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Chemical and biological characterization of paper: A case study using a proposed methodological approach

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ABSTRACT

One of the most important causes of paper degradation is biodeterioration, caused especially by fungi and bacteria under certain environmental conditions. This study was designed to check the presence of cellulolytic microorganisms on documents from the 19th century, and to verify any biodeterioration that may have occurred by using a series of predominantly non-invasive analyses in order to preserve their integrity. For the characterization of paper documents various non-destructive micro-analyses were performed in succession, so as to deepen our knowledge of these step by step and thus provide a logical approach for the general study of this type of artifact. The samples were analyzed by using light microscopy, histochemical tests, pH analysis, SEM–EDS observations, Fourier transform infrared (FTIR) spectroscopy in ATR, and GC–MS. Organic cultures were also made with the samples taken from the surfaces of the documents; in particular, four fungal species, *Aspergillus* sp., *Penicillium* sp., *Cladosporium* sp., and *Ulocladium alternarie*, have been identified, as well as the presence of bacteria and dust mites. Finally, relations/correlations between the results of chemical and biological analyses are discussed.

1. Introduction

Like all art materials, paper is subject to natural aging. Its deterioration can be caused by physiochemical processes, such as oxidation, hydrolysis, photo-deterioration, and fiber deformations (Carter, 1996; Kolar, 1997; Bogaard and Whitmore, 2002; Mantovani, 2002), but further degradation can have biological causes and in this case it is called biodeterioration.

Paper biodeterioration can be the result of many kinds of attack, which may occur directly or indirectly after natural events, such as earthquakes or floods, or after incorrect handling of the documents during consultation or transport (Altibrandi, 2002). Once it is produced, paper is a micro-habitat for microorganisms, as they can use it as a food source. In fact, with additional compounds such as animal or vegetable glue, inks, pigments, and bindings, paper is a very good source of organic carbon for many heterotrophic organisms and microorganisms. Thanks to its hygroscopicity, it favors the growth of microorganisms (Dhawan, 1986; Nyuksha, 1994; Pinzari et al., 2006). The main microorganisms that attack paper are bacteria and micro-fungi belonging to cellulolytic species and also non-cellulolytic species with a non-specific action.

The cellulolytic bacteria species responsible for degradation are Schizomycetes such as *Pseudomonas*, *Cellulomonas*, and *Cellvibrio*, Myxobacteria with *Cytophaga* and sporocytophaga, and finally Actinomycetes, with *Streptomyces* and *Nocardia* (Gallo, 1992; Altibrandi, 2002; Pasquariello et al., 2005).

The most frequent types of damage are caused by fungi, as they show great tolerance to a range of environmental conditions. They can germinate under conditions of low relative humidity (RH) from 60% upwards, and can tolerate a water content of the substrate much lower than that tolerated by bacteria. Some cellulolytic species of *Aspergillus* and *Penicillium* are able to grow on a substrate with a water content of 7–9%, which is the equivalent of an RH of 62–65% (Gallo, 1985). Most of the cellulolytic fungi belong to Deuteromycetes such as genera *Alternaria*, *Aspergillus*, *Fusarium*, *Myrothecium*, *Penicillium*, *Stachybotrys*, *Stemphylium*, *Trichoderma*, *Trichothecium*, and *Ulocladium*, and to Ascomycota such as *Chaetomium*, or Zygomycetes such as *Mucor* and *Rhizopus* (Gallo, 1985, 1992; Nyuksha, 1994; Zyska, 1997; Florian and Manning, 2000; Montemartini Corte et al., 2002; Pasquariello et al., 2005).

Microbial growth, whether fungal or bacterial, appears primarily as a discoloration, with the formation of patches of different colors (purple, yellow, brown, black, red, and green), shapes, and sizes, caused by the presence of pigmented mycelium or fungal spores or by exopigments produced by bacteria and fungi. Among discoloration processes, the best known is called foxing,

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and it is referred to by many authors (Press, 1976; Cain and Miller, 1982; Nol et al., 1983; Gallo and Pasquariello, 1989; Gallo, 1992; Nyuksha, 1994; Florian, 1996, 2000, 2002; Choisy et al., 1997; Arai, 2000; Florian and Manning, 2000; Montemartini Corte et al., 2002; Buzio et al., 2004; Pinzari et al., 2006; Zotti et al., 2007). This phenomenon occurs in the form of small isolated patches of discoloration that is typically rust, brown, or yellowish; it is usually limited in size, with sharp or jagged edges; it is often punctiform and sometimes circular. The observation of these spots under ultraviolet light shows them as fluorescent white, yellow, or blue (Matè, 2002).

Studies during the past 60 years have shown that it is not possible to single out a unique cause for the fox spots, but rather that their presence is influenced by various circumstances, such as the kind of paper or its conservation history. Current research focuses on four main possible causes of foxing: fungal activity, metal-induced degradation, general discoloration of paper caused by the synergy of moisture and cellulose, and multiple causes, such as interactions between the first two mentioned (Bertalan, 1994).

Structural alterations of the basic components of materials can also be present, caused by enzymes (cellulase, protease, and so on) produced by different types of microorganisms that contribute to the fragility of the paper and eventually cause its destruction. Finally, there may be changes in essential additive components of paper caused by microorganism attacks on substances such as plasticizers or adhesives, which may cause the loss of certain features of the media, to the point of making them unusable (Gallo, 1992).

By simple observations with an optical microscope it is possible to detect the presence of organic matter. Unfortunately this type of art medium, i.e., the paper, is very fragile and difficult to analyze with normal chemical–physical analyses. The preciousness of the manuscripts or documents also has to be considered, so it is often difficult to have enough material available for destructive analysis (such as GC–MS or FT-IR). For these reasons, this work suggests an initial cognitive approach based on the conservation status of the documents using non-destructive or micro-invasive techniques.

This research is concerned with the analysis of two documents that belong to the Royal Palace correspondence archives, stored in a binder in the archives of the Superintendent of Architectural, Natural, Historic, Artistic, and Ethno-Anthropological Heritages of Venice and its Lagoon, located in the Venetian Ducal Palace. They are both dated 1854 and are interesting due to their poor conservation state, with stains of many sizes and colors that are presumably of an organic origin. Several non-invasive or micro-invasive techniques were used, following a precize order so as to deepen our knowledge of these documents in a step-by-step fashion. Specifically, observations were made using optical microscopy, histochemical tests were carried out to identify the composition of paper items, and the pH of the documents was (Botti and Ruggiero, 2002). Analyses by SEM–EDS were used to elucidate the micro-anatomy of the document surfaces and reveal the possible presence of foreign substances (Florian, 1996, 2000; Arai, 2000; Pinzari et al., 2006; De Mico et al., 2008; Michaelsen et al., 2009). The examination of the documents using ATR FT-IR allowed the identification all the matrix components of the paper, including foreign substances, and their possible degradation products (Sistach et al., 1998; Garside and Wyeth, 2003; Buzio et al., 2004; Ganzerla et al., 2008; Zotti et al., 2008b), and, finally, GC–MS analyses highlighted the presence of degradation products of the paper material used (Colombini et al., 2002, 2007; Ganzerla et al., 2008).

Parallel to these chemical–physical analyses, microbiological tests were carried out to verify the presence, over the documents' surfaces, of cellulolytic microorganisms that could be implicated in the paper's degradation (Montemartini Corte et al., 2002; Zotti et al., 2007, 2008a; Michaelsen et al., 2009). Finally, we attempted to find relationships and correlations between the two types of analysis.

2. Materials and methods

2.1. Paper sample description

Two documents dated from the 19th century come from the archives of the Royal Palace, and are now preserved in the archives of the Superintendent of Architectural, Natural, Historic, Artistic, and Ethno-Anthropological Heritages in Venice and its Lagoon in the Ducal Palace of Venice, Italy.

The first document, called *Leaf 1*, is a double sheet with the heading "Imp. Regia Direzione delle Poste." It is from Padua and dated 1854. The second document, called *Leaf 2*, is a double sheet also dated 1854 with the heading "Palazzo Reale di Strà." These documents were stored together inside a large folder with other documents and are very interesting for this study in that they are heavily stained, with stains of different shapes, colors, sizes, and dispersion, on their surfaces.

For each document different samples were selected based on visual observation of the different colors and morphology (Figs. 1 and 2). Among these stains it was possible to recognize the fox spots typically associated with paper biodegradation (Table 1 and Fig. 3).

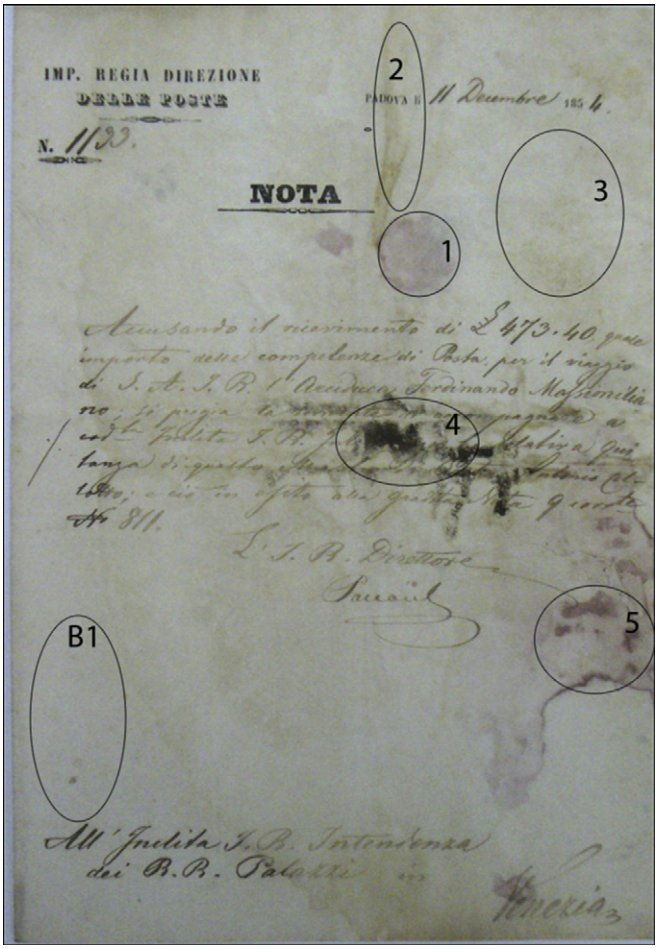

Fig. 1. Sampling areas of Leaf 1.

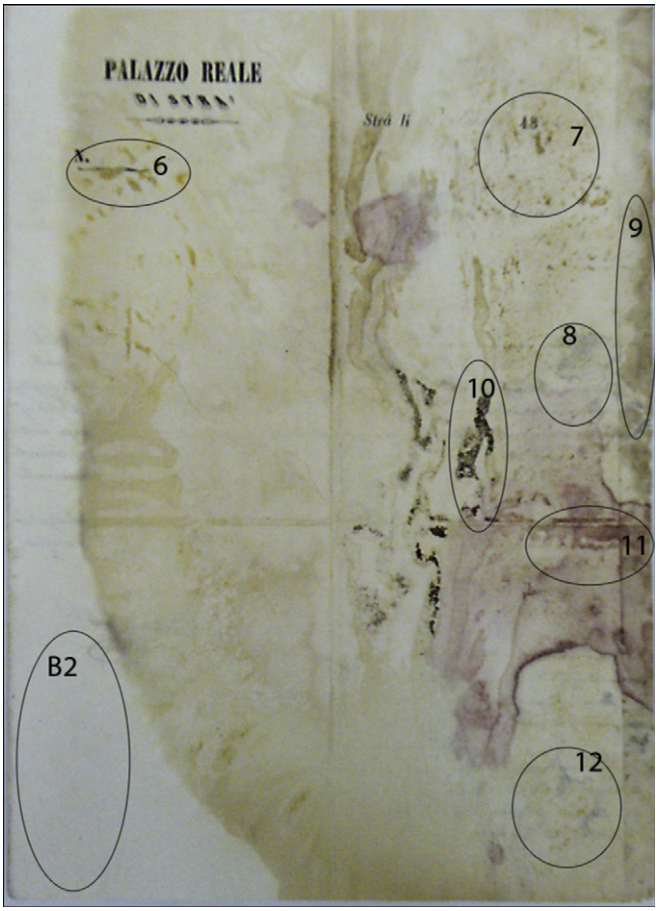

Fig. 2. Sampling areas of Leaf 2.

Every stain was subjected to chemical–physical and microbiological analysis.

2.2. Optical microscope observations

The optical microscope observations constituted a first step in identifying the composition of the papers, particularly their morphology, as well as characterizing the fiber colors, the encrusting substance, and the stains. They were also important in determining the presence of other materials, such as substances of organic origin.

Table 1
Descriptions of sampling areas of Leaf 1 and Leaf 2.

Document	Samples	Descriptions
Leaf 1	B1	Blank 1
	1	Circular pink halo
	2	Vertical brown strip
	3	Brown stains (fox-spots)
	4	Central dark brown stain
	5	Pink halo
Leaf 2	B2	Blank 2
	6	Ochre stains with black points
	7	Homogeneous brown stains
	8	Gray stain
	9	Dark brown right border
	10	Vertical dark brown strip
	11	Pink stain
	12	Sporadic ochre stains

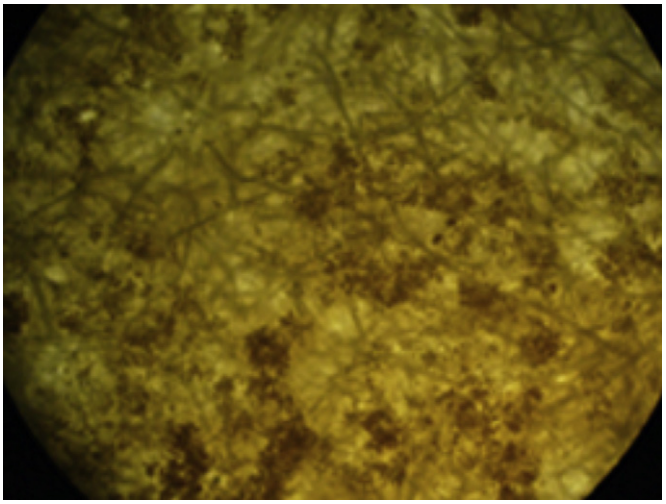

Fig. 3. Sample 3, foxing area, 100×. Brown-ochre covering with a dusty aspect. (For interpretation of the references to color in this figure legend,the reader referred to the web version of this article.)

To carry out these observations, every leaf was positioned inside the optical microscope Axio plan (Zeiss, Germany) and sampling areas were observed (100–400× magnification) without any pretreatments.

2.3. Histochemical tests

The histochemical analyses were based on colorimetric reactions and were carried out to identify the presence of certain constituent organic and inorganic substances and their positions inside every handmade artifact. These reactions occur directly on small areas of the paper surfaces. The presence/absence of lignin, starch, rosin glue, animal glue, and alum was checked by carrying out the following specific tests: Hertzberg, Graff's, Raspail, and Alizarina S (Sistach, 2002).

The Hertzberg test is performed with zinc chloride (Sigma–Aldrich, St. Louis, MO, USA), potassium iodide (JT Baker, Deventer, Netherlands), and iodine resublimed (Carlo Erba Reagents, Milan, Italy); it colors paper depending on the amount of lignin present: Chemical pulps from wood and most grasses stain blue; rag stains pink–purple; alpha-cellulose pulp stains a reddish color, and mechanical pulp stains yellow. Graff's test is performed with $CaSO_4$ (JT Baker, Deventer, Netherlands), NaOH (Carlo Erba Reagents, Milan, Italy), and C_2H_5OH (JT Baker, Deventer, Netherlands), and it turns the paper purple in the presence of gelatin (animal glue). The Raspail test is performed with sucrose (BDH, Milan, Italy) and H_2SO_4 (Fluka, Sigma–Aldrich, Milan, Italy) and in the presence of rosin it turns the paper red for a few seconds. Finally, the Alizarina S test is performed with Alizarin S (Sigma–Aldrich, St. Louis, MO, USA), NaOH (Carlo Erba Reagents, Milan, Italy), and CH3COOH (Carlo Erba Reagents, Milan, Italy) and it turns paper violet in the presence of alum.

2.4. Analysis of pH

Paper pH knowledge is a simple but very useful tool to define both the paper conservation status and the possible disinfection and restoration interventions to be carried out.

The method used determines by a potentiometric measurement the pH value of a thin water layer laid on a surface. The water allows the substances present in the superficial paper layer to cross into the water solution. These substances influence pH values.

The pH value was determined by using an HI 99171 portable pH meter (Hanna Instruments, Italy) with an HI 1414D flat membrane glass electrode. After calibration (buffer solutions at pH 7.01 and pH 4.01) measurements were carried out in different areas of both the leaves, by using an HI 70960 electrolytic solution (Hanna Instruments, Italy) to ensure the best contact between the probe and the paper surface.

2.5. Scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM–EDS)

Scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM–EDS) allowed the production of images of the sample surfaces at a very high magnification and at the same time the determination of the major elemental components of the sample. Small pieces of every sampling area were cut from the pages using sterile scissors and were placed, using sterile tweezers, face up on two-sided sticky tape placed on aluminum SEM stubs. Then, every sample was coated with gold using a Technics Hummer V sputter coater (Technics Inc., Baltimore, MD) and examined with a JSM-5600LV (JEOL, Tokyo) scanning electron microscope. The SEM images resulted from an SEI detector using high vacuum and a voltage of 20 kV for investigation of atomic elemental compositions.

2.6. Fourier transform infrared spectroscopy (FT-IR)

All sampling areas were considered and their FT-IR spectra were obtained in attenuated total reflection mode (ATR) using a Nicolet Nexus spectrometer (Thermo Scientific, Waltham, MA, USA) equipped with a single reflection ATR cell and a ZnSe crystal. All the spectra were acquired in the range of 4000–650 cm^{-1} with 96 scans at a resolution of 4 cm^{-1}.

Spectra were recorded and analyzed using OMNIC™ software (ver.6.0). The identification of some paper components was done by comparing the main features of the obtained spectra with those of selected standards and with known spectra (Buzio et al., 2004; Zotti et al., 2008b). The ATR mode allowed a complete non-destructive analysis of the paper.

To test for filler presence inside the leaves, two little samples from each leaf were treated with 1 M HCl (JT Baker, Deventer, Holland) and afterward they were analyzed with the ATR instrument.

2.7. Gas chromatography–mass spectroscopy (GC–MS)

This analysis was conducted on organic extracts of paper. Each sample (about 0.1 g) was extracted three times with 2 ml of 95% hexane (Romil Pure Chemistry, Cambridge, UK), in an ultrasonic bath, CP 104 (Vetrotecnica, Padua, Italy) for 6 min at 20 °C; then they were centrifuged (ALC 4217 centrifuge, MKII, Thermo Scientific, Waltham, MA, USA) for 4 min. The supernatant fractions were concentrated around 200 μl through flow of nitrogen (TurboVap® II, Caliper LifeSciences, Hopkinton, MA, USA).

The organic extracts were analyzed with a gas chromatograph 6890 N GC System (Agilent Technologies, Santa Clara, CA, USA) coupled to a quadrupole mass spectrometer 5973 (Agilent Technologies, Santa Clara, CA, USA). The chromatographic analysis was carried out using a capillary column (DB-5ms 60 m length, 0.25 inside diameter, and 0.25 μm thickness of the film, Agilent Technologies, Santa Clara, CA, USA), and 1 ml min^{-1} He flow was used as a carrier. Operating conditions are described in Table 2.

Table 2
GC–MS operative conditions.

Injector temperature	260 °C
Thermal program	• Beginning: 60 °C held for 5 min • Thermal gradient: 8 °C/min up to 280 °C • 280 °C held for 15 min

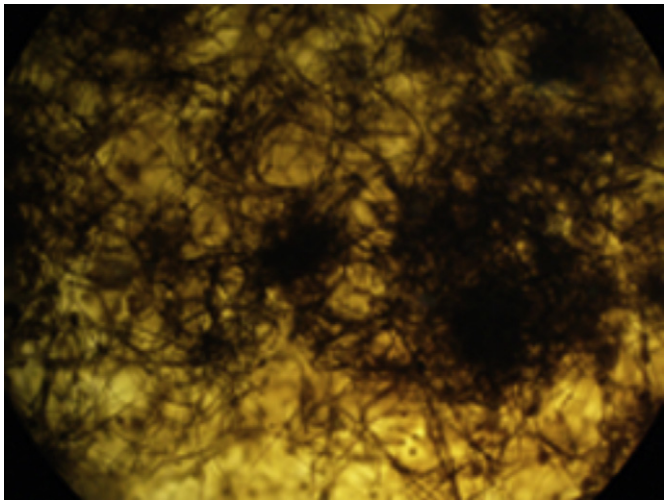

Fig. 4. Sample 4, brown stain, 100×. Dark brown fungal hyphae. (For interpretation of the references to color in this figure legend,the reader referred to the web version of this article.)

2.8. Isolation and identification of the fungi

Viable micro-fungi and bacteria were isolated from the sampling areas by dry-rub on the paper's superficial stains with a disposable sterile cotton swab; this was followed by inoculation on culture medium in sterile petri dishes with the plate loop technique. Petri dishes (9 cm dia.) were filled with about 20 ml of agar culture medium. In the beginning the rubbed material was inoculated on Sabouraud dextrose/maltose agar (SD/SM), specific medium for the fungal growth, and nutrient agar (NA) medium for fungi and bacteria growth. All petri dishes were put in a thermostatic incubator at constant conditions (26 °C, 80–90% RH).

From every stain, different fungi, moulds, and bacteria species were isolated, but only some fungal colonies were considered for identification. The choice of these colonies was made on the basis of their frequency inside many petri dishes, for their particularity, and for their representative status among all samples.

Fungal colonies' isolations were carried out taking the micro-fungi from the dishes through disposable sterile loops and transferring them onto specific media (Zotti et al., 2008a,b). For their growth, other more specific culture media were used, such as malt extract agar (MEA, for the isolation of general moulds and fungi), Czapek yeast autolyzate agar (CYA, for the recognition of *Penicillium* and *Aspergillus* genera), and potato dextrose agar (PDA, for the recognition of *Cladosporium* and *Ulocladium* genera). The fungal colonies isolated were identified following conventional mycology methods by observing their macro- and micro-morphological characteristics and their different trophic and physiological requirements, referring to Pitt (1979) for the genus *Penicillium*;

Table 3
Results of the histochemical tests.

Istochemical tests	Leaf 1	Leaf 2
Hertzberg	Pink–purple colour	Pink–purple colour
Graff's	Negative	Positive
Raspail	Positive	Negative
Alizarina S	Positive	Positive

Table 4
pH values.

Leaf	Sampling areas	pH values	T (°C)
Leaf 1	B1	5.93	18.8
	3	6.01	19.0
	4	6.25	19.0
	5	6.41	18.8
	Printing ink Leaf 1	6.02	19.0
	Indian ink Leaf 1	5.52	19.0
Leaf 2	B2	8.33	19.4
	7	7.01	19.2
	9	7.05	19.1
	10	6.85	19.1
	12	7.41	19.3
	Printing ink Leaf 2	6.84	19.4
	Indian ink Leaf 2	8.04	19.5

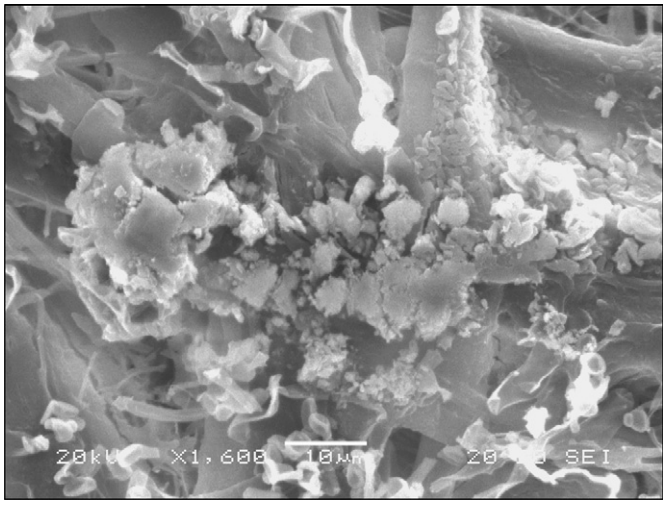

Fig. 5. SEM micrograph of sample 2, 1600×. Central aggregation probably composed of gypsum ($CaSO_4{}^*2H_2O$).

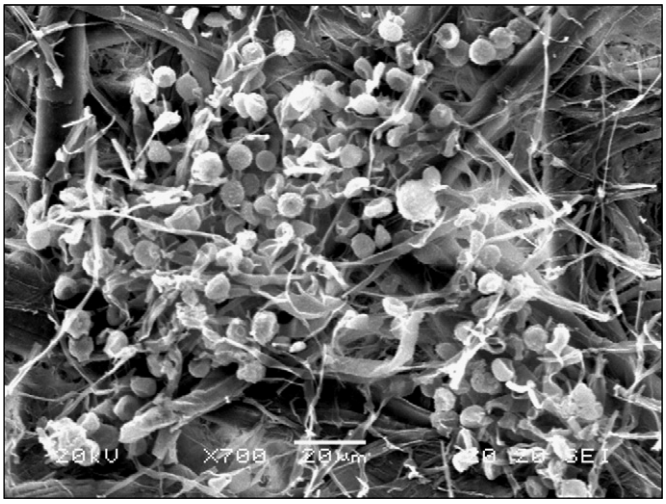

Fig. 7. SEM micrograph of sample 5, 700×. Several conidia, with different shape and dimensions on the paper surface.

Klich and Pitt (1994), Klich (2002) and Samson and Varga (2007) for the genus *Aspergillus*; and Ellis (1971), Domsh et al. (1980), and Crous et al. (2007) for the genus *Cladosporium*.

To verify the cellulolytic activity of the microorganisms previously isolated from paper samples, some of these fungi were subcultured on the same paper, but not infected with a biological attack. To ensure sterility, paper samples were exposed under UV light for 24 h and then placed in petri dishes, of which only half were supplied with culture media (Sabouraud dextrose agar and nutrient agar).

3. Results and discussion

3.1. Optical microscope observations

The optical microscopy observations enabled us to identify a biological presence on the paper surface of each sampling area for

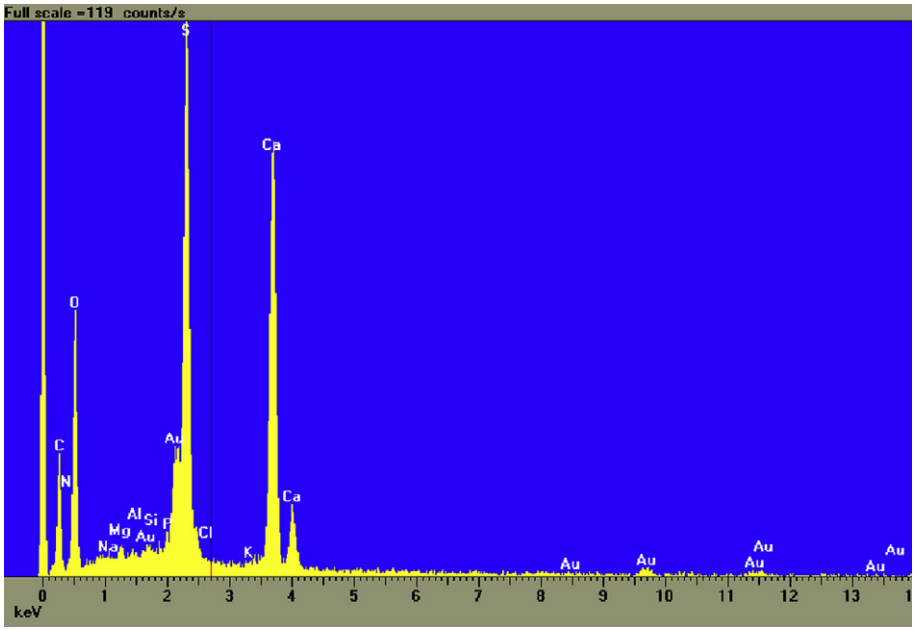

Fig. 6. EDS spectra of sample 2, gypsum aggregation.

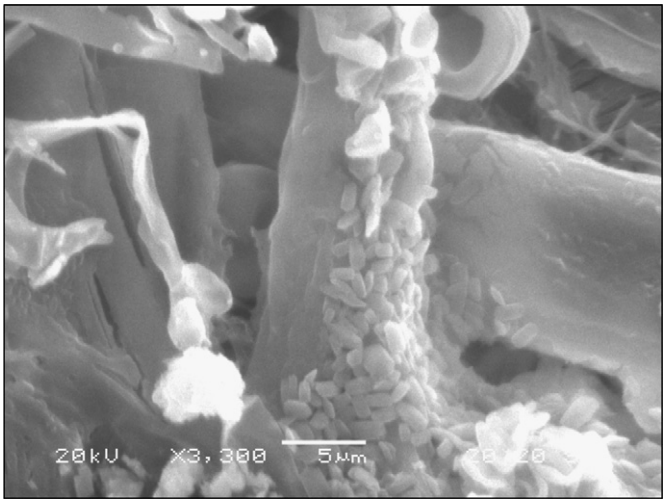

Fig. 8. SEM micrograph of sample 2, 3300×. Presence of bacterial bacilli on cellulose fibers.

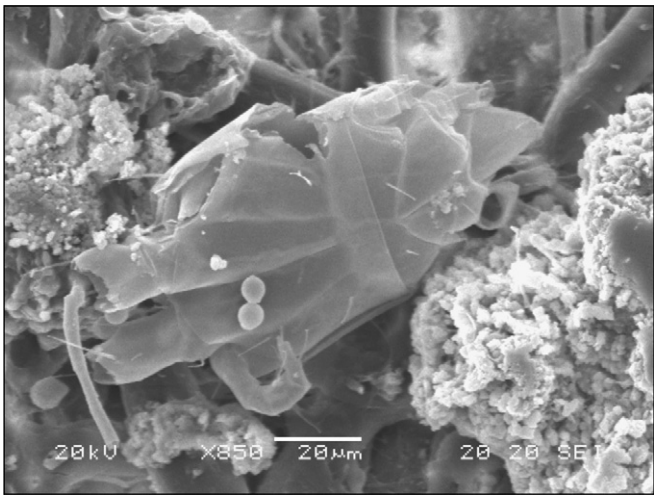

Fig. 9. SEM micrograph of sample 11, mite's carcass (Arthripoda, Chelicerata, Arachnids, Mite) seen ventrally, 850×.

both the leaves. The observation of sample 3, corresponding to a foxed area, on the other hand, in the brown stain of sample 4 a dark brown filamentous matrix was found, identifiable as fungal hyphae (Fig. 4).

3.2. Histochemical tests

The histochemical tests allowed us to identify the document's manufacture and the presence/absence of some compounds linked with the glue, and to establish the difference between the two documents. Histochemical analysis results are included in Table 3. The absence of lignin and the presence of rag materials in both the leaves were verified with the Hertzberg test; the semi-chemical origin of both paper mixtures was verified by obtaining a violet color. The second test, i.e., Graff's, revealed the presence of animal glue in Leaf 2, while the Raspail test revealed the presence of rosin sizing in Leaf 1. The last test, i.e., Alizarina S, revealed the presence of alum in both leaves, by making two red suspensions and by turning the two small paper pieces violet.

In Leaf 1, the presence of rosin and alum served to acidify the rosin alkaline suspension, thus favoring the re-establishment of free insoluble resin in order to make the fibers less hydrophilic, while in Leaf 2 (with animal glue) the alum favored paper glue hardening (Impagliazzo and Ruggiero, 2002).

3.3. pH analysis

The pH values of Leaf 1 all turned out to be below neutrality. Also the non-degraded area (B1) had a low pH value (5.93), probably because it had suffered a homogeneous superficial oxidative process and it didn't have an alkaline reserve; also, the presence of rosin and alum inside it (verified with the histochemical test, 3.1) had made the support acid. Low pH values represent an excellent condition for the development of microbiological attacks (Altibrandi, 2002; Maggi et al., 2005).

The pH values of Leaf 2 turned out to be lower than those of the first leaf. The non-degraded area (B2) had a high pH value (pH = 8.33), which means that this paper support was manufactured with a high alkaline reserve, presumably calcium carbonate, to prevent fast paper oxidation.

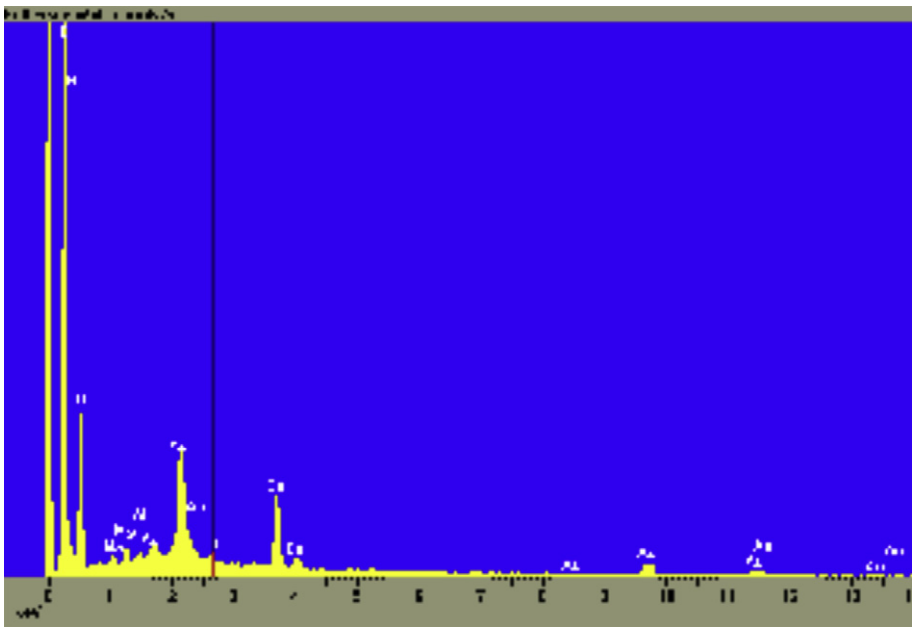

Fig. 10. EDS spectra of sample 11, Mite's leg.

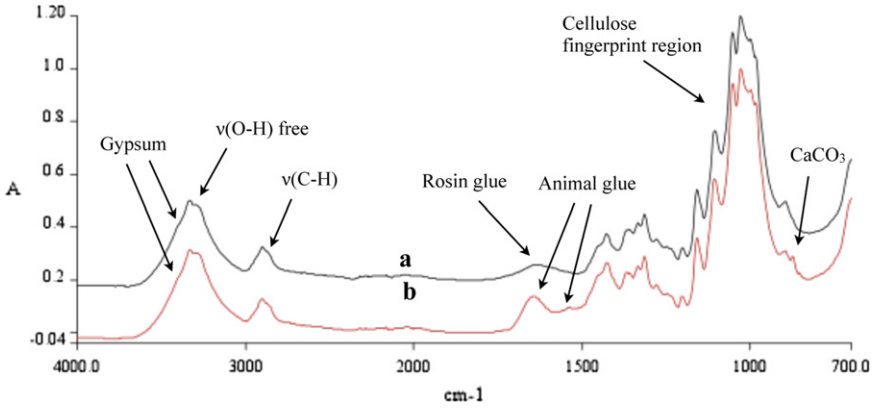

Fig. 11. ATR spectra: (a) sample Leaf 1; (b) sample Leaf 2.

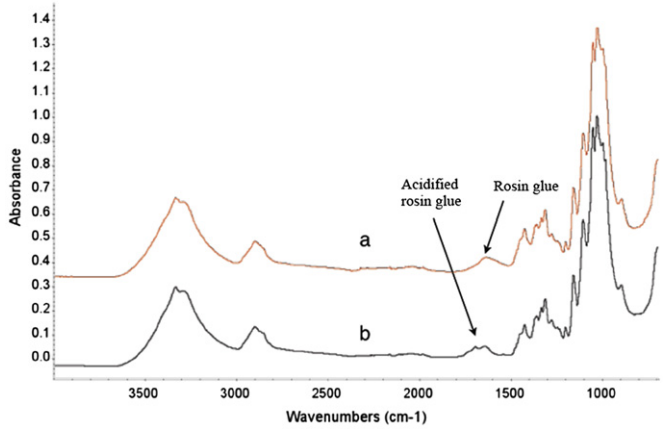

Fig. 12. ATR spectra of Leaf 1: without pre-treatment (a) and with acid treatment (b).

Only in the strong chromatic alteration (sampling area 10 in Leaf 2), and in the printing ink, were low pH values noted. All pH results are reported in Table 4.

3.4. SEM–EDS

The SEM observations of the samples allowed us to establish the unequivocal presence of microorganisms on the paper surfaces, and to bring into focus the morphology of two different paper mixtures. Leaf 1 appeared to consist of a homogenous fibrous mixture with medium-length rag fibers and mixture glue, which we assume was rosin. Leaf 2, also composed of a homogenous mixture of rag fibers, appeared to have superficial glue, typical of animal glue (verified with the histochemical test, 3.1). The presence in both leaves of inorganic fillers (talc, calcium carbonate, gypsum) (Fig. 5) and sodium chloride (probably due to the high water level to which these documents were exposed during their conservation) were found and detected by EDS spectra (Fig. 6).

The presence of foreign organic materials, such as fungal spores, hyphae, and bacteria, was detected on the entire surface of the paper samples. Their presence was heterogeneous, crowded in some areas and limited in others.

Fungal spores and hyphae of different size and morphology were identified, and this means that there were different micro-fungal species on the paper surfaces, although probably not active; in fact no type of conidiophores were found. Some of the spores were oval-shaped, with smooth walls or with spore scars, or they had a saggy shape (Fig. 7). Several spores instead had a spherical shape and rough walls. They resembled *Aspergillus* and *Penicillium* genera spores, but they couldn't be unequivocally identified. In fact, it was demonstrated that sample preparations prior to SEM observations affect hydration of fungal spores, as well as their size and forms (Florian, 1996, 2000). Bacteria like bacilli, with the typical elongated shape and grouped separately, were identified in the cellulosic fibers of paper (Fig. 8).

In sample 11, the presence of mite carcasses were identified. These organisms normally eat human skin fragments and dandruff, but may also eat fungi, some bacteria and insect fragments (Fig. 9). For these reasons, their presence was not

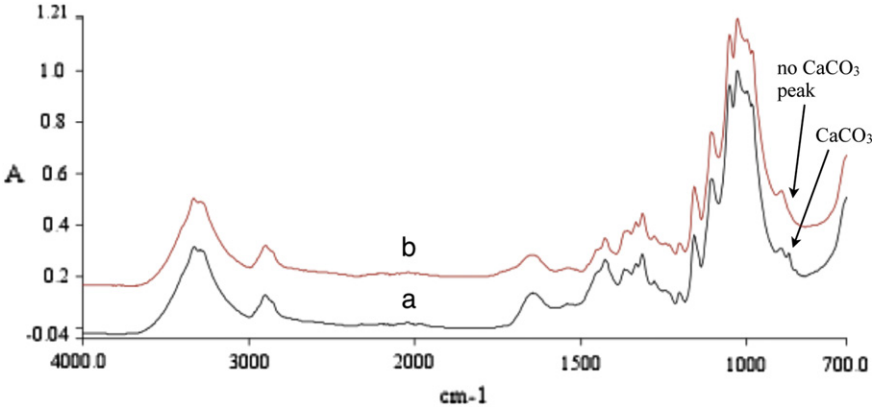

Fig. 13. ATR spectra of Leaf 2: without pre-treatment (a) and with acid treatment (b).

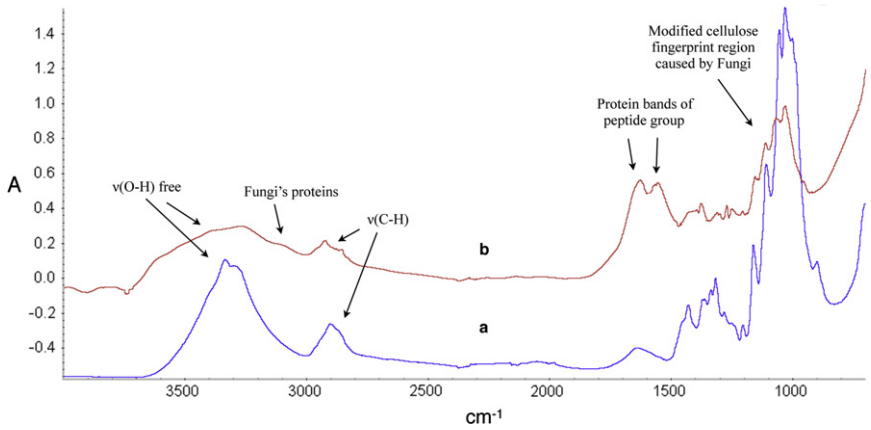

Fig. 14. ATR spectra of Leaf 1 (a) vs. sample 4 (b).

surprising. Florian and Manning (1999, 2000) have also detected the presence of two snout-nosed mite-like animals, which they called fox-trotter, but they are different from the mite carcass found in this case. An EDS spectrum of a mite leg (Fig. 10) revealed the presence of carbon, oxygen, and nitrogen, probably linked with the chitin structure, the main component of the cell walls of fungi, as well as of the exoskeletons of arthropods. Moreover, the test also showed a calcium peak, probably due to the presence of calcium carbonate that, if available in the environment, is fed upon by the arthropods with soft cuticles (such as mites) to reinforce their exoskeleton (Omodeo, 1983).

3.5. FT-IR spectroscopy

Fig. 11 shows the spectrum corresponding to the paper mixtures of Leaf 1 (a) and Leaf 2 (b). In these spectra, it is possible to recognize the peaks of cellulose, especially in the range 850–1500 cm^{-1}, called the fingerprint region; on the other hand, there are no peaks of lignin (1510 and 810 cm^{-1}) present in either leaf, and this observation is relevant to the knowledge of the raw materials used for the paper. Paper without lignin is made from semi-chemical or chemical mixtures, from which lignin is removed by specific chemical agents.

The bands at 3000 – 2800 cm^{-1} represent the vibration of stretching of the C–H link of cellulose, while the bands at 3650–3000 cm^{-1} belong to OH of cellulose/water. The shoulder bands at 3550–3450 cm^{-1} are due to the presence of gypsum in both leaves.

Leaf 1 presents a shoulder of about 1600 cm^{-1}, attributable to rosin glue; Leaf 2, by contrast, shows the calcium carbonate peak at about 870 cm^{-1} and the peaks of animal glue at 1644 and 1550 cm^{-1}.

After the treatment with 1 M hydrochloric acid, the spectra of the leaves showed some difference. In the spectrum of the treated Leaf 1 (Fig. 12), the presence of glue at about 1600 cm^{-1} became a broad shoulder at about 1730 cm^{-1} with a little peak at 1696 cm^{-1}, typical of acidified rosin glue.

After the acid treatment of Leaf 2 (Fig. 13), the peak of calcium carbonate at about 870 cm^{-1} disappeared.

The spectra of the sampling of the degradation area showed a different trend, similar to those observed by Zotti et al. (2007): In fact, in Fig. 14 the spectrum of Leaf 1 (a) is compared with that of sample 4 (b). The presence of protein bands produced by typical fungal attack corresponding to glue absorptions was noticed. The peaks at 1622 and 1543 cm^{-1} and the shoulder at 3105 cm^{-1} are due to protein bands of peptide groups. Also, the cellulose fingerprint region around 1033 and 1066 cm^{-1} is adjusted because of the fungal presence.

3.6. GC–MS

Analysis with GC–MS only characterized Leaf 1 (sample B1), identifying the presence of abietic acid and its degradation products, dehydroabietic acid and 7-oxo-dehydroabietic acid (Fig. 15 and Table 5).

The absence of lignin in both documents was further confirmed, which is in agreement with the results of previous analytical techniques (see Sections 3.2 and 3.5).

The recognition of abietic acid and its degradation products were made by direct comparison of mass spectra of the same products synthesized in the laboratory.

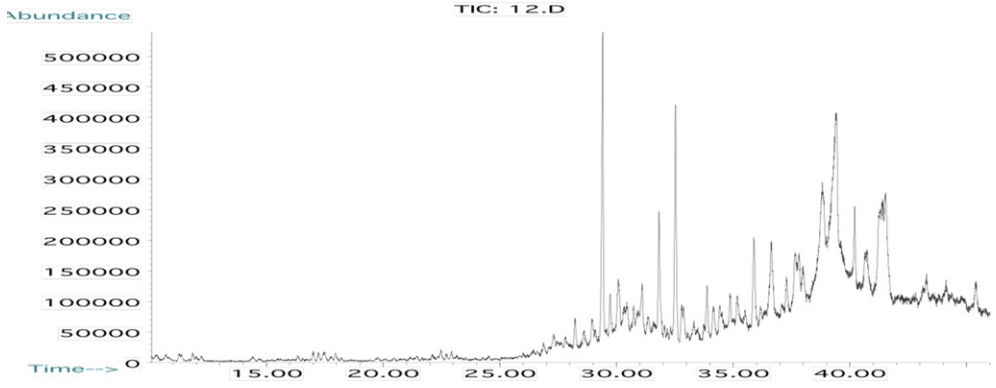

Fig. 15. GC–MS chromatogram of sample B1.

Table 5
Peaks' attribution observed in chromatogram of sample B1.

Retention times (min)	Compounds
22	Di-ter-butylphenol
29.3	Phthalate (contamination)
31	Carboxylic acid
34.1	Unsaturated carboxylic acid
34.5	Saturated carboxylic acid
34.8	Saturated carboxylic acid
37.6	Deidroabietic acid
38	Carboxylic acid
38.8	Abietic acid
39.2	7-oxo-deidroabietic acid
40.2	Phthalate (contamination)

The presence of abietic acid in sample B1 confirms the presence of resin, but it proves that the fibrous matrix is composed of coniferous wood. The detection of a degradation product (dehydroabietic acid) confirms the oxidation of paper by the light. Even exposure to sunlight only produces this kind of compound. However, the additional presence of the 7-oxo-deidroabietic acid could be connected to an attack carried out by micro-enzymes (Bicho et al., 1995; Liss et al., 1997; Martin et al., 1999; Noguera, 2007).

With regard to the GC–MS analysis of a sample derived from Leaf 2, no degradation products of lignin and abietic acid were detected. The first absence was due largely to the matrix paper, consisting of a semi-chemical mixture, while the second confirmed the presence of a different glue (gelatin).

3.7. Fungi isolated and identified

Fungal entities, selected and cultivated in specific culture media, belong to the four main genera that damage archival materials: *Aspergillus* sp., *Penicillium* sp., *Cladosporium* sp., and *Ulocladium* sp. (Gallo, 1992; Nyuksha, 1994; Zyska, 1997; Altibrandi, 2002). In Table 6, the results are presented schematically: The first column indicates the areas where fungal species have been identified, the second column refers to the specific medium used, and the third refers to the micro-fungi isolated and identified.

In particular, fungi belonging to the genera *Penicillium* and *Aspergillus* were identified; unfortunately their identification was restricted to the genera level, but it was still significant. These genera, in fact, are often present in libraries and archives and their infections are the most widespread (*Aspergillus* sp. at about 30% and *Penicillium* sp. at about 30%) (Gallo, 1992). All the species that were isolated had a tendency toward xerophilia, osmophylia, and/or osmotolerance; indeed, they were capable of surviving with low concentrations of water and were therefore able to cause the deterioration of paper media of different compositions (Nol et al., 1983; Rambelli and Pasqualetti, 1996; Arai, 2000; Montemartini Corte et al., 2002; Zotti et al., 2008b).

However, it should be considered that only 5% of micro-fungal colonies in nature can grow in vitro and this does not mean to say that the isolated species are the ones actually responsible for the deterioration of our documents.

Table 6
Results of fungal isolation from sampling areas.

Sampling areas	Specific culture medium	Fungal colonies and isolated species
1a	MEA	Mycelium
3a	CYA	*Aspergillus* sp. Sez. Candidi
4a	PDA	*Ulocladium alternariae* (Cooke) E.G. Simmons
5a	PDA	*Cladosporium* Link sp.
B2a	MEA	*Penicillium* sp.
7a	PDA	*Cladosporium* Link sp.
8a	MEA	*Penicillium* sp.
9a	MEA	*Penicillium* Link sp.
11a	CYA	*Aspergillus* P. Micheli ex Link sp. Sez. Circumndati
12a	MEA	*Aspergillus* sp.
12b	MEA	*Aspergillus* sp.
12c	MEA	*Penicillium* Link sp.
12e	MEA	*Penicillium* Link sp.

4. Conclusions

In this case study a logical methodology was implemented and followed that was suitable for an archive environment. Two 19th Century documents, with strong biodeterioration, were examined, called Leaf 1 and Leaf 2.

Starting with observations of the paper surface by light microscopy, it was already possible to identify the presence of biological material. On the other hand, with the histochemical test, the two documents were characterized in terms of the glue used in their manufacture and the pH analysis allowed us to note a high alkaline reserve.

The SEM–EDS have ascertained the presence of several foreign microorganisms and the state of conservation of the paper's cellulose fibers, while FT-IR spectra have enabled us to confirm the general absence of lignin and to highlight the presence of biological material in the sampling areas, although it was not possible to trace these back to the relevant species (Zotti et al., 2007).

The GC–MS gave us only information about the type and condition of the sizing of the documents in particular, one glued with rosin. As we did for the cases where it was possible to work with a greater availability of samples, we plan to supplement the GC–MS with Py-GC/MS and HPLC-MS/MS (Ganzerla et al., 2008).

Fungal strains isolated from the documents' spots belonged to genera commonly isolated from paper materials: *Aspergillus*, *Penicillium*, *Cladosporium*, and *Ulocladium*. These fungal species are also commonly found in the air and their presence was also confirmed by an aerobiological analysis carried out in the Superintendent's Archives (data not shown), which makes all the archival material potentially subject to their decay action.

With the results obtained, we can still say that the conservation status of the documents studied is not ideal, having confirmed the presence of cellulolytic microorganisms in a dormant state on the samples and, given a change in the environment, they could start to grow and potentially damage the paper. This information is very important in evaluating the general condition of the archives and it can be used as a starting point for a restoration project and/or optimal conservation to prevent the possible proliferation of this kind of fungi.

The analysis sequence was designed in such a way as to deepen the knowledge of paper by using non-destructive techniques. We have seen that the results of one technique were then confirmed by another technique, and this is important in that more invasive techniques, such as GC–MS, can be avoided. Finally, as hoped for, there was a correspondence between the results of physical–chemical and biological techniques.

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