Katja Sterflinger,* Jörg Ettenauer,* Christian Voitl* and Patricia Engel**

Books, Fungi and Men and their dislike of ethylene oxide – Final Report

Abstract

The European project "Men and Books" aimed to analyze the short term and long term effect of ethylene oxide fumigation on the material and the fungi of contaminated books. Bound volumes from the archives of the Church of Peace in Świdnica (Poland) were sampled by non-destructive methods, fungi were isolated and identified based on DNA sequencing. Three years after ethylene-oxide fumigation, 13 species of viable fungi were found on the volumes. Among these isolates were cellulolytic and keratinolytic fungi contributing to the decay of all materials including paper, leather and parchment. From the results of this study it was concluded that the fumigation with ethylene oxide neither has the effect of total sterilization – especially in bound volumes where access of the gas to the inner parts of the books is limited – nor it has a long term preventive effect against re-colonization. Because also dead fungal spores have an allergenic potential, mouldy material in used libraries as it is the case in Świdnica, has to be cleaned mechanically in order to avoid the dispersal of the spores into the air and the inhalation of spore by visitors and readers of the books. From the microbiological-hygienic point of view the volumes in Świdnica have to be cleaned or can only be used wearing gloves and respiration masks. Because of the limitation of the biocidal action of ethyleneoxide

* University of Natural Resources and Life Sciences Vienna, Department of Biotechnology

** European Research Centre Horn

on the one hand and the strong cancerogenic effect on the other hand, we do not recommend the use of ethyleneoxide in further decontamination actions.

1 Introduction

Fungi play a considerable role for the deterioration of cultural heritage. Due to their enormous enzymatic activity and their ability to grow at low a_w values fungi are able to inhabit and to decay paintings, textiles, paper, parchment, leather, oil, casein, glue and other materials used for historical art objects.[1] Especially for books, fungi are one of the most detrimental microorganisms threatening bound volumes, folios and single sheets in libraries and collections.[2] Besides of their biodeteriorative action, fungi are a potential health risk for humans. Fungi produce numerous toxins and allergens and for immune-compromised persons they can even be life threatening due to lung infections. Some of the physical methods used for disinfection – as e.g. Gramma radiation or heat – are incompatible with the materials – paper, parchment or leather – albeit being efficient to kill fungi and their spores. Chemical methods bear plenty of risks for the materials – as e.g. discolourations or oxidations – and might even be toxic for humans too. Ethylene oxide – a gaseous toxin – was used for disinfection of museum material including books. However, virtually nothing is known about the long term effects of the fumigated books for the reader and about the persistence of the toxin inside of the materials. In the special literature the use of ethylene oxide for book-disinfection is discussed controversially.[3]

1 Sterflinger (2010)

2 Sterflinger/Pinzari (2011)

3 EN 1422 and EN 550, which allow the use of ethylene oxide for sterilization of certain medicine materials. Since 1981 Germany law which has forbidden to fumigate food with ethylene oxide. For books no regulations exist. See also Meier/Petersen (2006).

It still remains an open question whether or not ethylene oxide is a good choice for book-fumigation. The material in Świdnica is unique and a treasure for the Pan-European history.

The Świdnica library is not a museum, it is a living library, the books are used by historians and other readers and thus must be handled. Nevertheless the books in Świdnica were fumigated with ethylene oxide. The last fumigation was carried out in 2010. This was performed by the Ossolineum in Wrocław. Due to this rather recent fumigation, the books are perfect candidates to be studied and answer the following questions:

- Was the fumigation in 2010 effective and is there any prophylactic effect of the fumigation preventing the re-colonization of fungi?
- What is the risk for the reader concerning fungal spores?

To investigate how fungal colonization contributes to the ageing of the materials used for books as e.g. parchment, leather and paper.

Thus for the project 76 books have been selected, 44 manuscripts and 32 prints. The volumes selected for the microbiological analysis are listed in Tab. 1.

2 Materials and methods

2.1 Isolation of fungi from books

Isolation of fungi was carried out in June 2013 when the books were stored in the Buchzentrum Horn for further analysis. Fungal spores and mycelial fragments were isolated from the books (Tab. 1) by using sterile membranes of nitrocellulose (0.45 mm pore-size, Millipore; 47 mm in diameter). The membranes where gently pressed for 10 s over mycelial spots (Fig. 1A, B), then immediately transferred to the surface of 9 cm Petri dishes containing dichloran 18% glycerol (DG 18) agar and a second membrane for each spot on 2% malt extract agar.

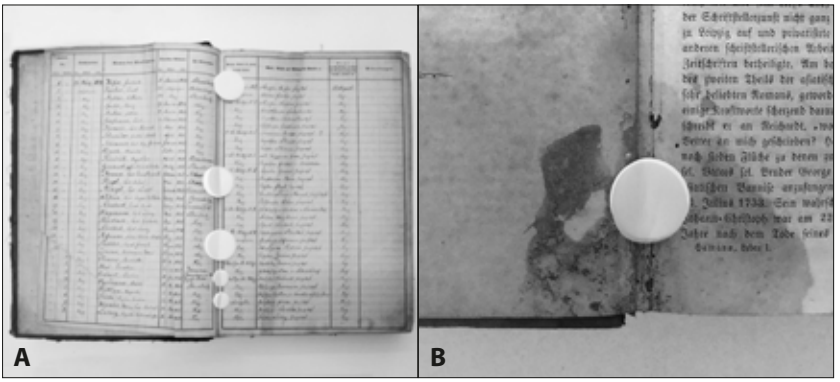
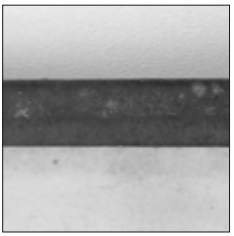
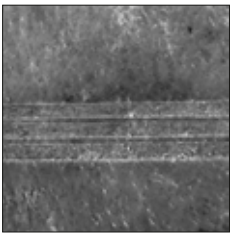
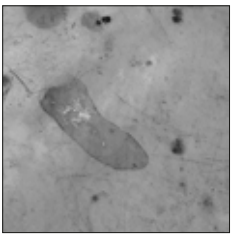
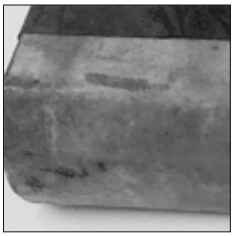
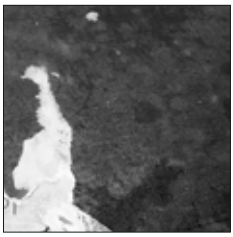
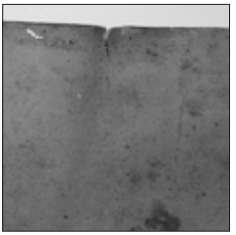
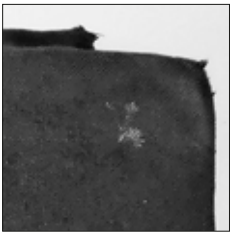
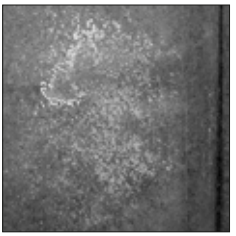
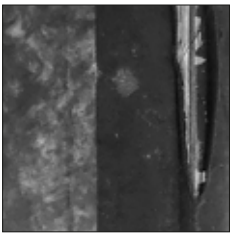
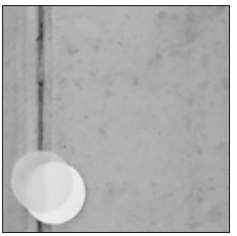


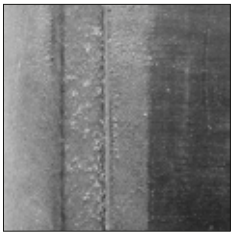
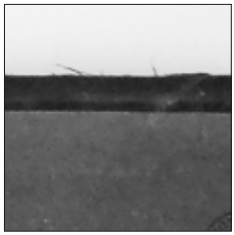
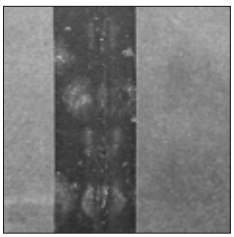
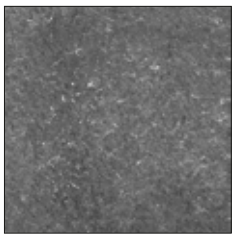
Fig. 1 A/B: Sampling of fungal spores and mycelial fragments by nitrocellulose membranes.

Volumes brought to the Buchzentrum Horn for analysis of fungi and ethyleneoxide (Tab. 1).

sign. date	cover	block	biology samples No. / description
00029 1927	leather, plant tanned	paper	  1 / leather inside with white mycelial colonies 2 / leather cover, white mycelial colonies
R0029 1708	parch-ment	Hadern paper	 16 / parchment cover, white dense colony

sign. date	cover	block	biology samples No. / description
02426 1875	textile	paper	 10 / paper in block, strong dark colonization, insects
08122 1919	Parch-ment, paper	paper	 17 / parchment, spine, dark spots
R0127 1842	textile	Hadern paper	 15 / textile cover, white layers
01695 1852	leather, plant tanned	paper	 19 / paper in block

sign. date	cover	block	biology samples No. / description
R0103 1733	textile	Hadern paper	  9 / paper cover page, dark spots 18 / textile cover, white discolouration
R0232 1929	parch-ment, textile	paper	  13 / paper under cover, yellow colonies 14 / textile cover, white mycelia
R0176 1839	leather, tanned	Hadern paper	  11 / paper in block, dark spots 12 / paper under cover, dark spots

sign. date	cover	block	biology samples No. / description
R0184 1930	leather, tanned	paper	  5 / suede leather, spine, yellow colonies 6 / leather, cover inside, white layers   7 / textile glued, white discolouration 8 / paper under cover, ochraceous colonies
00084 —	leather	/	3 / leather under cover 4 / leather cover
			20 / reference

Tab. 1 Books sampled for microbiological analysis.

2.2 *Identification of fungi by ITS sequencing*

Fungi cultures grown from filters on agar plates were purified by several transfers onto 2% MEA 2% and DG 18 (Merck, Austria). Pure cultures were identified based on their morphology and sequencing of rDNA (ITSI-5.8S-ITSII).

DNA extraction was performed directly from all material samples using the method previously described by Sert and Sterflinger[4] with the following modifications: Pieces of different materials (20–50 mg

4 Sert/Sterflinger (2010)

for mummies material and 50–100 mg for wall material) together with 500 µl extraction buffer I [50 mM Tris-HCl, 150 mM NaCl, 50 mM EDTA and 0.3% SDS (w/v), pH 8.0] were added to the lysing matrix E tubes (MP Biomedicals, Illkrich, France). After vortexing, the sample was processed twice in the Fast Prep FP120 Ribolyzer (Thermo Savant; Holbrook, USA) for 40 seconds at speed 6 (m/sec). Between these ribolyzing steps the sample was incubated at 65 °C for 1 hour at 800 rpm. After centrifugation for 10 minutes at 10,000 rpm (all centrifugation steps at 4 °C), the supernatant was transferred into a new microfuge tube. Further DNA extraction was done with 1 : 1 Vol. chloroform-isoamyl alcohol (24 : 1 v/v; Roth). During vortexing a white interface formed and after centrifugation for 5 minutes at 13,000 rpm the aqueous supernatant was transferred into a new tube. This step was repeated using the same volume (1 : 1 Vol) phenol/chloroform/isoamyl alcohol (25 : 24 : 1, v/v; Roth). Prior centrifugation after the addition of chloroform and phenol, tubes were incubated at 5 °C for 5 min. After a centrifugation step (5 minutes at 13,000 rpm), the supernatant was transferred to a new microfuge tube and further purified using the QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer. The final elution step was repeated twice with 100 µl of 80 °C preheated ddH_2O (Sigma Aldrich, St. Louis, USA). The purified DNA was used directly for PCR amplification. The concentration and quality of the DNA extracts was assessed using a NanoDrop® ND-1000 Spectrophotometer (peqLab Biotechnologie GmbH, Linz, Austria). The analyses were performed according to the manufacturer's protocol and the extracted DNA was analysed in duplicate.

PCR amplification of extracted DNA

For all PCR reactions 2× PCR Master Mix from Promega (Vienna, Austria) [50 units/ml of TaqDNA Polymerase supplied in a appropriate reaction buffer (pH 8.5), 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, 3 mM $MgCl_2$] was diluted to 1×, and 12.5 pmol/µl of each primer (stock: 50 pmol/µl, VBC-Biotech, Austria) were added. In a total volume of 25 µl, 400 µg/ml BSA (stock: 20 mg/ml; Roche, Diagnostics Gmbh, Germany) and 2.5 µl DNA template were added. PCR were performed in a MJ Research PTC-200 Peltier Thermal Cycle.

For the analysis of fungal sequences, fragments of 450–600 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]

Identification was done by using the BLAST nucleotide homology search tool.[6]

2.3 Inoculation of dummy materials with fungal strains

Spore suspensions with 10^6 spores of *Aspergillus niger, Alternaria alternata* and *Penicillium chrysogenum* were prepared. 50 ml of spore suspension were further diluted in two liter of sterile tab water and samples were inoculated by submerging them in the suspension bath for 5–10 seconds (Fig. 2). The samples were air dried and incubated in a humid chamber at 80% RH at ambient temperature for four weeks. Samples were packed and distributed the projects partners (Horn, Graz) for further processing.

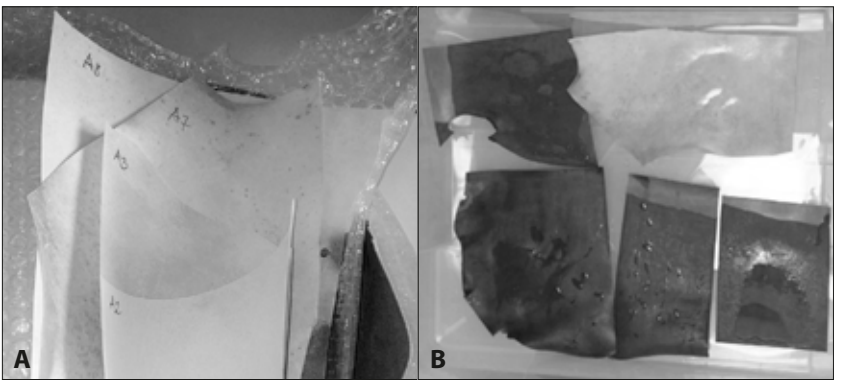


Fig. 2 Dummy materials were inoculated with fungal spore suspensions

5 White et al. (1990)

6 http://blast.ncbi.nlm.nih.gov/Blast.cgi

2.4 Isolation of fungi from dummy materials after test series

Isolation was carried out using surface contact plates (2% MEA, DG 18) and by sampling with sterile cotton swabs with direct inoculation of agar plates (2% MEA, DG 18). Incubation was seven days at room temperature.

3 Results

3.1 Fungi on books

The books under investigation showed three main phenomena of biodeterioration:

1. the dominant phenomenon were white fungal mycelia with different density. Those were found on all materials including paper, leather, parchment and textiles;
2. dark spots and colonies were less frequent. They were observed both on paper and parchment. They may also be present on leather but are not clearly visible because of the dark coloured background;
3. traces of paper destroying insects were found in one volume but the insects were not studies in the frame of this project.

The fungi isolated from the different books and materials are listed in Tab. 2. In total 13 different species were found.

Signature	Material type	Fungal isolates (according to ITSI, 5.8S, ITS II sequencing data)
00029	leather inside	*Cladosporium cladosporioides, Davidiella tassiana, Acremonium polychromum, Penicillium citrinum*
	leather outside	*Cladosporium cladosporioides*
R0029	parchment	*Eurotium appendiculatum, Aspergillus proliferans*
02426	paper	*Davidiella tassiana*
08122	parchment	*Alternaria alternata*
R0127	textile	*Penicillium chrysogenum, Arthrinium sacchari*

Signature	Material type	Fungal isolates (according to ITSI, 5.8S, ITS II sequencing data)
01695	paper	*Phoma medicaginis*
R0103	paper	no growth
	textile	*Aspergillus fumigatus, Penicillium chrysogenum*
R0232	paper	no growth
	textile	*Cladosporium ossifragi*
R0176	paper	no growth
	paper	*Davidiella tassiana*
R0184	leather	*Davidiella tassiana, Cladosporium cladosporioides*
	leather inside	*Eurotium appendiculatum, Penicillium commune*
	textile	no growth
	paper	*Penicillium chrysogenum*
00084	leather inside	*Davidiella tassiana*
	leather outside	no growth
Reference	table, sampling environment	*Cladosporium cladosporioides, Davidiella tassiana*

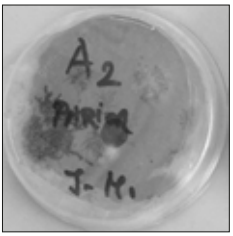
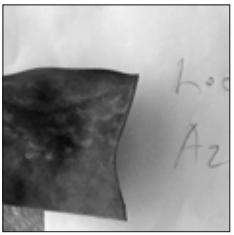
Tab. 2 Fungi isolated from books. Identification is based on sequencing of ITSI/5.8S and ITSII regions and subsequent nucleotide homology search using the Blast search tool.

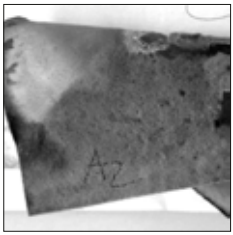
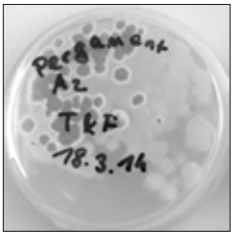
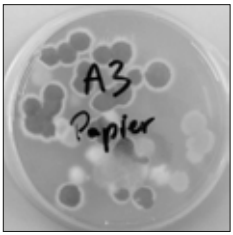
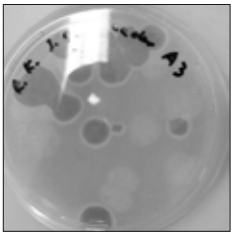
Cladosporium cladosporioides and *Davidiella tassiana* are ubiquitous fungi frequently found in indoor and outdoor air. However, both species have their original habitat on plant material and are able to degrade cellulose. *Acremonium polychromum* is a soil fungus with cellulolytic activity. *Alternaria alternata* is a plant pathogen but is also frequently found in indoor environments and on humid cellulose materials (wall paper, books). Species of *Penicillium*, as e.g. *P. commune* or *P. chrysogenum*, are extremely wide spread and occur on any type of material as soon as enough humidity is available. Those fungi grow very fast, depending on light conditions they may form white thin mycelia (in the dark) or mycelia covered with colourful masses of green to blue green spores. *Eurotium appendiculatum* was isolated from parchment and leather. This fungus is able to grow with very low levels of water and the species

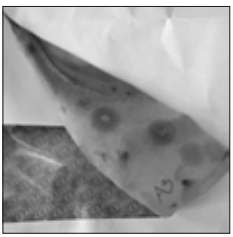
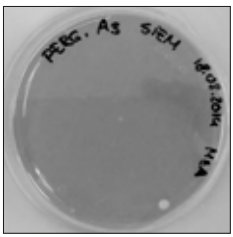
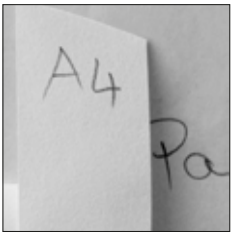
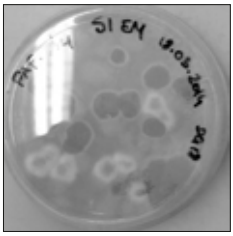
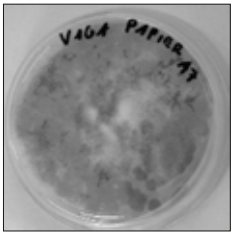
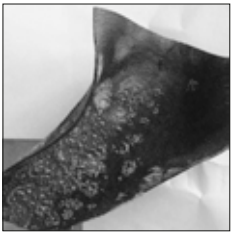
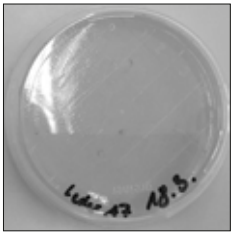
was originally isolated from fumigated sausages, thus showing a clear assignment of this fungus to the keratinous substrate. *Arthrinium sacchari* commonly occurs as a saprobe on grasses, and also on leaves, stems and roots of a range of different plant substrates and thus has a clear association with cellulose as a substrate.

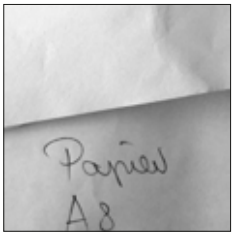
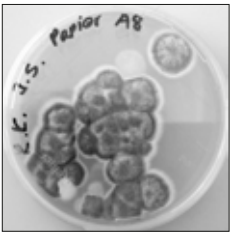
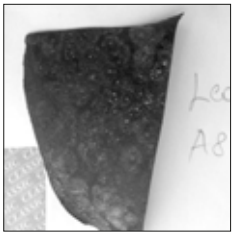
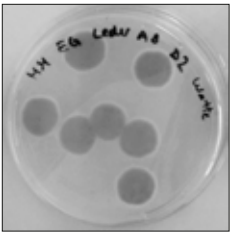
3.2 Fungi on dummy materials

The growth of fungi after re-colonization is shown in Tab. 3. Independently from the treatments – fumigation (series A1, A3, A8) versus non fumigated samples (A2, A4, A7) *Penicillium chrysogenum* was isolated from the samples. Although dark discolouration indicate colonization by the formerly inoculated fungi *A. alternata* and *A. niger*, these fungi could not be re-isolated after the treatments neither from fumigated nor from non-fumigated dummy material.

Sample	Macroscopical observation	Fungal growth	Identification
A2 paper	 Blackish-brown fungal colonies	 +	*Absidium sp.* *Penicillium chrysogenum*
A2 leather	 Ochraceous, widespread colonies	 + (weak)	*P. chrysogenum*

Sample	Macroscopical observation	Fungal growth	Identification
A2 parchment	 Light ochraceous colonies, some dark spots	 +	*Crysosporium sp.* *P. chrysogenum*
A3 paper after ethylene-oxide	 Brown discolourations and spots	 +	*P. chrysogenum*
A3 leather after ethylenoxide	 Ochraceous, widespread colonies	 +	*P. chrysogenum*

Sample	Macroscopical observation	Fungal growth	Identification
A3 parchment after ethylene-oxide	 Light ochraceous colonies, some dark spots	 / (one bacterial contamination)	*P. chrysogenum*
A4 paper reference	 No visible growth	 +	*P. chrysogenum*
A7 paper	 Brown discolourations and spots	 +	*P. chrysogenum* *Mucor sp.*
A7 leather	 Ochraceous, widespread colonies	 –	/

Sample	Macroscopical observation	Fungal growth	Identification
A8 paper after ethylene-oxide	 Brown discolourations and spots	 +	*P. chrysogenum*
A8 leather after ethylene-oxide	 Ochraceous, widespread colonies	 +	*P. chrysogenum*
A8 parchment after ethylene-oxide	 Light ochraceous colonies, some dark spots	 +	*P. chrysogenum*

Tab. 3 Isolation of fungi from dummy materials

4 Discussion

Hyphomycetous fungi – so called "mould" – are the most important agents of biodeterioration in museums, their storage rooms, in libraries, collections and restoration studios. Fungi are able to live at low water activities, they are perfectly adapted to indoor environments and thrive in microclimatic niches caused by condensation, lack of ventilation or water retention by hygroscopic materials. Fungi deteriorate valuable pieces of art by aesthetical spoiling, by mechanical action, by chemical attack and by degradation of organic components. Historical material made of paper and oil paintings with high amounts of organic binders are especially susceptible to fungal deterioration. Mould always was and still is threatening materials including historical and contemporary material of objects of art in libraries and in museums.[7] Books and documents are composite objects mainly, but not only, made of organic compounds. Paper is based on cellulose which in natural environments represents the major source of energy for microorganisms,[8] and parchment is made of collagen which is rich of nitrogen and therefore easily degradable by microorganisms, like filamentous bacteria and proteolytic fungi. The storage of books and documents inside structures destined to their preservation has created new, manmade environments for fungal and microbial species to inhabit.[9] Fungal attack on library materials inevitably occurs on paper and parchment as part of that natural process that human eagerness can only delay. From the biodeterioration point of view the fungi on Cultural Heritage materials can be divided in two main functional groups:

1. Opportunistic fungi that are growing on practically all types of materials if there is sufficient humidity. These fungi are not able to degrade the material enzymatically and to use it as main source of carbon, and

7 Sterflinger (2010); Sterflinger/Pinzari (2011); Allsopp et al. (2004); Nittérus (2000a); Capitelli et al. (2009); Mesquita et al. (2009); Pangallo et al. (2009); Koestler et al. (2003)

8 Florian (2002)

9 Kowalik (1980); Zyska (1997); Nitterus (2000a)

2. real "material pathogens" that are substrate specific and able to degrade specific materials of works of art as e.g. cellulolytic fungi on paper and keratinolytic fungi on leather, hair and feathers.[10]

Both groups may cause serious deterioration but only fungi belonging to the second group can decay the material itself. In this study real "material pathogens" – cellulolytic and keratinolytic fungi – have been isolated from the Świdnica volumes. In spite of an ethyleneoxide fumigation carried out in 2010, only three years later, deteriorative fungi like *Cladosporium, Davidiella, Acremonium, Alternaria, Phoma* and *Eurotium*, able to grow and to germinate were detected on the volumes. Also the tested dummies show that fungi were able to grow after fumigation with ethyleneoxide although with a clear dominance of *P. chrysogenum*. The survival and growth of this fungus on all materials and in all test series can probably be explained by its ability to form an enormous amount of spores which. Thus, even if most of the spores are killed by a treatment, the survival of only an extremely small percentage of spores is enough to guarantee the survival of the species on and in the materials. Nevertheless, from the results of this study it can be clearly stated that the fumigation with ethylene oxide neither has the effect of total sterilization – especially in bound volumes where access of the gas to the inner parts of the books is limited – nor it has a long term preventive effect for re-colonization. Generally it must be stated that no biocide that is on the marked by now has a sufficient sporocidal effect or a long term preventive effect. From this point of view enthyleneoxide is not worse or better that other biocides or treatments. However, it must be pointed out that ethyleneoxide, due to its ability to intercalate with DNA, has a high cancerogenous potential for humans. For disinfection of a recent and progressive fungal damage a limited range of physical and chemical methods are available.[11] Chemical treatments include liquid biocides and fumigation with gases. The choice of the appropriate biocide is limited by the European Biocide directive.[12] Topical treat-

10 Blyskal (2009); Meier/Petersen (2006)

11 Allsopp et al. (2004); Sterflinger/Pinar (2013)

12 http://ec.europa.eu/environment/biocides/index.htm

ments with chemicals can be useful but the efficacy depends on the sensitivity of the single fungal species and the tolerance of the treated support. Biocides frequently used in restoration are:

1. formaldehyde releasers.
2. quaternary ammonium compounds with an optimal chain length of C14-C16
3. Isothiazolinone, a more recent biocide, was documented to be effective and even preventive on paper objects and
4. the most common disinfectant used in microbiology: 70% ethanol.

Ethanol can also have a good fungitoxic effect if the contact time is at least 2–3 minutes.[13] A broad spectrum of chemical and non-chemical mass treatments has been utilized to kill microfungi attacking paper-made objects in an attempt to inhibit degradation.[14] Ethyleneoxide fumigation – for the reasons mentioned above – is banned in some countries because extremely toxic, but it is still regarded the most efficacious system for mass treatment of mouldy library materials. Gamma-radiation is very effective against fungi and their spores. Since the dose for fungi has to exceed 10–20 KGy[15] this method also affects many materials and application is restricted. The application of gamma rays can result in cumulative depolymerisation of the underlying cellulose and in severe ageing characteristics.[16] Sterflinger/Pinar[17] and Sterflinger/Querner[18] already pointed to the fact that (a) biocide treatments bear numerous of pitfalls that might even harm Cultural heritage and that (b) prevention and cleaning are inevitable and the most important measures to prevent biodeterioration.

Fungi in libraries, museums and their storage rooms can seriously threaten the health of the restorers, of the museum personnel and of the visitors due to their allergic potential, due to the production of my-

13 Nittérus (2000b)

14 Magaudda (2004)

15 Nittérus (2000a)

16 Butterfield (1987); Adamo et al. (1998)

17 Sterflinger/Pinar (2013)

18 Sterflinger/Querner (2013)

cotoxins but also due to their ability to cause systemic infections in humans.[19] Airborne fungal spores in storage rooms of museums can well reach levels of more than 8,000 per m^3 including fungal pathogens like *Aspergillus flavus* and *Stachybotrys chartarum*.[20] The health risk for restorers and other museum personnel is evident is such cases. Both, the deteriorative and pathogenic potential of fungi have consequences for the handling of objects, their conservation, their cleaning and their storage as well as for the occupational safety and health of museum personnel.[21] Concerning the health threatening action of fungal spores it must clearly be stated that the allergenic potential of spores and mycelial fragments is not effected by a biocide treatment. Also a spore that has been killed and is no longer able to germinate, has an allergenic potential. In consequence this means that mouldy material in used libraries as it is the case in Świdnica, has to be cleaned mechanically in order to avoid the dispersal of the spores into the air and the inhalation of spore by visitors and readers of the books.

Summary

Three years after ethylene-oxide fumigation, 13 species of viable fungi were found on the volumes from Świdnica.

In this study real "material pathogens" – cellulolytic and keratinolytic fungi – have been isolated from the Świdnica volumes. These fungi contribute to the decay of all materials including paper, leather and parchment.

From the results of this study it can be clearly stated that the fumigation with ethylene oxide neither has the effect of total sterilization – especially in bound volumes where access of the gas to the inner parts of the books is limited – nor it has a long term preventive effect against re-colonization.

Because also dead fungal spores have an allergenic potential, mouldy material in used libraries as it is the case in Świdnica, has to be cleaned mechanically in order to avoid the dispersal of the spores into the air

19 Crook/Burton (2010)

20 Sterflinger, unpublished data

21 Sterflinger/Pinzari (2013)

and the inhalation of spore by visitors and readers of the books. From the microbiological-hygienic point of view the volumes in Świdnica have to be cleaned or can only be used wearing gloves and respiration masks (filterclass 3).

Because of the limitation of the biocidal action of ethyleneoxide on the one hand and the strong cancerogenic effect on the other hand, we do not recommend the use of ethyleneoxide in further decontamination actions.

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