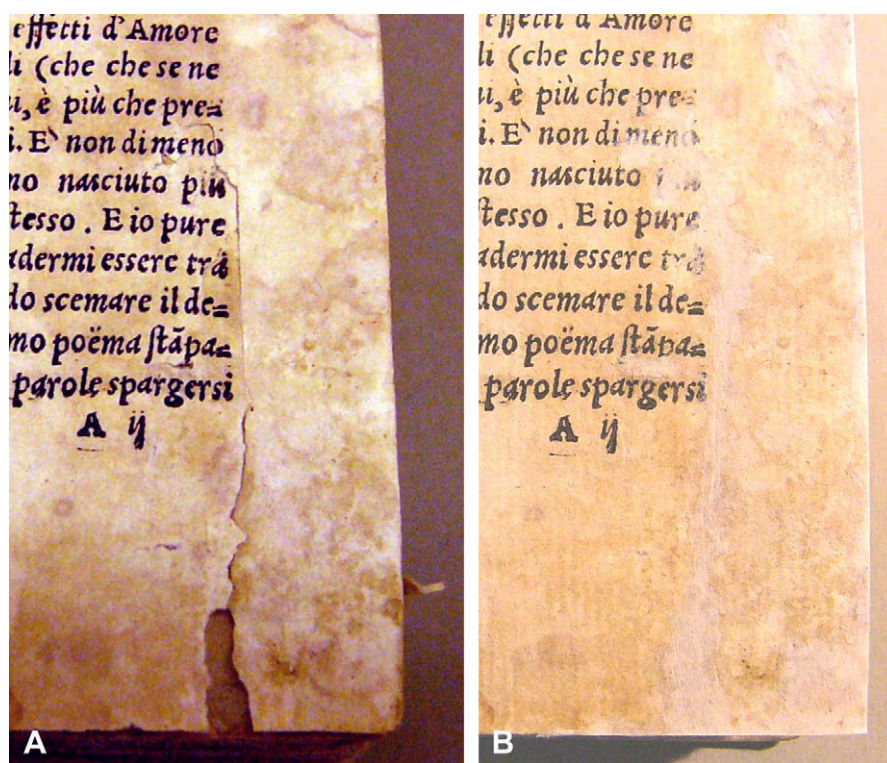


Fig. 9. The restoration of the copy of "Le Stanze del Bandello" by Matteo Bandello was performed according to the methodology of "in situ repair" which involves limited operations to treat limited damages. Paper sheets were locally reinforced with partial interventions in order to reintegrate empty spaces 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.

Acknowledgements

The authors are grateful to Federico Botti (restorer at the Restoration Laboratory at the ICPL (Rome), and person in charge of the restoration of the book, for the supply of materials, and his kind collaboration. The molecular analysis included in this project, and A. Michaelsen, were financed by the Austrian Science Fund (FWF) within the framework of project P17328-B12.

References

- Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, J.D., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* 25, 3389–3402.
- Barrett, T., 1989. Early European Papers/Contemporary Conservation Papers. *The Paper Conservator* 13, 1–108.
- Botti, F., Scimia, A., 1996. Velature con carte giapponesi pretrattate operando direttamente con solventi. In: *Cabnewsletter*, 1. Istituto Centrale per La Patologia del Libro, Roma.
- Ciferri, O., Tiano, P., Mastromei, G., 2000. Of Microbes and Art. The Role of Microbial Communities in the Degradation and Protection of Cultural heritage. Kluwer Academic/Plenum Publishers, New York.
- Coley-Smith, J.R., Verhoeff, K., Jarvis, W.R., 1980. The Biology of *Botrytis*. Academic Press, London.
- Di Lenna, P., Marciano, P., Magro, P., 1981. Comparative investigation on morphological and physiological features of three isolates of *Botrytis cinerea*. *Phytopathology* 100, 203–211.
- Domsch, K.H., Gams, W., Anderson, T.H., 1993. *Compendium of Soil Fungi*. IHW-Verlag, Eching, Germany.
- Federici, C., Munafò, P. (Eds.), 1996. International Conference on Conservation and Restoration of Archival and Library Materials. Erice, 22nd–29th April. GP Palumbo, Palermo.
- Florian, M.L.E., 1999. Fungal fox spots and others, in: Ciferri, O., Tiano, P., Mastromei, G., (Eds.), *On Microbes and Art. Proceedings of an International Conference on Microbiology and Conservation (ICMC)*. Kluwer Academic/Plenum Publishers, New York, pp. 135–151.
- Fog, N.K., 2003. Mycotoxin production by indoor molds. *Fungal Genetics and Biology* 39, 103–117.
- Gallo, F., 1985. Biological Factors in the Deterioration of Books. Technical Notes. ICCROM, Rome.
- Gopinath, S.C.B., Anbu, P., Hilda, A., 2005. Extracellular enzymatic activity profiles in fungi isolated from oil-rich environments. *Mycoscience* 46, 119–126.
- Hills, R.L., 1992. Early Italian papermaking, a crucial technical revolution. In: Cavaciocchi, S. (Ed.), *Produzione e Commercio della Carta e del Libro secc, XIII–XVIII*. Istituto Internazionale di Storia Economica “F. Datini”, Prato, Firenze, Italia, pp. 73–97.
- Hunter, D., 1974. *Papermaking, the History and Technique of an Ancient Craft*. Courier Dover Publications, New York.
- Korpi, A., Pasanen, A.-L., Pasanen, P., Kallioikoski, P., 1997. Microbial growth and metabolism in house dust. *International Biodeterioration & Biodegradation* 40, 19–27.
- Kowalik, R., 1980. Microbiodegradation of library materials. Part 2. Microbiodecomposition of basic organic library materials. *Restaurator* 4, 135–219.
- Krogh, K.B.R., Mørkeberg, A., Jørgensen, H., Frisvad, J.H.C., Olsson, L., 2004. Screening genus *Penicillium* for producers of cellulolytic and xylanolytic enzymes. *Applied Biochemistry and Biotechnology* 114, 389–401.
- Kurtzman, C.P., Fell, J.W., 1998. *The Yeasts: A Taxonomic Study*, fourth ed. Elsevier Science, Amsterdam.
- Marshall, W.A., 1997. Seasonality in Antarctic airborne fungal spores. *Applied and Environmental Microbiology* 63, 2240–2245.
- Michaelsen, A., Pinzari, F., Ripka, K., Lubitz, W., Piñar, G., 2006. Application of molecular techniques for the identification of fungal communities colonising paper material. *International Biodeterioration & Biodegradation* 58, 133–141.
- Muyzer, G., De Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology* 59, 695–700.
- Nakase, T., Suzuki, M., 1985. Taxonomic studies on *Debaryomyces hansenii* (Zopf) Lodder et Kreger-Van Rij and related species. II. Practical discrimination and nomenclature. *Journal of General and Applied Microbiology* 31, 71–86.
- Nyuksha, Y.P., 1983. Some special cases of biological deterioration of books. *Restaurator* 5, 177–182.
- Pitt, J.I., Hocking, A.D., 1985. *Fungi and Food Spoilage*. CSIRO – Division of Food Research. Academic Press, Australia, Sydney.
- Pitt, J.I., 1979. The Genus *Penicillium* and its Teleomorphic States *Eupenicillium* and *Talaromyces*. Academic Press, London.
- Raper, K.B., Fennell, D.I., 1973. *The genus Aspergillus*. R.E. Krieger Publishing Company, Huntington, NY, 686 pp.
- Reese, E.T., Downing, M.H., 1951. Activity of the *Aspergilli* on cellulose, cellulose derivatives, and wool. *Mycologia* 43, 16–28.
- Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. *Molecular Cloning: A Laboratory Manual*, second ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, USA.
- Samson, R.A., Hoekstra, E.S., Frisvad, J.C., Filtenborg, O. (Eds.), 2002. *Introduction to Food- and Airborne Fungi*, sixth ed. Centraalbureau Voor Schimmelcultuur, Utrecht, The Netherlands.
- Samson, R.A., Pitt, J.I. (Eds.), 1985. *Advances in Penicillium and Aspergillus Systematics*. Plenum Publishers, London and New York.
- Samson, R.A., Pitt, J.I. (Eds.), 2000. *Integration of Modern Taxonomic Methods for Penicillium and Aspergillus Classification*. Harwood Academic Publisher, The Netherlands.
- Schabereiter-Gurtner, C., Piñar, G., Lubitz, W., Rölleke, S., 2001. An advanced strategy to identify bacterial communities on art objects. *Journal of Microbiological Methods* 45, 77–87.
- Szczepanowska, H., Lovett, C.M., 1992. A study on the removal and prevention of fungal stains on paper. *Journal of American Institute for Conservation* 31, 147–160.
- Turner, W.B., 1971. *Fungal metabolites*. Academic Press, London.
- Zonneveld, B.J.M., 1988. Morphology of initials and number of nuclei initiating cleistothecia in *Aspergillus nidulans*. *Transaction of the British Mycological Society* 90, 369–373.
- Zyska, B., 1997. Fungi isolated from library materials: a review of the literature. *International Biodeterioration and Biodegradation* 40, 43–51.