Assessing the microbiological risk to stored 16th Century parchment manuscripts: a holistic approach based on molecular and environmental studies

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Abstract

The microbial risk for the conservation of seven 16th century parchment manuscripts, which showed brown discolouration putatively caused by microorganisms, was evaluated using non-invasive sampling techniques, microscopy, studies of surface-associated and airborne microflora with culture-independent molecular methods, and by measuring repository thermo-hygrometric values. Microscopic observations and ATP assays demonstrated a low level of contamination, indicating that the discolouration was not related to currently active microbial colonisation. Nevertheless, a culture-independent molecular approach was adopted to fully characterise surface-associated communities searching for biodeteriogens that could grow under appropriate thermo-hygrometric conditions. Indeed, potential biodeteriogens and microorganisms that are ecologically related to humans were found, suggesting the need to control the conservation environment and improve handling procedures. Microbial loads of air and thermo-hygrometric measurements showed that the repository was not suitable to prevent the microbial deterioration of parchment. A holistic approach to the assessment of risk of microbial deterioration of documents and heritage preservation is proposed for the first time.

Keywords

Parchment, biodeterioration, microbiological risk, airborne microorganisms, culture-independent methods, environmental conditions.

Introduction

Since early times, humans have strived to transmit thoughts, information and knowledge to other people and to future generations. Evidence of the intellectual and cultural efforts of the human race can be found in historical documents, including those made of parchment. The major component of parchment is collagen, an organic polymer that is susceptible to deterioration by various microorganisms, especially bacteria and fungi, and which serves as an energy and carbon source (Cappitelli et al. 2005; Michaelsen et al. 2009; Jurado et al. 2010; Sterflinger & Pinzari 2012). The microbial degradation of parchment causes various kinds of damage: fungi and bacteria with collagenolytic and proteolytic activities can hydrolyze collagen fibres and other proteinaceous molecules of parchment, and they can also act by modifying inorganic components or produce pigments causing discolouration (Pinzari et al. 2012).

The biodeterioration of historical parchment is a cause of great concern for libraries and archives all over the world (Cappitelli et al. 2010). Although the microbial attack of parchment can occur as part of a natural process, today's scientific research aims at preventive and active conservation that is aimed at slowing down the rate of deterioration significantly. Considering the health hazards of microbial contamination, the low cost of prevention compared to recovery costs, and human health and environmental concerns raised by the use of chemicals for disinfection treatments, the modern trend is to focus on preventive measures (Florian 2002; Cappitelli et al. 2005). Researchers are united in considering the following key steps as crucial to the assessment of the level of actual or potential biological risk, and to properly plan long-term conservation for historical documents: i) non-invasive sampling techniques, ii) quantification of microbial colonisation and of airborne populations in the conservation environment, iii) identification of potential biodeteriogens on surface and air by highly sensitive molecular methods, and iv) control of environmental conditions, particularly temperature and relative humidity (Michaelsen et al. 2006; Cappitelli et al. 2010; Sterflinger & Pinzari 2012). As the culture-dependent methods traditionally used in conservation detect only small amounts of effective surface-colonising and airborne organisms (Michaelsen et al. 2006), today's challenge in microbial investigations on historic documents is to characterise both airborne and superficial communities using culture-independent molecular approaches that do not affect the documents' integrity (Cappitelli et al. 2010). To date, no work has been done to face the conservation problem with such a holistic approach. In recent years, investigations of surface-associated microflora have been focused mainly on paper manuscripts (Michaelsen et al. 2006, 2009, 2010, 2012; Principi et al. 2011), while the few studies on parchment were conducted using only culture-based approaches (Pinzari et al. 2012; Kraková et al. 2012) or

invasive sampling (Jurado et al. 2010). In any case, all these studies were performed without considering either climate control or aerobiological investigations.

The present study evaluated microbial risk in the conservation of seven 16th century manuscripts written on finely illuminated parchment, reporting liturgical music used in religious ceremonies. An initial inspection of the parchment revealed two types of discolouration putatively caused by microbial colonisation: (1) brown stains all over the pages, and (2) brown rings on the bottom edges of the sheets (Figure 1).

The aims of this work were: (a) to clarify any relationship between the presence of an active microbial community and discolouration, (b) to study microbial air quality and environmental conditions in the repository, and (c) to investigate the relationship between airborne and surface-associated microbial communities. In this way it was possible to: i) supply exhaustive guidelines for the correct conservation of manuscripts and ii) set up, for the first time, a holistic and routine method to assess the risk of microbial deterioration in documentary heritage preservation, taking into consideration studies of microorganisms on the parchment surface (using non-invasive sampling) and in the air, and the environmental parameters of the repository.

This is the first time that the microbial community on historical parchment has been investigated by both non-invasive sampling and fully culture-independent approaches. Furthermore, it is the first time that a surface-associated community study was coupled with aerobiological monitoring, again by an exclusively biomolecular approach.

Materials and Methods

Sampling

Thirty-three samples from areas of discolouration, putatively caused by microorganisms, and apparently non-discoloured areas (7 samples) were collected by sterile nitrocellulose membranes following the non-invasive method reported by Principi et al. (2011), and subjected to molecular analysis. In brief: nitrocellulose membranes (Sartorius AG, Göttingen, Germany), 47 mm in diameter (corresponding to an area of 17.34 cm^2) and handled with sterile forceps, were gently pressed for 30 s onto the surface of the manuscript using sterile swabs, then immediately transferred into tubes containing phosphate buffered saline (PBS, Sigma Aldrich, Milan, Italy) and transported to the laboratory for processing.

Adhesive tape strip (Fungi Tape™, DID Milan, Italy) was used to collect samples of biological structures from stained and apparently non-discoloured parchment as described by

Michaelsen et al. (2012). The adhesive tapes were stored on sterile plates and transferred to the laboratory for microscopic analysis.

Airborne microorganisms were collected at five sites in the repository (Figure 2) in both summer and winter. Three replicates of 120 l of air for each sample were collected with a MAS-100 portable bioaerosol sampler (Merck), flow rate 100 l min^{-1}, onto Petri dishes containing two different media viz. plate count agar medium (PCA, Merck) and potato dextrose agar medium (PDA, Merck), to determine the microbial charge of aerobic heterotrophic bacteria and fungi respectively. The agar plates were kept at 28°C for 48 h. After growth, the colonies were counted and the results expressed as colony forming units per cubic meter of air (CFU m^{-3}). Two replicates of 275 l of air for each site were collected also with an AGI-30 impinger in 25 ml of sterile PBS, rate flow 4.55 l min^{-1}, as reported by Polo et al. (2012), and transported to the laboratory for molecular processing. During the air sampling days, the temperature and relative humidity were monitored with the sensor Hygrolog-D (Rotronic AY, Swiss).

Table 1 shows the details of the samples, code, location, type of discolouration and performed analyses. The sample codes are presented in the form XY.Z.(T), where X indicates the sample source (P for parchment and A for air), Y represents the manuscript (numbered 1 to 7) for samples from parchment, or the sampling season for air samples (S for summer and W for winter), Z indicates the sampling page (for samples from parchment) or the sampling sites in the repository (for air samples) (see Figure 2). Only for the samples taken from the manuscripts, T represents the page number.

Microscopic analysis of the chromatic changes

Adhesive tape strips were mounted on a microscope glass slide and fixed in 4% paraformaldehyde solution (Sigma-Aldrich) in 0.1 M PBS pH 7.2 for 2 h on ice. After three PBS washing steps, a portion of the tape confined by *in situ* frames (1 cm^2 area; Eppendorf) was stained with 100 µl of 10 mM SYTO 9, a green-fluorescent nucleic acid stain (Invitrogen) and 100 µl of 0.4 mg ml^{-1} Fluorescent Brightener 28 (Sigma-Aldrich) for 20 min in the dark at room temperature, to label bacteria and chitin in the walls of fungi, respectively. After three washes with demineralized water, all samples were observed by epifluorescence microscopy with a Leica DM 4000 B (Leica Microsystems, Milan, Italy) and images were acquired by the CoolSNAP CF camera (Photometrics Roper Scientific, Rochester, USA).

Viability assessment of colonising community

The viability of the surface-associated microbial community was assessed *in situ* by measuring the relative light units per second (RLU s^{-1}), using a surface hygiene test kit (Promicol) and a FB 14 Vega bioluminometer (Berthold Detection Systems). The surfaces studied were 17.34 cm^2 for each site investigated. The sampling was conducted by means of a circular frame corresponding to the area of the cellulose-membrane used for the non-invasive sampling of the discolourations. For each manuscript, one site apparently without discolouration was used as a control. The ATP content was measured according to the manufacturer's protocol. The RLU s^{-1} values were converted to ATP concentrations (nmol) using an ATP Standard Kit (Promicol) as standard, and thus in nmol cm^{-2}, by dividing by sampled surface values. Viability measures of each site (both with and without discolouration) were performed in duplicate. For each manuscript, the mean values, the standard error of the mean, and analysis of variance (ANOVA) were calculated using GraphPad Prism 4 to assess the significance of differences in nmol ATP cm^{-2} among several of the surfaces investigated. Differences were considered significant with P-values < 0.05. Individual comparisons were made *post hoc* with the Tukey-Kramer test.

DNA extraction and amplification

On the same day as sampling, the nitrocellulose membrane filters with the sampled cells were vortexed for 15 min to detach cells from the membrane, and centrifuged at 6000 rpm for 30 min to concentrate cells. The pellet was resuspended in 1ml of lysis buffer (EDTA 40 mM, Tris HCl 50 mM pH 8, sucrose 0.75 M) and vortexed for 10 min. Both replicates of each air sampled by impinger were filtered through a sterile polycarbonate membrane (pore size 0.2 mm), and then put into tubes with 1.8 ml of lysis buffer and vortexed for 10 min in order to detach the cells. All samples were stored at -20°C. Total DNA was extracted directly from the surface and air samples as described by Ausubel et al. (1994), with the addition of three thermal cycles -80°C/ +70°C before the addition of lysozyme to break the cellular walls.

Bacterial communities were analysed by amplifying 16S rRNA gene fragments with primers 357 F (3'-ACGGGGGGCCTACGGGAGGCAGCAG-3') and 907 R (5'-CCGTCAATTCCTTTGATGTTT-3') with the following chemical conditions: 1X of PCR run buffer, 1.8 mM of $MgCl_2$, 0.2 mM of dNTP mix, 0.3 µM of each primer, 2 µg $µl^{-1}$ of bovine serum albumin (BSA) and 1.25 U of Taq DNA polymerase (GoTaq, Promega), and a thermal cycling program as reported by Polo et al. (2010). Fungal communities were analysed by amplifying the 18S rRNA gene fragments by semi-nested PCR performed as follows: a first amplification step using the combination of primers NS1 (CCAGTAGTCATATGCTTGTC) and EF3 (5'-TCCTCTAAATGACCAAGTTTG-3') with 1X of PCR buffer, 1.8 mM of $MgCl_2$, 0.2 mM of dNTP

mix, 0.5 μM of each primer and 0.3125 U of Taq DNA polymerase (GoTaq, Promega); the cycling program consisted in an initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 45 s and extension at 72°C for 3 min, and a final extension at 72°C for 10 min. The second amplification step was performed using the first PCR product as template, with the primers NS1-GC (5'-CCAGTAGTCATATGCTTGTC -3' with GC clamp CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGCACGGG) and NS2 (5'-GAATTACCGCGGCTGCTGGC-3'). The reaction mixture was identical to first-step PCR except for 0.625 U of Taq DNA polymerase. The cycling program consisted in an initial denaturation at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 45 s and extension at 72°C for 2 min, and a final extension at 72°C for 10 min. All the PCR were performed in a final volume of 25 μl.

Denaturing gradient gel electrophoresis (DGGE) and profile analysis

The obtained amplicons were analysed by DGGE, as previously described by Polo et al. (2010). Amplicons from both superficial and air samples were loaded in the same gel to make the DGGE profiles comparable. DGGE gels were performed with 40–60% and 30–55% denaturant gradients for the bacterial and fungal communities, respectively. After excising, the DGGE bands were eluted in 50 μl milli-Q water by incubation at 37°C overnight and re-amplified with the same conditions as above, except for the absence of the GC clamp for primers. Reamplified PCR products of excised DGGE bands were purified with a QIAquick PCR purification kit (Qiagen) according to manufacturer's instructions and identified by sequencing (Primm, Milan). The sequences were analysed in September 2012 using BLASTN software (www.ncbi.nlm.nih.gov/BLAST).

The DGGE gels were run simultaneously and 16S rDNA and 18S rDNA band profiles were converted into computer digital images using the gel imaging system GelDoc (Biorad). Lanes were normalized to contain the same amount of total signal after background subtraction, and individual lanes of the gel images were straightened and aligned using Adobe Photoshop (Adobe Systems, Inc. Mountain View, CA, USA). The DGGE images were then transformed into line plot profiles using the ImageJ software (Rasband 2008), and then imported into an Excel file as x/y values. The X-axis represented distance along the line and the Y-axis was the pixel intensity. The matrix of x/y values of DGGE line profiles was analyzed using the principal component analysis (PCA). Multivariate investigations were conducted by XLSTAT (version 7.5.2 Addinsoft, France). The PCA type used during the computations was the Pearson's correlation matrix. The significance of the PCA-analysis model was tested by a cross-validation procedure.

Results

Epifluorescence microscopy

Tape samples from both the discoloured and non-discoloured surfaces showed a few microbial cells of filamentous shape. In general, most of the cells were stained with Fluorescent Brightener 28 (blue fluorescence) ascribable to eukaryotic microorganisms. Figure 3 shows the detail obtained from sample P2.139.2 (brown stain) and an apparently non-discoloured area (sample P2.139.1).

Viability assays

On most of the surfaces of the manuscripts studied, cellular activity on the discoloured surfaces showed no significant changes compared to non-discoloured surfaces (P-values > 0.1), the exception being P1.52.1 (brown ring), P5.49.1 and P7.41.1 (brown stains) where the P-values were < 0.031[2]. The ATP values were between $6.9 \cdot 10^2$ and $3.0 \cdot 10^3$ nmol cm^{-2}.

DGGE, sequencing and community profile analysis

Figure 4 shows the DGGE profiles and Table 2 reports the strains identified from sampling the manuscripts and the air. To assess the role of the microflora evidenced by 16S and 18S DGGE, the microbial communities on discoloured and non-discoloured areas were compared by PCA-analysis. A plot of the two-dimensional scores for PCA-analysis from superficial samples accounted for 83.3% of the variability in the input data for bacteria and 60.2% for fungi. The bacterial PCR product from discoloured surfaces was obtained only from samples collected on manuscript 1, the exception being sample P2.139.2 from manuscript 2. PCA-analysis showed that the bacterial communities on discoloured and non-discoloured (control samples P2.139.1 and P1.108.3) surfaces were statistically different, the exception being samples P1.10.1 and P2.139.2 (Figure 5a). The fungal PCR product was obtained only from 14 samples collected on all the manuscripts. Whilst samples from manuscripts 2-7 and control samples from non-discoloured surface presented statistically significant similar fungal communities, samples P1.1.1, P1.10.2, P1.21.2, P1.32.1, P1.52.2, P1.108.2, P2.139.2 and P3.69.1 presented separate clusters (Figure 5b).

PCA-analysis of 16S and 18S DGGE profiles from both superficial and air samples was adopted to study the relationship between airborne and surface-associated microbial communities. The plot of the two-dimensional scores for PCA-analysis from both superficial and air samples accounted for 71.8 % of the variability in the input data for bacteria and 73.4% for fungi. Whilst control samples and samples P1.32.1, P1.108.1, P2.139.2, AS.2, AW.2, AW.4 and AS.1 presented statistically significant similar bacterial communities, the other samples from air and manuscript 1

showed statistically different bacterial communities (Figure 6a). No similarity was found between fungal airborne and surface associated communities (Figure 6b).

Airborne community charges and microclimatic parameters

Cultural analyses of airborne communities showed that heterotrophic bacteria and fungi were present in the repository air. The microbial airborne loads (CFU m^{-3}), temperature (T °C) and relative humidity (RH %) monitored during the air sampling campaigns are reported in Table 3. In the semi-confined environment outside the repository (sampling site 1) the microbial airborne loads for heterotrophic bacteria and fungi were, respectively, 213±155 and 533±103 CFU m^{-3} in winter, and 486±332 and 869±164 CFU m^{-3} in summer. Inside the repository (sampling sites 2-5) the microbial airborne loads for heterotrophic bacteria were between 73 ±12 and 267±99 CFU m^{-3} in winter, and between 817±92 and 1461±141 CFU m^{-3} in summer; for fungi they were between 420 ±191 and 480±72 CFU m^{-3} in winter, and between 211±54 and 481±51 CFU m^{-3} in summer.

Discussion

In order to assess the role of the microflora dwelling on the discoloured areas, the microbial component of discoloured and non-discoloured surfaces was evaluated by microscopic observations and an ATP bioluminescence assay. The adhesive tape strip technique was chosen for microscopic analysis, as it has been shown to be a useful sampling method for monitoring microbial colonisation as well as the spatial distribution of microorganisms (Urzì & De Leo 2001; Villa et al. 2009). However adhesive tape could cause the removal of fragments from physically damaged parchment (Cappitelli et al. 2010), therefore, for the present study, only three surfaces were chosen as representative of the seven manuscripts: P4.1.2 and P2.139.2 as brown stain on page (P2.139.2 was the surface with the largest stain) and P2.139.1 as non-discoloured surface. No sample was collected by fungal tape from a brown ring as the parchment on the lower-external edges of the sheet was physically damaged (Figure 1b). Epifluorescence microscopy showed that, although in low number, both filamentous and circular structures, respectively ascribable to fungal hyphae and spores, dominated compared with bacteria. The small number of cells detected by epifluorescence microscope on both the discoloured and non-discoloured surfaces was the same, demonstrating that the discolouration on the surfaces studied was not caused by current microbial colonisation.
The determination of ATP via the firefly bioluminescence assay has been applied previously in the cultural heritage field for the detection of viable fungal spores contaminating paper documents (Rakotonirainy et al. 2003) and graphic documents (Rakotonirainy & Arnold 2008). In the present

work, no statistically significant differences of ATP content on discoloured and non-discoloured surfaces were observed for most of the surface studied. It is important to note that ATP assay kits available on the market have been developed essentially for bacteria, from which ATP is more easily extracted compared to from fungi (Rakotonirainy et al. 2003). Thus the ATP results are more representative of the bacterial component of the community. At present, ATP extraction methods for fungal cells have been devised, but have been only applied to artificially contaminated paper or fragments cut from old documents (Rakotonirainy et al. 2003; Rakotonirainy & Dubar, 2013). The ATP data are in agreement with the few bacterial cells observed by microscopic investigation. Although samples P1.52.1, P5.49.1 and P7.41.1 showed cellular activities that were significantly higher than the control surfaces, the changes were less than one order of magnitude and the mean values of ATP per cm^2 did not differ from those detected in the other manuscripts on both discoloured and non-discoloured surfaces.

Microscopy and ATP results confirmed that the discolouration on the parchment was not related to current active microbial colonisation as the primary source of damage. However, the biological origin of discolouration due to past microbial activity cannot be excluded and it is known that microbial attack can result in loss of structure and irreversible distortion and staining (Szczepanowska 2013). In addition, the cause of the discolouration may not related to biological agents. Prior to the development of the printing press, manuscripts were often written using iron gallotannate inks. Iron ions can leach from the ink to the substrate, causing brown discolouration (Brown & Clark 2002). Furthermore, degradation of the iron gallotannate ink complex can introduce a yellow to brown colour associated with oxidation to quinoid structures (Ciglanská et al. 2013).

Despite the low contamination level, it is important to study microbial communities on parchment surfaces as identification can highlight potential biodeteriogens, which could grow under favourablethermo-hygrometric conditions (Cappitelli et al. 2010; Principi et al. 2011).
A culture-independent molecular approach based on PCR-DGGE from DNA directly extracted from environmental samples was adopted to fully characterise the surface-associated communities, as reported in several works in the cultural heritage field (Michaelsen et al. 2006; Polo et al. 2012). Indeed, Jurado et al. (2010) reported the use of a culture-independent molecular method for a microbiological study on parchment, but an invasive sampling procedure was applied. In this study, nitrocellulose membranes were used to collect cells because they provided a non-invasive sampling procedure, which has already been successfully applied on frescoes and paper (Pitzurra et al. 1999; Principi et al. 2011). To date, no study coupling a non-invasive membrane-sampling procedure with a culture-independent molecular method has been reported for parchment. The statistical

approach based on PCA-analysis was used to study the structural changes in the bacterial and fungal communities between discoloured and non-discoloured surfaces evidenced by the 16S and 18S DGGE profiles, as previously reported by Principi et al. (2011). On manuscript 1, the bacterial community of the greater part of the samples from both brown stains and brown rings was dissimilar to that of the non-discoloured surface. Fungal communities were detected on all the manuscripts, in agreement with the microscopy analysis. Only the fungal samples from manuscript 1 presented statistically significant differences, compared to the non-discoloured surface. Of the manuscripts studied, manuscript 1was the only one that had recently undergone conservation treatment. As conservation treatments involving organic materials (eg as adhesives and consolidants) could potentially support the growth of microorganisms, thus accelerating the biodeterioration process (Cappitelli et al. 2010), the results suggested that the conservation treatment might have contributed to conditions favourable for microbial growth. Although in the present investigation work no data were available for the microbial communities prior to the conservation work, such information should be taken into account in further studies. The sequences obtained in this study were phylogenetically most closely related to bacteria belonging to *Burkholderia thailandensis*, Betaproteobacteria, *Methylobacterium* sp., *Microbacterium* sp., *Lactobacillus* sp., *Sphingomonas* sp. and *Aeribacillus* sp., and fungi belonging to *Aspergillus* sp. and *Candida* sp.. Although species belonging to Betaproteobacteria have been mainly isolated from soil and water (Wongprompitak et al. 2008), *Burkholderia thailandensis* has been isolated from breathing apparatus (Glass et al. 2006), therefore its presence on the surface of manuscript 1 could be of human origin. Members of the genus *Methylobacterium* are aerobic phototrophic bacteria distributed in a wide variety of natural habitats, including soil, dust, air and fresh water as well as in man-made environments. Because of carotenoid and photopigments production (Hiraishi et al. 1995), *Methylobacterium* strains could potentially be responsible for discolouration. Strains belonging to the genus *Microbacterium* have been isolated from coloured stains on historical documents made of parchment (Kraková et al. 2012). Furthermore, they have proteolytic properties (Kraková et al. 2012) and can colonise subsurface layers along collagen fibres (Petushkova & Koesler 1999). Fermenting bacteria belonging to the genus *Lactobacillus* are common inhabitants of the human gastrointestinal tract as well as of the oral cavity (Müller et al. 2001; Walter & Ley 2011), so their presence on both the sheets and the external edges of the manuscript could be of human origin. *Sphingomonas* have been isolated from biofilm on deteriorated bas-relief walls (Lan et al. 2010) and mural paintings (Heyrman & Swings 2001), and they were reported as being responsible for the degradation of ceramic tiles covered by a green and/or black patina (Coutinho et al. 2013) and waterlogged archaeological wood (Landy et al. 2008; Palla et al. 2013). Bacteria

belonging to *Aeribacillus* sp. have never been isolated on cultural heritage and historical documents. However, the role of *Aeribacillus* spp. in the fermentation of starch, a substance often used in parchment conservation, makes these bacteria potentially dangerous (Fratkin & Adams 1946; Woods 1995). Fungi belonging to the genus *Candida* are commonly isolated from human skin and mucosal surfaces as harmless commensal organisms (Samaranayake & MacFarlane 1990; Heo et al. 2011) and their presence on manuscript 1 can be ascribed to the hand of readers as the manuscript was in religious ceremonies and has, in the past, been subjected to conservation treatment. *Aspergillus* species are common biodeteriogens of organic and synthetic materials (Cappitelli & Sorlini 2005) and are frequently associated with paper spoilage as they are able to degrade cellulosic materials and cause discolouration (Pinzari et al. 2006; Zotti et al. 2008; Principi et al. 2011). Members of the genus *Aspergillus* are also known as the most active biodeteriogen agents on both ancient and modern parchments (Polacheck et al. 1989; Matè 2002). Aspergillus spp. secrete a range of pigments and proteolytic enzymes that respectively cause aesthetic and chemical damage, whilst hyphal growth exerts mechanical pressure on the substrate, causing weakness (Cappitelli & Sorlini 2005; Michaelsen et al. 2010; Kraková et al. 2012). *Aspergillus* spp. were isolated from both brown stains on all the manuscripts and from the brown rings of manuscript 1. *Aspergillus fumigatus* is one of the most ubiquitous airborne saprophytic fungus. Being a xerophilic and xerotolerant fungus, it has often been isolated from the indoor aeromycoflora of libraries (Zielinska-Jankiewicz et al. 2008), museums (Gaüzère et al. 2013) and hospitals (O'Gorman, 2011). The proteololytic activity of *A. fumigatus* represents a potential risk for library materials as it might hydrolyse different complex proteins available as substrates, including collagen (Lee & Kolattukudy, 1995; Farnell et al. 2012). Besides all these potential effects on manuscript, handling mould-contaminated objects constitutes a health risk as *A. fumigatus* is an opportunistic human pathogen (O'Gorman, 2011; Pinheiro et al. 2011). Interestingly, there are other case studies in the literature reporting the identification of only one fungal genus, i.e. *Aspergillus* or *Penicillium*, in the air of archives (Borrego et al. 2010; 2012).

Although in this research the suitability and usefulness of using non-invasive sampling methods and molecular techniques to determine the presence and diversity of bacteria and fungi on heritage material has been demonstrated, these methods also have some drawbacks. The molecular approach can be time-consuming, requires skilled personnel and is often expensive (Cleeland et al. 2013) and a major intrinsic limitation of non-invasive techniques is that microorganisms growing in the substrate without producing emerging structures cannot be collected (Cappitelli et al. 2010). The study of the microbial communities on the manuscript surfaces was undertaken together with the study of the microbial airborne communities and the environmental conditions in close proximity to

where the manuscripts are stored as well as outside the repository. It is widely reported that microbial cells reach surfaces mainly through transport in air (Borrego et al. 2010) and that a close relationship exists between culturable airborne microorganisms and the microflora colonising paper documents (Cappitelli et al. 2010). Aerobiological and thermo-hygrometric investigations of conservation environments are therefore helpful in choosing interventions aimed at preventing the microbiological deterioration of historical documents. In the present work, the air-sampling procedure and the study of airborne microbial community was carried out without any cultivation step, in order to fully investigate the source of surface-associated biodeteriogens. This is the first time that such an approach has been proposed for historical document repositories. The microbial community fingerprints from DGGE gels were compared by PCA-analysis to investigate the relationship between surface-associated and airborne communities. The analysis showed the lack of a clear relationship between bacterial communities in air and on manuscript 1, and although some samples from both the brown stains and the rings were similar to some airborne communities detected inside and outside the repository during both winter and summer, most samples showed no such similarity. In addition, PCA-analysis excluded the presence of a relationship between fungal communities in air and on manuscript surfaces. This lack of similarity was in contrast to data based on conventional cultural-dependent methods (Cappitelli et al. 2010). Indeed, Polo et al. (2012) in a culture-independent investigation excluded a close similarity between airborne and surface-associated microflora on stone surfaces in an outdoor environment. There could be several reasons for this: the culture-based approach greatly limits the microflora that can be studied; the diluted concentrations of airborne microorganisms make detection difficult; environmental factors cause important quantitative and qualitative changes of airborne communities in space and time and the chemical and physical features of surfaces select colonising microorganisms.

The sequencing of bands of airborne microflora demonstrated that in addition to microorganisms belonging to *Burkholderia*, Betaproteobacteria, *Methylobacterium*, *Microbacterium*, *Sphingomonas* and *Aeribacillus* identified on the discoloured surfaces, airborne communities also included bacteria belonging to *Mesorhizobium*. *Mesorhizobium* sp. is a soil bacterium (Gonzàlez-Lòpez et al. 2005) and is most likely present because the repository is located in a rural area. *Aspergillus* was the only fungal genus detected inside and outside the repository in both winter and summer. *Aspergillus* spp. have been isolated from the air of indoor and outdoor environments (Arya et al. 2001; Borrego et al. 2010; Vanhee et al. 2010; Docampo et al. 2011; Borrego et al. 2012), and are considered primary colonizers: when the relative humidity increases, and indoor environments are without ventilation for long periods of time, conidia can be deposited quickly over documents and deteriorate them (Borrego et al. 2012).

Airborne *Methylobacterium*, *Microbacterium*, *Sphingomonas* and *Aspergillus* spp. are potential biodeteriogens. However, air microbiota are known to coexist with document collections without causing significant damage under suitable conditions of temperature and relative humidity. However, if there is an increase in thermohygrometric values, microorganisms can accelerate deterioration (Cappitelli et al. 2009; Borrego et al. 2012). Furthermore, parchment absorbs water, expanding when the relative humidity rises and shrinking when it falls (Pavlogeorgatos 2003; Giacometti et al. 2012). Therefore suitable thermo-hygrometric conditions inside the repository are crucial. The UNESCO program 'Memory of the World' (UNESCO 1995) suggests 18 °C (maximum daily variability of 2°C) and 50-60% relative humidity (maximum daily variability of 5%) as optimal values to prevent the deterioration of parchment (UNESCO 2000). At present, the repository environment is not suitable to protect the parchment as in both winter and summer the temperatures are respectively below and above the threshold values. In addition, the microclimatic parameters are subject to marked seasonal change, possibly because the windows of the repository are not fully insulated, allowing the outdoor environment to contribute to temperature and humidity fluctuations.

Microbial loads inside and outside the repository (confined and semi-confined environment, respectively) were determined, because current museum regulations consider only microbial load thresholds in order to establish air contamination. Indeed, the museum standards set down by the Italian Ministry of Cultural Heritage (MIBAC 1998) recommend less than 750 CFU m^{-3} of heterotrophic bacteria and less than 150 CFU m^{-3} of fungi for museum indoor environments. The aerobiological monitoring that was carried out showed that in winter, the bacterial loads did not exceed the limits either inside or outside the repository; instead the fungal loads always exceeded the threshold values at all the sampling sites (both in the semi-confined and confined environment). During the summer, the microbial load (both bacteria and fungi) exceeded the threshold values at every sampling site, with the exception of the bacterial load outside the repository door. The microbial load outside the repository door during both summer and winter were about one order of magnitude less than that reported by other similar investigations conducted in outdoor environments (Cappitelli et al. 2009). Instead, inside the repository the microbial counts agreed with those reported in investigations conducted in other indoor environments (Cappitelli et al. 2009; Borrego et al. 2012).

In summary, microscopic and viability assays currently demonstrate that biodeterioration does not represent a threat to the conservation of the manuscripts. Nevertheless, to ensure long-lasting conservation of the manuscripts, the marked differences in the presence of microbes between winter and summer (probably due to inadequate controlled environmental conditions), the presence

of potential biodeteriogens and an opportunistic human pathogen and the retrieval of microorganisms related to the human body led to the following proposals: i) environmental remediation of the repository; ii) routine monitoring of air and surfaces; and iii) improvement of handling procedures. In order to prevent seasonal fluctuations in thermo-hygrometric conditions, the door and windows of the repository should be insulated and adequate climate control equipment installed. Furthermore the microclimatic parameters and the microbial contamination on the surface of heritage objects, and the surrounding air should be monitored from both the quantitative and qualitative points of view at least twice a year, corresponding to the winter and summer seasons. Finally, from now on, the manuscripts should be handled only using gloves.

While in this specific case study the number of microorganisms detected was only small, it is possible that there could be extensive colonization of parchment surfaces by a microbial community that could actively attack collagen. To obtain information about the biodegradation potential of the microbial communities present on objects, molecular techniques could be used. Indeed, bacteria and fungi capable of degrading parchment produce a group of enzymes called collagenases (Talwar & Srivastava 2003). A molecular approach, based on the use or design of primer/probe sets specific for collagenase gene identification could be adopted to evaluate the potential ability of the community to degrade collagen (Tsuruoka et al. 2003; Sadikot et al. 2005). Today, the need for integrated microbiological risk management, supported by well-managed information, is crucial to collection institutions where human and financial resources are often limited. The coupling of the fast detection of viable microbial colonization with more selective molecular techniques has proved effective for a quick and exhaustive inspection of both surfaces and air quality. In this respect, the present work promotes the proposal of guidelines for the correct management of historical documents, in order to preserve them from microbiological attack and, in turn, to ensure long-lasting conservation.

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Figure 1. Brown stain on pages (a) and brown ring on lower-external edges of sheets (b).

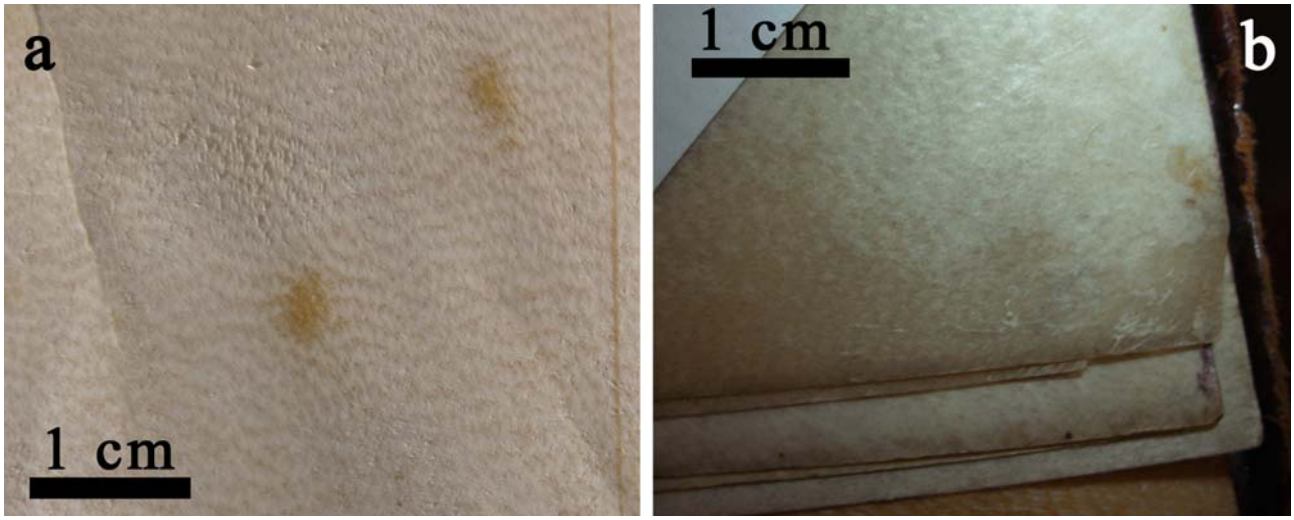

Figure 2. Repository plan. The rectangle inside the repository indicates the bookcase. Numbers indicate the air sampling sites both outside (1) and inside (2-5) the repository door.

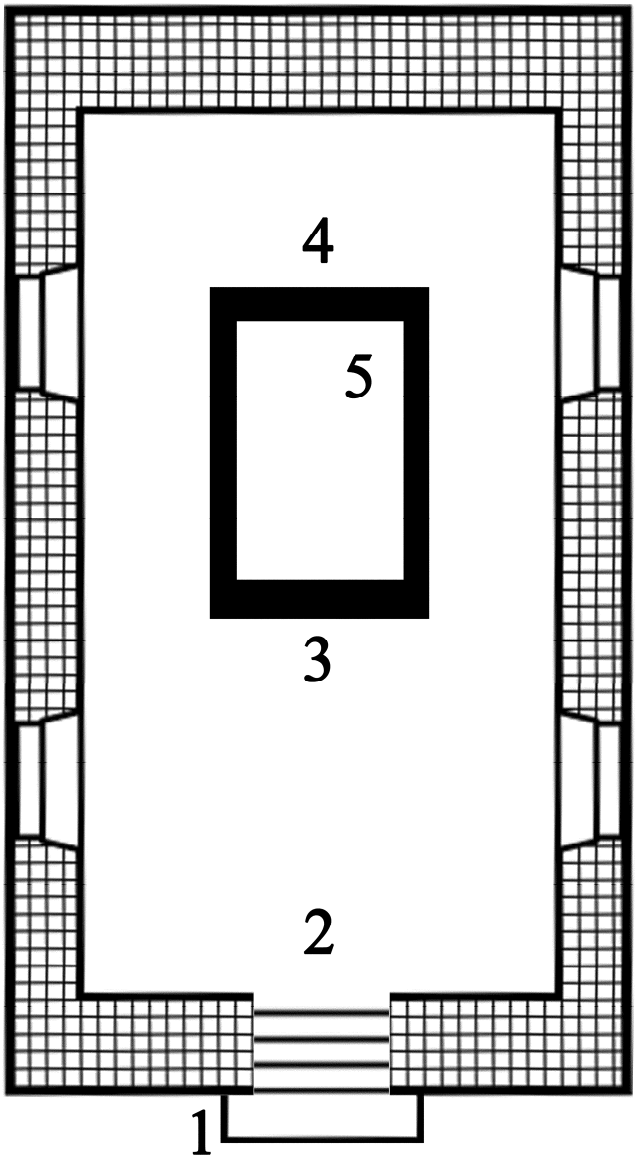

Figure 3. Cells stained with Fluorescent Brightener 28 (blue fluorescence) and SYTO 9 (green fluorescence) on tape samples: bright field (a) and epifluorescence (b) microscope images of apparently non-discoloured area (sample P2.139.1); bright field (c) and epifluorescence (d) microscope images of P2.139.2 showing brown stain.

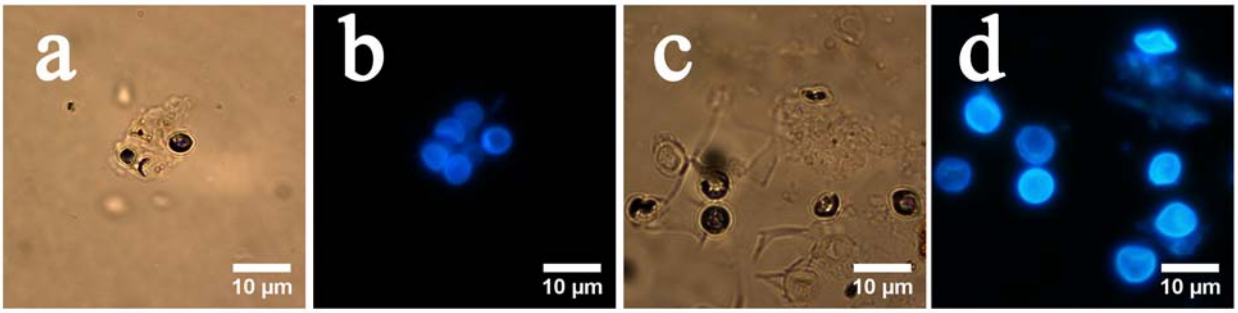

Figure 4. 16S (a) and 18S rRNA gene (b) DGGE profiles of the samples (P2.139.2, P1.21.1, P1.21.2, P1.32.2, P1.42.1, P1.103.3, P1.52.1, P1.52.2, P1.108.1, P1.108.2, AW.1, AW2, AW.3, AW.4, AW.5, AS.1, AS.2, AS.3, AS.4, AS.5, P1.108.3 and P2.139.1 for 16S DGGE profiles; P2.139.2, P3.69.1, P6.1.3, P5..49.1, P4.1.1, P6.1.2, P2.139.1, P1.10.1, P1.1.1, P1.21.2, P1.32.1, P1.52.2, P1.108.2, AW.1, AW.2, AW.3, AW.4, AW.5, AS.1, AS.2, AS.3, AS.4 and AS.5 for 18S DGGE profiles).

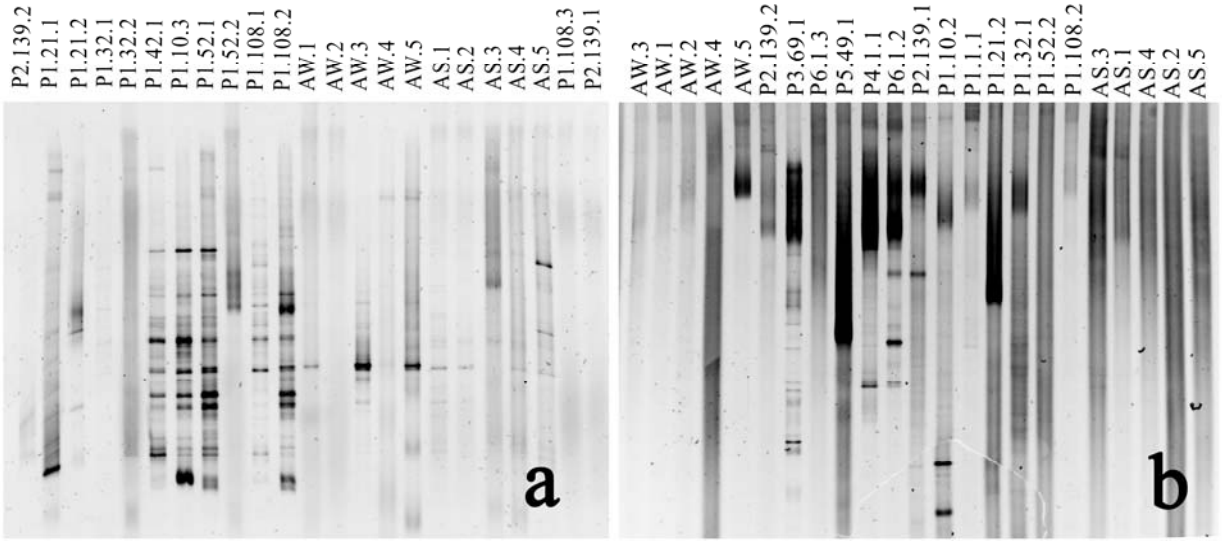

Figure 5. bacterial (a) and fungal (b) DGGE band patterns from samples collected on parchment surface. In panel (a) ■ represents sample P1.108.3 from the non-discoloured surface of manuscript 1 and P2.139.1 from manuscript 2; in panel (b) ■ represents the samples from non-discoloured surface of the manuscripts from 1 to 7.

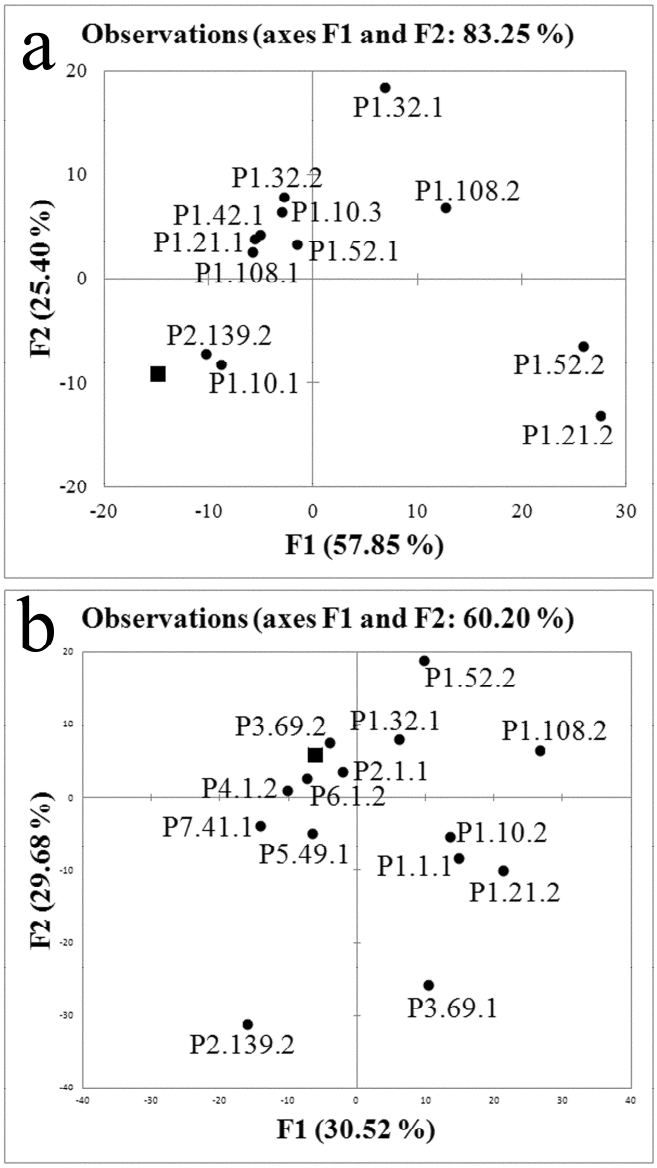

Figure 6. PCA-analysis of bacterial (a) and fungal (b) DGGE band patterns from parchment surface and air samples. In panel (a) ■ represents the sample P1.108.3 from the non-discoloured surface of manuscript 1 and P2.139.1 from manuscript 2; in panel (b) ■ represents the samples from non-discoloured surfaces of the manuscripts from 1 to 7.

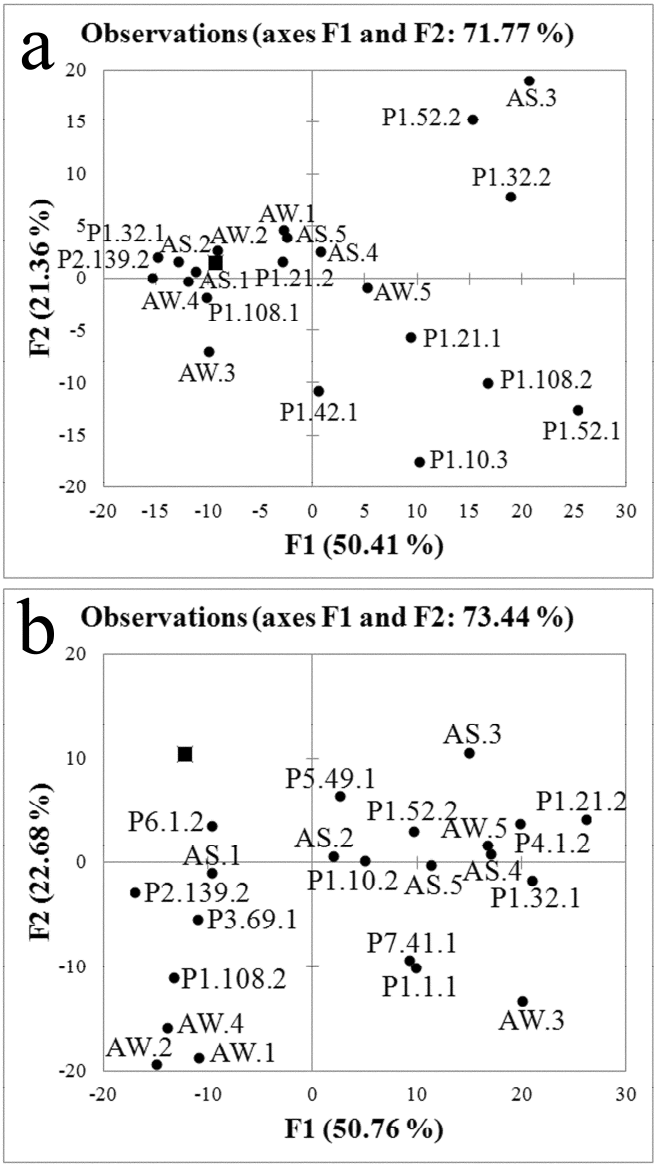

Table 1. Samples collected for microbiological analyses: sample code, type of discolouration, location and analyses. The sample codes are presented in the form XY.Z.(T), where X indicates the sample source (P for parchment and A for air), Y represents the manuscript (numbered 1 to 7) for samples from parchment, or the sampling season for air samples (S for summer and W for winter), Z indicates the sampling page (for samples from parchment) or the sampling sites in the repository (for air samples). Only for the samples taken from the manuscripts, T represents the page number.

TYPE OF SAMPLE (CODE XY.Z.(T)), POSITION AND ALTERATION				TYPE OF ANALYSIS			
SURFACE			AIR				
ON THE PAGE		LOWER-EXTERNAL EDGES OF SHEET		MICROSCOPY	CELL VIABILITY	MOLECULAR METHODS	MICROBIAL LOAD
NON-DISCOLOURED SURFACE	BROWN STAIN	BROWN RING					
		P1.1.1 P1.10.1 P1.21.1 P1.32.1 P1.42.1 P1.52.1 P1.108.1 P2.1.2 P2.139.3 P3.69.3 P4.1.1 P5.30.2 P5.49.2 P6.1.1 P7.41.2 P7.77.22			X	X	
	P1.10.2 P1.10.3 P1.21.2 P1.32.2 P1.52.2 P1.108.2 P2.1.1 P3.69.1 P3.69.2 P5.30.1 P5.49.1 P6.1.2 P7.41.1 P7.77.1				X	X	
	P2.139.2 P4.1.2			X	X	X	
P2.139.1				X	X	X	
P1.108.3 P3.69.4 P4.1.3 P5.30.3 P6.1.3 P7.77.3					X	X	
			AS.1 AS.2 AS.3 AS.4 AS.5 AW.1 AW.2 AW.3 AW.4 AW.5			X	X

Table 2. Identification of 16S and 18S gene sequences of DGGE profiles from brown stains (BS) and brown ring (BR) on several manuscripts (P1-P7), and air during summer and winter, both inside (in) and outdoors (out) of the repository. (B) indicates the control sample from a non- discoloured area. X and - indicate, respectively, presence or absence of the strain.

Samples																		BlastN reference strains			RDP tassonomic Classifier	
P1		P2		P3		P4		P5		P6		P7		Air summer		Air winter		B	Closest relative strain	Accession number	Similarity (%)	Most probable taxon	Similarity (%)
BS	BR	BS	BR	BS	BR	BS	BR	BS	BR	BS	BR	BS	BR	in	out	in	Out						
X	X															X			*Burkholderia thailandensis*	DQ388537	99	*Burkholderia*	100
	X															X	X		Uncultured Betaproteobacterium	JQ937380	98	Betaproteobacteria	100
																X	X		Uncultured *Methylobacterium* sp.	HM565053	96	Rhizobiales	100
																X	X		Uncultured *Mesorhizobium* sp.	GU271769	96	Rhizobiales	100
X	X															X	X		*Methylobacterium* sp.	EU303272	99	*Methylobacterium*	100
X	X																X		*Microbacterium* sp.	JF279926	99	Microbacteriaceae	100
X	X																		*Lactobacillus sanfranciscensis*	JN863669	98	*Lactobacillus*	87
X	X																X		*Microbacterium* sp.	JX434128	98	Micrococcineae	100
	X													X		X			*Sphingomonas* sp.	JQ917912	99	Sphingomonadaceae	100
X	X														X	X			Uncultured *Methylobacterium* sp.	JF274019	98	*Methylobacterium*	100
X	X														X	X			*Aeribacillus* sp.	KC551235	100	*Aeribacillus*	98
X	X	X		X		X		X		X		X		X	X	X	X	X	Uncultured *Aspergillus* sp.	AJ635506	99		
X	X																		*Candida* sp.	HM161746	99		
	X																		*Aspergillus* sp.	HE814598	99		
X	X													X	X	X			*Aspergillus fumigatus*	JQ665711	100		
X	X																		*Candida albicans*	JN940588	99		

Table 3. Microbial airborne loads (CFU m^{-3}), Temperature (T°C) and Relative Humidity (RH%) values registered at the air sampling sites (see Figure 2) during sampling days in winter and summer.

Sampling site	Winter				Summer			
	Bacteria (CFU m^{-3})	**Fungi (CFU m^{-3})**	**T (°C)**	**UR (%)**	**Bacteria (CFU m^{-3})**	**Fungi (CFU m^{-3})**	**T (°C)**	**UR (%)**
1	213±155	533±103	6.7	27.4	486±332	869±164	24.5	67.7
2	73±12	420±191	5.2	45.5	1461±141	481±51	24.3	64.6
3	147±81	480±72	4.0	49.9	1292±258	394±5	24.3	62.4
4	207±136	440±80	2.1	61.2	1397±59	364±34	27.7	60.8
5	267±99	420±92	4.1	54.6	817±92	211±54	24.4	62.9