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

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ABSTRACT

Fungi are among the most degradative organisms inducing biodeterioration of paper-based items of cultural heritage. Appropriate conservation measures and restoration treatments to deal with fungal infections include mechanical, chemical, and biological methods, which entail effects on the paper itself and health hazards for humans. Three different conservation treatments, namely freeze-drying, gamma rays, and ethylene oxide fumigation, were compared and monitored to assess their short- (one month, T1) and long-term (one year, T2) effectiveness to inhibit fungal growth. After the inoculation with fungi possessing cellulose hydrolysis ability — *Chaetomium globosum*, *Trichoderma viride*, and *Cladosporium cladosporioides* — as single strains or as a mixture, different quality paper samples were treated and screened for fungal viability by culture-dependent and -independent techniques.

Results derived from both strategies were contradictory. Both gamma irradiation and EtO fumigation showed full efficacy as disinfecting agents when evaluated with cultivation techniques. However, when using molecular analyses, the application of gamma rays showed a short-term reduction in DNA recovery and DNA fragmentation; the latter phenomenon was also observed in a minor degree in samples treated with freeze-drying. When RNA was used as an indicator of long-term fungal viability, differences in the RNA recovery from samples treated with freeze-drying or gamma rays could be observed in samples inoculated with the mixed culture. Only the treatment with ethylene oxide proved negative for both DNA and RNA recovery. Therefore, DNA fragmentation after an ethylene oxide treatment can hamper future paleogenetic and archaeological molecular studies on the objects.

1. 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 (Nol et al., 2001; Corte et al., 2003; Cappitelli and Sorlini, 2005; Michaelsen et al., 2006, 2009; Rakotonirainy et al., 2007; Zotti and Ferroni, 2008; Mesquita et al., 2009) and, in a minor way, bacteria (De Paolis and Lippi, 2008; Jurado et al., 2010; Michaelsen et al., 2010), resulting in both structural and aesthetic damage.

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, it is crucial not only to control active fungal growth, i.e., hyphae, mycelium, or mould, but also to remove or reduce the amount of fungal ascospores and conidia. The structural nature of ascospores as progenitors of future growth allows the fungi to survive severe conditions, and ascospores are consequently harder to inactivate than the vegetative hyphae. In unfavorable 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 should be directed toward the spores, as the vegetative hyphae are relatively easy to control by physical removal and through monitoring of the storage climate, in terms of temperature, relative humidity, and activity of water (Nitterus, 2000). Fungi are an increasing and dominant problem for archives and museums and thus the prevention of their growth and the development of appropriate treatment measures for contaminated objects are a challenge for restorers, curators, and scientists (Sterflinger, 2010).

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A broad spectrum of chemical and non-chemical components has been used to sanitize microfungi attacking paper-made objects in an attempt to inhibit degradation (Magaudda, 2004). In this study we chose three different conservation treatments for paper to compare their effectiveness over the short- and long-term, namely freeze-drying, gamma rays, and ethylene oxide fumigation.

A commonly used strategy in paper conservation is freeze-drying, a method in which the water is frozen and then removed by sublimation, i.e., it goes from the solid phase to water vapor, bypassing the liquid phase. Sublimation allows the water to be removed without the effects of water evaporative forces, which can cause dimensional changes. In addition, freeze-drying with dehydration can kill hydrated conidia and germinating conidia and it stops the growth of fungal mycelia and bacterial cells (Sussman, 1966; Mazur, 1968; Florian, 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; Walsh, 1988; Parker, 1989; Florian, 1990).

The application of gamma rays in conservation science dates back to the 1960s, when the radio-resistance of significant mould fungi from goods of cultural value was tested (Belyakova, 1960). 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). Gamma irradiation is successfully used to sterilize laboratory and hospital utensils and food, but it can have unwanted side effects when applied in paper conservation where high irradiation doses, which are often required in repeated doses, can result in cumulative depolymerization of the underlying cellulose in the paper. Severe aging characteristics, such as lowered folding endurance and tear resistance, increased yellowing, and general embrittlement, have been reported in paper treated with gamma rays (Butterfield, 1987; Adamo et al., 1998), whereas more recent studies have suggested that the damage in terms of mechanical–physical properties is not significant (Adamo et al., 2001; Gonzalez et al., 2002). The observed effects of gamma rays on fungi from paper have confirmed that radiation treatment of books and documents is effective, as there was no fungal growth detectable in cultivation studies (Jörg et al., 1992; Da Silva et al., 2006).

Finally, ethylene oxide (EtO) has been widely used for sterilization of objects of cultural heritage since 1933 (Ballard and Baer, 1986). Ethylene oxide does not require activation energy, is used at room temperature, and expresses the high reactivity and diffusivity required 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). This ability to act as an alkylating agent was widely taken to indicate a carcinogenic potential, an assumption that has subsequently been proven for EtO and some of its residuals (Bolt, 1996; Angerer et al., 1998), leading to a ban on EtO for the practice of paper conservation 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). In this study, the effects of these three conservation treatments on fungal spores and mycelium viability and activity were investigated by culture-dependent and -independent strategies. The main goals were: (a) to compare the efficacy of molecular versus cultivation techniques as models for the monitoring of conservation treatments, (b) to evaluate massive and aggressive disinfecting treatments on DNA integrity, and (c) to use rRNA analyses as a method of determining long-term viability of fungi.

With this end, 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 monitored over the short- and long-term after the treatments to determine time-dependent effects of the treatments on fungal viability. These effects were examined by culturing and on a molecular level by generating DNA-denaturing gradient gel electrophoresis (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 of the viability of the respective fungi after the samples had been left for a year for eventual regrowth. This strategy is commonly used in bacterial ecology and is based on the fact that metabolically active species transcribe more rRNA for ribosome synthesis than do 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 a strategy for detecting the most 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.

2. Materials and methods

2.1. Sample preparation and cultivation

Fungal growth was induced in vitro by inoculating two types of paper with three fungal strains previously isolated: *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 ash content, and paper D was a “Mezzofino” type paper with a high lignin content, namely a naturally aged rag paper produced by the Istituto Poligrafico dello Stato in 1976 (Gallo et al., 1999), No. 200953, consisting of bleached cellulose (45%), wood pulp treated with sulphites (25%), wood pulp from softwood (20%), glue (3%), aluminum sulfate (5%), and kaolin (2%), pH 4.5. Each paper sample existed as a set of small discs (about 1 cm diameter) cut with a puncher (12.5 mm disc punch n. T5443, Agar Scientific Ltd., Stansted, Essex, England).

Fungal strains were grown on malt extract agar (2%) to obtain colonies with mature fruiting bodies or reproductive structures. Conidia and ascospores 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^{-1}. The water vapor-sterilized paper samples were inoculated with 50 μl of broth each to provide a physiological starter for germination. Each inoculum was applied to the sample disc in a single spot. About 100% relative humidity (RH) 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 (*C. cladosporioides* and *T. viride*), and 14 days (*Ch. globosum* and mixed inocula).

Chaetomium is a genus of filamentous fungi (phylum *Ascomycota*, class *Sordariomycetes*) encompassing species that typically possess densely setose, ovoid to pyriform ostiolate ascomata, clavate asci, and pigmented, one-celled ascospores (Samson et al., 2000). Species

of *Chaetomium* are important in the decomposition of cellulose-rich materials. *T. viride* is the conidial stage of *Hypocrea rufa*. Several strains of *Trichoderma* have been developed as biocontrol agents against fungal diseases of plants. The various mechanisms include antibiosis, parasitism, inducing host-plant resistance, and competition. The *T. viride* strain used in the production of paper samples was characterized by producing only conidia, and not the ascospores typical of its prefect state (Hypocrea). *Cladosporium* is a dematiaceous (pigmented) mould widely distributed in air and rotten organic material. The genus *Cladosporium* is one of the largest genera of dematiaceous hyphomycetes, and is characterized by conidia in acropetal chains and *Davidiella* teleomorphs. *C. cladosporioides* has conidiophora distinct from vegetative hypahe and conidia that are one-celled, thick-walled, and finely roughed, produced in branched chains (Samson et al., 2000).

One replica of each set was used as a control sample and not subjected to any treatment. The other replicas were treated with freeze-drying, gamma rays, or EtO. Viability 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.

2.2. Treatments of paper samples

To avoid contamination from airborne fungi after the treatment, all samples were placed into sterile paper air-permeable envelopes before being treated and left therein until further testing. Sample envelopes were stored in petri dishes in dark conditions at room temperature. Gamma irradiation and EtO fumigation are hazardous and consequently conducted by specialists and approved companies.

2.2.1. 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 five paper leaves was interposed between each envelope and a 2-cm layer of paper leaves was 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 5-h cycle with warming temperatures of 50 °C maximum.

2.2.2. 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 exposure time of 1 h and 3 min (Adamo et al., 2001).

2.2.3. Ethylene oxide

Fumigation treatment was performed by Spix Italia s.r.l. in a vacuum cell device with a mixture of EtO (10%) and CO_2 (90%) for 48 h at a temperature between 20 and 22 °C. After gas abatement, 20 washing cycles with air were performed. The treatment was verified with chemical (EO stripes n. 6615 EtO chemical indicator, VWR International PBI Srl) and biological (EO vials n. 3166 EtO biological indicator, VWR International PBI Srl) standard indicators (Ballard and Baer, 1986).

2.2.4. Control samples

Sets of inoculated paper samples that developed fungal mycelia were air-dried in sterile polystyrene ventilated petri dishes at about 21 °C and 50% RH for one week to stop active growth of fungal mycelia, but to keep conidia and ascospores alive. Following natural dehydration the samples were sterilely introduced into paper envelopes and kept in petri dishes in the same manner as the treated samples.

2.2.5. Culturing of treated and untreated samples

The treated and the untreated control samples were tested for viability of the fungal spores by means of the 25 points inoculum method: Sets of inoculated and treated paper, three each, were washed three times in sterile water and divided into 25 subsamples that were inoculated directly on solid nutritive agar (malt extract agar, 20 g l^{-1}) distributed according to a geometric scheme (Fig. 1). The frame consists of a grid with 25 nodes spaced out equally. The development of the fungal mycelium in all or a fraction of the 25 inoculum points allows for a statistical comparison between different treatments and untreated samples (Dix and Webster, 1995, after adaptation on cellulosic materials). One-way ANOVA was computed and followed by the Fisher test according to Massart et al. (1998) using XLSTAT statistical software (Addinsoft, Paris) to determine the significant differences between the treatments. The inocula on agar were performed directly after the treatments and 1 year later (long-term monitoring, T2).

2.3. DNA extraction from paper material

DNA was extracted directly from the prepared small paper discs (about 1 cm in diameter) by using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, 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 pre-treated 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 (short-term monitoring, T1) and again one year later (long-term monitoring, T2) to test the efficiency and long-term effects of the different treatments on the DNA of the inoculated fungi.

2.4. DNA amplification and DGGE analyses

All PCR reactions were executed using PCR Master Mix (Promega, Mannheim, Germany). Fragments of about 500–700 bp size

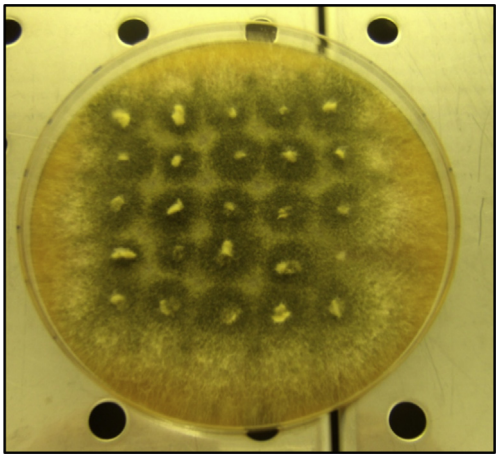

Fig. 1. Example of a 25-point inoculum of *Chaetomium globosum* on paper A taken from control paper samples. The development of mycelium in the points allows for a statistical comparison between different treatments and untreated control samples. In the control sample all the 25 fragments of paper produced a colony after the inoculation on nutritive agar.

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 (White et al., 1990; Muyzer et al., 1993). All reactions were carried out as described in Michaelsen et al. (2006) and PCR products were visualized 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 × 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 °C and 75 V for 14 h, stained with ethidium bromide, and visualized using a Bio-Rad Gel Doc imaging system (Bio-Rad Laboratories Pty., Ltd, Australia).

2.5. 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 manufacturer's protocol. One disc per treatment was placed in a Lysing Matrix E tube (MP Biomedicals, Solon, OH, USA) and 600 μl of buffer RLC from the RNeasy Plant Mini Kit was added. The tubes were processed on the FastPrep beadbeater for 30 s 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 manufacturer's instruction. An on-column DNAase treatment was performed using the Qiagen RNase-free DNase set (Qiagen, Australia). The final elution step was carried out twice with the provided water and stored at −80 °C. RNA extraction was performed one year after the treatment of samples (long-term monitoring, T2) to allow conclusions on the viability and actual growth of the inoculated and treated strains to be compared with the DNA results.

In all cases, PCR controls with the crude RNA extracts were performed to exclude DNA contamination.

2.6. RNA amplification

RT-PCR was carried out using 2 μl RNA template for the initial denaturing step with 1 μl of reverse primer 18Sr (5′-GATCCT TCTGCAGGTTCACCTAC-3′) for 5 min at 65 °C. 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 in a 10-μl reaction volume with molecular grade water (all Qiagen, Australia) for 60 min incubation at 37 °C.

The cDNA was used to amplify approximately 250 bp products of the 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 AmpliTaqTM10× 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 °C for 2 min, followed by 30 cycles of 95 °C for 30 s, 61 °C for 30 s, and 72 °C for 30 s, and a final extension cycle of 10 min at 72 °C. 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 30–50% urea/formamide denaturing gradient using the DGGE 2401 system (C.B.S. Scientific Co., USA). Gels were run at 60 °C and 55 V for 17 h, stained with ethidium bromide, and visualized using a Bio-Rad Gel Doc imaging system (Bio-Rad Laboratories Pty., Ltd, Australia).

3. Results and discussion

3.1. Cultivation of fungal spores from treated and non-treated control samples

The development of fungal mycelium in the 25-spot grid enabled statistical comparisons between the treatments and the control samples and among the control samples as presented in Fig. 1 and Table 1, according to the analysis of variance (ANOVA) method and Fisher test. Non-treated control samples showed 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. *C. cladosporioides* appeared to be no longer cultivable and the recorded growth for *T. viride* was low and statistically different from both the control sample and *Ch. globosum*. No statistical difference was found for *Ch. globosum* and mixed inocula samples with respect to the control samples. Both gamma irradiation and EtO fumigation did suppress fungal growth of all single species and the mixed inocula over the short-term and showed full efficacy as disinfecting agents when evaluated with cultivation techniques (Table 1).

Long-term monitoring showed differences in the growth of the singly inoculated fungal strains stored without treatment (T2). *T. viride* and *C. cladosporioides* were not able to grow after one year at room temperature. *Ch. globosum* did not behave in a statistically different manner compared to the sample one year before. These results can be explained by the higher resistance of *Chaetomium* ascospores with respect to the conidia of *Trichoderma* and *Cladosporium* strains. Ascospores are typically thick-walled and protected in a fruiting body, as in *Chaetomium* spp. (Deacon, 2005).

Comparing the two types of paper tested, statistically significant differences (Table 1) were also noted in a general favor of paper A, the Whatman paper, for fungal growth after treatment. However, as

Table 1
Statistical analysis of the 25-inoculum growth test. Three replicates of 25 inoculum grids were considered (25 × 3 inoculum points for each treatment/paper/fungus). Average value ± standard deviation. Different letters indicate significant differences (Fisher, $p < 0.05$) between the means of independent replicates. Paper grades: A (Whatman) and D (Mezzofino). Treatments: nt T1, control after 1 month from fungal inoculums; nt T2 control after 1 year of natural drying; O ethylene oxide treatment; γ, gamma rays treatment; L, freeze-drying treatment. Fungi: Ch = *Chaetomium globosum*; Cl = *Cladosporium cladosporioides*; Tr = *Trichoderma viride*; Mix = the three strains together.

Treatment	Average paper A ± St. Dev.	Average paper D ± St.Dev.
nt T1_Ch	25.0 ± 0.0 A	23.3 ± 1.5 A
nt T1_Cl	25.0 ± 0.0 A	25.0 ± 0.0 A
nt T1_Mix	24.0 ± 1.7 A	24.3 ± 1.2 A
nt T1_Tr	19.0 ± 1.0 B	17.3 ± 0.6 B
nt T2_Ch	25.0 ± 0.0 A	7.7 ± 4.9 D
nt T2_Cl	0.0 ± 0.0 E	0.0 ± 0.0 E
nt T2_Mix	24.0 ± 1.7 A	2.7 ± 0.6 E
nt T2_Tr	0.0 ± 0.0 E	0.0 ± 0.0 E
O_Ch	0.0 ± 0.0 E	0.0 ± 0.0 E
O_Cl	0.0 ± 0.0 E	0.0 ± 0.0 E
O_Mix	0.0 ± 0.0 E	0.0 ± 0.0 E
O_Tr	0.0 ± 0.0 E	0.0 ± 0.0 E
γ_Ch	0.0 ± 0.0 E	0.0 ± 0.0 E
γ_Cl	0.0 ± 0.0 E	0.0 ± 0.0 E
γ_Mix	0.0 ± 0.0 E	0.0 ± 0.0 E
γ_Tr	0.0 ± 0.0 E	0.0 ± 0.0 E
L_Ch	25.0 ± 0.0 A	12.3 ± 1.5 C
L_Cl	0.0 ± 0.0 E	0.0 ± 0.0 E
L_Mix	16.7 ± 3.8 B	0.3 ± 0.6 E
L_Tr	0.0 ± 0.0 E	2.3 ± 1.5 E

the same effect was observed for the control samples of paper A, it indicates that cotton linter generally supported fungal growth better than rag paper D, the Mezzofino paper made from cellulose and wood pulp, and the differences evaluated can be independent of the treatments.

3.2. DGGE fingerprints to monitor and compare the effect of the treatments

Denaturing gradient gel electrophoresis is the technique most often used to study the structure of the microbial communities colonizing artworks (Gonzalez and Saiz-Jimenez, 2004). Furthermore, it has recently been implemented to monitor structural changes in microbial communities colonizing stone-works after conservation treatments (Piñar et al., 2010; Ettenauer et al., 2011). Hence, in this study we applied DGGE for the first time to monitor paper samples that were inoculated with fungal spores and further subjected to different conservation treatments. This allowed monitoring of changes on the DNA level of different fungal species in relation to the treatments, and determination of the treatments' efficiency.

To this end, the internal transcribed spacer region 1 (ITS1) was amplified from template DNA extracted directly from the treated paper samples as well as from non-treated control samples and subsequently separated in DGGE (Michaelsen et al., 2006).

Interestingly, our results show only minor effects on the recovery of fungal DNA from samples treated with freeze-drying or gamma rays, as minor differences between the DGGE profiles of treated and non-treated control samples are mostly restricted to the mixed inocula samples (Fig. 2). This fact may indicate a result of the competition between fungal strains during incubation.

3.2.1. Control samples

Denaturing gradient gel electrophoresis fingerprints derived from untreated control samples one month after the treatment (T1) and one year later (T2) are nearly identical, as shown in Fig. 2a. DNA from *T. viride* (lanes 5 and 6) was not detected in any sample when inoculated in a mixture with *Ch. globosum* and *C. cladosporioides* independently of the nature of treatment. However, it should be considered that *T. viride* was initially not competitive enough and was outgrown by the mycelium of the other two fungi, probably due to the antifungal effect of *Chaetomium* (Kanokmedhakul et al., 2002). The amount of fungal spores and mycelium from *T. viride* on the samples was below the detection limit of our PCR and DGGE conditions.

DGGE bands representing *C. globosum* (lanes 3 and 4) were observed in both single and mixed inoculated control samples of both paper types, at both T1 and T2, whereas *T. viride* only showed clear bands when inoculated as a single strain at both time points monitored. Unexpectedly, no DGGE band could be obtained for *C. cladosporioides* inoculated as a single strain on paper D throughout the experiment, probably due to the difficulty of this species to grow as a single strain on the lignin containing/acidic paper. The conidia of this fungus deposited on paper D with the inoculum did not germinate but as conidia were possibly around they were probably below the detection limit of our PCR and DGGE conditions. Therefore, this sample is not shown in Figs. 2 and 3.

3.2.2. Samples treated with freeze-drying

Temperature has an effect on the growth and survival of fungi, as it dictates the rate of the reactions catalyzed by enzymes, but it is important to note that a temperature below which no growth occurs should not be understood as a lethal temperature for fungi (Nitterus, 2000). Reducing the temperature to subzero values, as happens in freeze-drying cycles, reduces the viability of many fungi and can finally exhaust even dormant spores if these temperatures are 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 should be killed at temperatures below 0 °C (Nitterus, 2000). But our results show that, on the contrary, cultivation and molecular techniques both produced positive results for fungal growth or activity on samples subjected to freeze-drying, which are comparable with those of 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 (Florian, 1990, 2002).

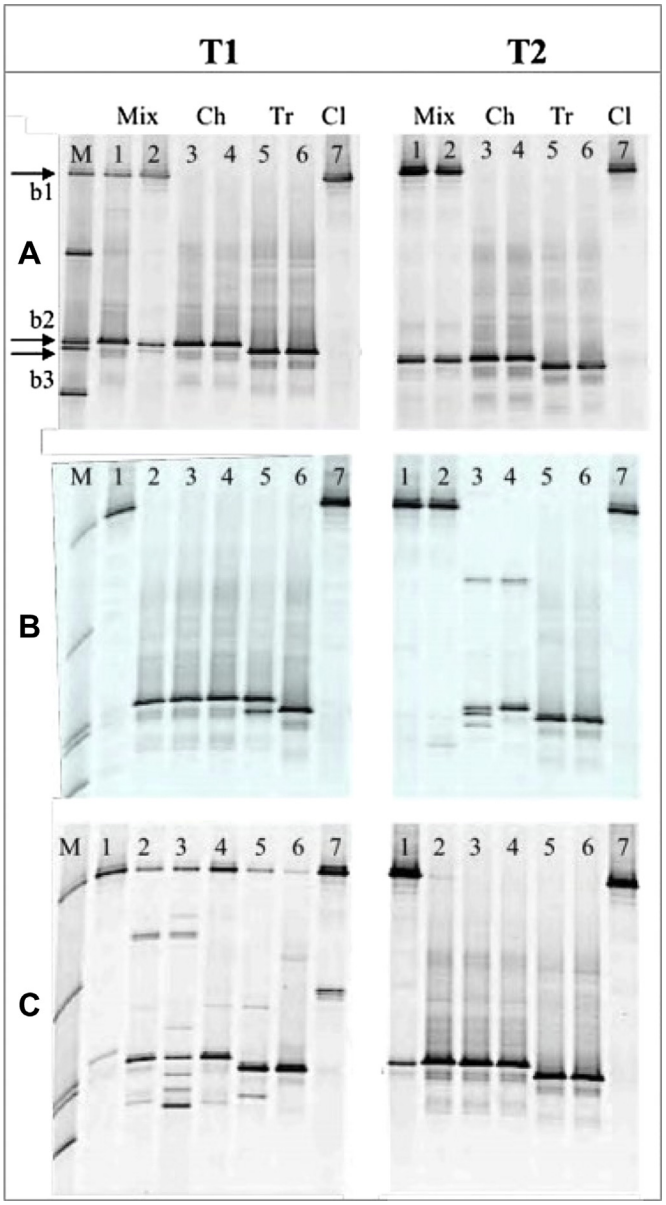

Fig. 2. DGGE profiles generated from ITS1 derived DNA of untreated control (a), freeze-dried (b), and gamma ray- (c) treated paper samples extracted after one month of the application of treatments (T1) and one year later (T2). Lanes contain marker (M), mixed inocula (Mix) on paper types A and D (lanes 1 and 2), *Chaetomium globosum* (Ch) on paper types A and D (lanes 3 and 4), *Trichoderma viride* (Tr) on paper types A and D (lanes 5 and 6), and *Cladosporium cladosporioides* (Cl) on paper type A (lanes 7). No DNA was obtained for any sample treated with EtO. The marker contains, among others, fragments from *C. cladosporioides* (b1), *Ch. globosum* (b2), and *T. viride* (b3).

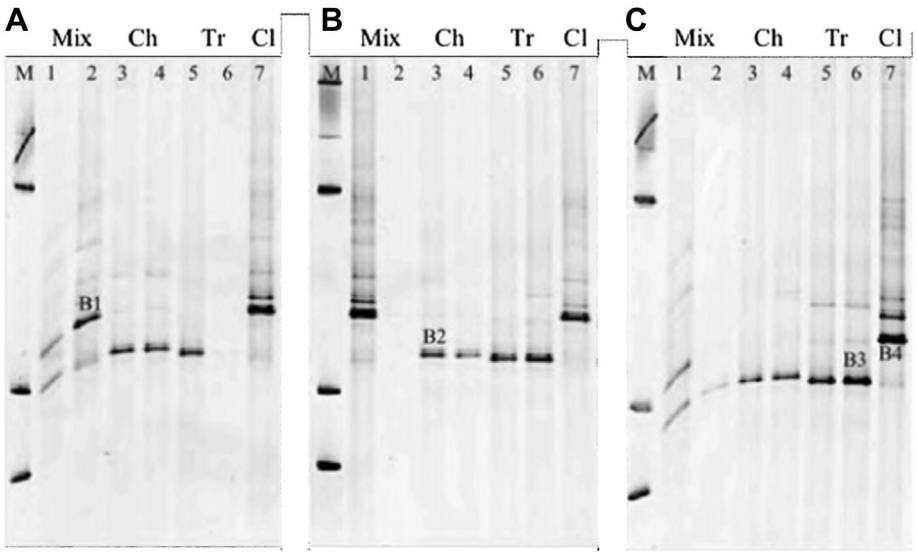

Fig. 3. DGGE profiles generated from 18S cDNA of untreated control (a), freeze-dried (b), and gamma ray- (c) treated paper samples one year after the application of treatments (T2). Lanes contain marker (M), mixed inocula (Mix) on paper types A and D (lanes 1 and 2), *Chaetomium globosum* (Ch) on paper types A and D (lanes 3 and 4), *Trichoderma viride* (Tr) on paper types A and D (lanes 5 and 6), and *Cladosporium cladosporioides* (Cl) on paper types A (lane 7). Marked bands indicate the position of each fungus on DGGE: *C. Cladosporioides* (B1, in A and B4 in 3C), *Ch. globosum* (B2), and *T. viride* (B3).

DGGE fingerprints derived from samples treated with freeze-drying showed some differences compared to the control samples (Fig. 2b). One month after the treatment (T1) the fingerprints derived from samples inoculated with the mixed culture differed from the control and between paper A, Whatman, and paper D, Mezzofino. The *C. cladosporioides* was represented by a strong band on paper A (T1, lane 1) but it was missing on paper D (T1, lane 2). By contrast, *Ch. globosum* appeared as a strong band on paper D but was very faint on paper A, whereas *T. viride* was not detected in any mixed inoculated sample, as observed in the control samples. Mixed inocula showed a dominance of *Ch. globosum* that exhibited a strong antifungal tendency against *T. viride*. The bands representing each single fungus inoculated as pure culture did not show differences due to treatment.

Long-term effects due to the freeze-drying treatment were only observed for *Ch. globosum* when inoculated as a single strain, showing an unspecific band-pattern (Fig. 2b, T2, lanes 3 and 4). Furthermore, no DGGE band corresponding to this fungus was detected when it was inoculated in a mixture with the other two fungi (Fig. 2b, T2, lanes 1 and 2). The other fungal bands showed patterns in accordance with the control samples.

3.2.3. Samples treated with gamma rays

Different functions of the paper or its components, such as cellulose, are affected by gamma irradiation. Furthermore, its germicidal power on fungal microflora has been reported to be dose-dependent (Magaudda et al., 2000; Adamo et al., 2001). A dose of about 5 kGy, as applied in our experiment, was found to cause statistically significant variations on almost all paper properties, such as tensile strength, tearing resistance, and folding endurance, but also reduced the fungal population down to the "blank" samples level (Adamo et al., 2001; Magaudda, 2004). Relatively low irradiation doses of 2–3 kGy delivered comparable decontaminating power without the increasingly depolymerizing effects of gamma rays on cellulose.

Samples treated with gamma rays showed DGGE patterns with numerous unspecific bands one month after treatment (Fig. 2c, T1). Bands corresponding to the individual fungi *Ch. globosum*, *T. viride,* and *C. cladosporioides* were still observed, but their identities were indecisive in some samples. In addition, new faint bands were observed. These patterns indicate a mixed population of intact DNA from surviving fungi and DNA artifacts, as a consequence of DNA fragmentation due to the irradiation, from affected fungi.

One year later (Fig. 2c, T2), no more visible effect of gamma radiation on the DNA of pure strains, compared to the control samples, was observed. This indicates that the surviving fungal population recovered with time and only the intact DNA of this population was detected. For paper samples inoculated with mixed inocula, only the band for *C. cladosporioides* was shown to be weak on paper D (Fig. 2c, T2, lane 2).

3.2.4. Samples treated with EtO fumigation

The observation that EtO can significantly reduce or erase the amount of amplifiable DNA on small items has been reported before. Ethylene oxide is successfully applied in forensics to remove DNA contamination on items used both at crime scenes and in the forensics laboratory, without significantly affecting any needed downstream DNA analysis when used to sterilize equipment (Shaw et al., 2008). The gaseous EtO is considered a radiomimetic agent, which means it is similar to ionizing radiation by virtue of its ability to induce the same biological end-points, such as 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, resulting in PCR not delivering any evidence of fungal DNA or RNA on all samples submitted to fumigation. In accordance with this, 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).

As Shaw et al. (2008) did, 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 sterilizing agent compared to gamma radiation was explained by Chovanec et al. (2001) as a combination of differences in the nature of damage caused and the distribution of the target. Ionizing radiation

can react more locally and focused with free radicals following tracks along covalent bonds, whereas small molecules such as EtO can diffuse quite freely in the cell nucleus, causing damage on a broader scale. Although EtO application is banned for use in conservation, it should still be recommended in certain cases where the careful restoration and conservation of valued material is assessed.

The 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).

3.3. RNA as an indicator for metabolically active fungi

As we confirmed the presence of DNA on most samples, with the exception of those treated with EtO, the next consequent step was to investigate if the fungi inoculated on the samples were still metabolically active. Several methods are available to test fungal and microbial viability, such as the quantitation of cell's ATP content (Rakotonirainy et al., 2003) or fluorescein diacetate (FDA) and acridine-orange stainings that provide a qualitative observation of a microorganism's active structures by means of an epifluorescent microscope, equipped with specific filters (Pinzari et al., 2011).

In this study we decided to use nucleic acids as markers of fungal viability. The existence of ribosomal-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). Thus, if responses of microbial 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). To test the viability of the inoculated fungi, we targeted the fungal rRNA directly, as it allows the detection of 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.

In this study, RNA was extracted from paper samples one year after the application of the respective treatments (T2), and subsequently transcribed into cDNA for further DGGE analyses. The DGGE profiles generated with 18S primers from the fungal cDNA are shown in Fig. 3. Untreated control samples and those subjected to freeze-drying and gamma irradiation were all positive for amplification of fungal 18S cDNA, whereas no positive RNA extraction or PCR could be established for EtO-treated samples. Strong signals could be observed for all pure strains, with the exception of *T. viride* on paper D, in the control samples (Fig. 3a). The pattern of single bands obtained for the pure inoculated strains indicated the presence of only one fungus on each paper sample. On samples inoculated with the mixed cultures, the DGGE band corresponding to *T. viride* was not observable, a pattern that is congruent with the DNA-generated gels.

RNA analyses revealed a minimal long-term impact of either gamma rays or freeze-drying treatments on the viability of the fungal spores, when inoculated as single strains (Fig. 3b and c, lanes 3–7). All samples deliver clear bands with a pattern that matches the expectations and consistent for the different fungi. However, when fungi were inoculated as mixed cultures, no RNA band at all or very faint signals were observed for paper D after freeze-drying and gamma ray treatments, respectively (Fig. 3b and c, lane 2).

Unfortunately, an amplification of the cDNA with ITS primers, as used for the DGGE analysis of DNA, failed. Therefore we used primers targeting the 18S rRNA to obtain DGGE profiles of cDNA. ITS sequences are the most popular choice for species identification of fungi in environmental DNA pools (White et al., 1990), 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). Even if there is evidence that the ITS regions can be used for the detection of active fungi, as was done by Anderson and Parkin (2007), it is generally 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 samples. We assumed, during the experimental work, that there was more rRNA available in the soil samples themselves, as soils are active environments compared to the paper environment, which resembles starving conditions. While the present paper was under review, a paper by Rajala et al. (2011) was published, which demonstrated that internal transcribed spacers (ITS) precursor rRNA are a better marker of active fungi than small subunit rRNA. This is because the ITS sequences, after being excised from the subunits turnover rapidly, and small and large subunit rRNA particles are relatively stable in the environment. The work by Rajala et al. (2011) may be important for follow-up elaboration of the present work.

3.4. Comparison of methods used for the monitoring of treatments — cultivation versus molecular methods

Our molecular results generally contradicted the cultivation results obtained in this study. By using culture-dependent techniques, samples subjected to freeze-drying showed growth in the case of *T. viride*, but is was recorded as low and statistically different from the control sample, whereas no statistical difference was found for *C. globosum* and the mixed inocula sample with respect to the control samples (see Table 1). However, by using culture-independent techniques we could observe some differences on samples inoculated with the mixed culture samples one month after the freeze-drying treatment (T1) compared to the control samples (see Fig. 2b). DGGE bands representing each single fungus inoculated as pure culture did not show differences due to treatment, but a long-term effect was only observed for *Ch. globosum* when it was inoculated as a single strain, showing an unspecific band-pattern.

Samples subjected to gamma irradiation and EtO fumigation showed no fungal growth, either of all single species or the mixed inocula. Therefore, the last two treatments indicated a full efficacy as disinfecting agents when evaluated with cultivation techniques (see Table 1). However, when using culture-independent techniques, gamma rays were not able to eliminate fungal colonization of paper even if relatively high doses (5 kGy) were applied, but they could potentially reduce or slow down the growing process. DGGE profiles obtained from DNA extracted shortly 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 was observed, indicating the recovering of the surviving fungal fraction. 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 et al. (2006) determined the efficiency of the radiation treatment on fungal spores by cultivation attempts only, and therefore described a higher degree of reduction than our DNA- and RNA-based studies. Our cultivation results are in accordance with these observations, but are proven wrong when molecular data are included.

Only in the case of EtO fumigation did both culture-dependent and -independent techniques indicate the total inactivation of the inoculated fungi.

Comparing DNA and RNA recovery, we could observe no differences in the case of samples inoculated with pure strains, being able to recover both DNA and RNA, which indicates that the fungi were indeed viable. However, differences in the RNA recovery from samples treated with freeze-drying or gamma rays were observed when samples were inoculated with the mixed culture, especially on paper type D, indicating the inactivation of the fungi on this kind of paper and the higher sensitivity of RNA- versus DNA-based analyses for the detection of viability.

4. Conclusions

The differences between culture and molecular results obtained in this study highlight the importance of molecular tools to complement conventional techniques, as they display higher sensitivity and bypass culturing methods. Retrieval of fungal-specific rRNA directly from paper can, in fact, be successfully used as an indicator for the viability of fungi after disinfecting treatments.

Information on the effects of mass treatments on the DNA of paper-spoiling fungi is also provided. Our results show, in fact, that the freeze-drying treatment displays only minimal effect on DNA recovery over both the short- or long-term when compared to control samples, whereas the application of gamma rays shows a short-term slight reduction of DNA recovery and DNA fragmentation but no significant difference in long-term effect.

When RNA was used as an indicator for long-term fungal viability, differences in the RNA recovery from samples treated with freeze-drying or gamma ray-treated samples were observed when samples were inoculated with the mixed culture, specially on paper type D. Samples inoculated with the single strains showed no difference in the RNA recovery compared to control samples. Only treatment with ethylene oxide proved negative for both DNA and RNA recovery. These results show the higher sensitivity of RNA- over DNA-based molecular analyses for the detection of viability of fungi on disinfected paper materials.

Considering our results, we can assume that gamma rays can be used to treat large amounts of paper simultaneously and without subsequent chemical hazard, 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 at under the danger threshold is crucial in paper conservation, and it can be achieved with gamma radiation, whereas freeze-drying can be applied only to stop heavy mould before further treatment.

Furthermore, our results point to ethylene oxide fumigation as the only effective and long-lasting treatment when the sterilization of a fungal infected paper object is needed. Subsequent contamination is, however, possible, as ethylene oxide is not a chemical that remains as a preventive biocide on materials.

It is worth emphasizing 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 characterization and diagnostics, including paleogenetic and archaeological molecular studies. Ethylene oxide fumigation was used by museums and libraries in the recent past to treat insect or mould infestations, but it is often not traceable or not clearly mentioned in the reports that accompany the treated objects. Consequently, misleading diagnostics could be produced in subsequent molecular studies. What is needed is improved documentation of the conservation history of objects of cultural value, in order to account for treatments that can alter future studies and analysis and to avoid any impediments by past treatment based on EtO.

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

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