# STUDY OF THE CONTAMINATING MICROBIOTA OF OLD PAPER SUPPORTS

***Simona Isabela Dunca, Associate Prof. PhD***
***Catalin Tanase, Prof. PhD***
***Claudia Padurariu, PhD***
***Tiberius Balaes, PhD***
Faculty of Biology, "Alexandru Ioan Cuza" University of Iasi, Romania
***Elena Ardelean, Lecturer PhD***
***Nicoleta Melniciuc Puica, Prof. PhD***
Faculty of Orthodox Theology, "Alexandru Ioan Cuza" University of Iasi, Romania

**Abstract**

Biodeterioration has drawn the attention of different specialists who started to cooperate and to understand the need of joint research to have a picture as comprehensive as possible of the degradation agents and the measures that can be taken to salvage the heritage assets. This paper contains a description of the microbiota identified in two types of old paper supports part of a private collection from North Moldavia (Romania), namely a church book from the end of the 19th century for which the paper was obtained manually from textile fibres, and a book from 1870 with paper from cellulose pulp derived from wood. To the purpose of identifying the types of bacteria and fungi present on the supports examined, several comments were made related to the morphology of the colonies developed on the growth medium, which provided important indications for the determination based on the microscopical examination as well. Bacterial contamination (of the genera *Bacillus, Clostridium*, *Pseudomonas* and *Micrococcus*) was detected in most samples collected. The diversity of the fungi isolated from the paper supports (the genera *Penicillium*, *Alternaria*) is the result of the fact that since they are highly hygroscopic materials, they have the capability to retain water more easily, which stimulates fungal growth. The investigations made to determine the presence of microorganisms responsible for degradation and their identification allowed the discovery and acertainment of the real and justified need to find ways to prevent biodeterioration or to inactivate and destroy biodeteriogens by non-invasive, eco-friendly chemical and physical treatments.

**Keywords:** Old book, contamination, microbiota, bacteria, fungi

## Introduction

Since biodeterioration is the result of either the combined or separate activity of several organisms on different substrates and under varied ecological conditions, the understanding and stoppage of its effects require the concurrence of the different branches of biology, biochemistry, chemistry, physics-chemistry, etc. Paper is mainly made of cellulose and secondary substances such as lignin, hemicellulose, pectin, waxes, tannins, proteins and mineral constituents. In time, paper undergoes processes of deterioration, which cause the scission of macromolecular chains, an irreversible process called „paper ageing". The chemical, physical or microbial deteriorations can affect the cellulose fibers of the chemical composition of paper, depending on the raw material and the manufacturing procedure used.

The chemical destructions are principally caused by the oxidation of the cellulose chains. Generation of free carbonyl groups and redox reactions can involve all paper constituents. The ink, glue and impurities as well as other organic or inorganic matters can negatively alter the process of paper ageing. Most often, the physical deterioration caused by light, temperature, humidity can enhance the biodegradation processes. The chemical hydrolysis of cellulose can stimulate the attack of microorganisms (Michaelsen *et al*., 2010). Paper microbial deterioration leads to different types of losses/destructions depending on the organism involved (Michaelsen *et al*., 2010). Of the varied range of biological agents, three main categories can be distinguished: filamentous fungi, cellulolytic bacteria and insects.The literature estimates there are over 200 species of fungi and bacteria causing paper degradation. Some of these are found in the raw materials, while others contaminate paper when coming into contact with air. Degradation caused by microbial growth occurs in general as: chromatic alterations in the form of stains in a wide variety of colours (e.g. purple, yellow, brown, black, red etc.), shape and size due to the presence of a pigmented mycelium, of spores or, in certain circumstances, pigments produced by bacteria or fungi; structural alterations of the main components caused by enzymes (e.g. cellulases, proteases etc.) produced by different types of microorganisms, which ultimately lead to paper embrittlement or even its partial destruction; alterations to the essential added components (e.g. adhesives, plasticisers, antioxidants etc.) due to the attack of microorganisms (Pasquariello *et al*., 2005). The presence of bacteria is indicated by appearance of isolated spots of different colours of low intensity at first. Once the attack begins, such spots acquire bigger sizes and deep hues: red, brown, black etc., depending on the species, pH value and so on. Fungi can colonize and degrade an extensive range of heritage assets. They secrete destructive acids and are able to depolymerize cellulose fibers. Many widely-spread species, such as *Aspergillus* sp., *Alternaria* sp., *Chaetomium*sp., *Penicillium* sp. can degrade cellulose and are often associated with the holdings of libraries (Konkol *et al*., 2009). The appearance of foxing (an extensively studied phenomenon lately) also was attributed to the species belonging to the *Bacillus* genera, as the causing agent (De Paolis and Lippim, 2008), even though this degradation process is described as being triggered by the fungal attack as well. Species of actinomycetes and bacteria secreting strong, staining pigments and organic acids causing destruction – *foxing* (Strzelczyk *et al*., 2004) were identified. Still from *foxing* stains, Michaelsen *et al*. (2010) isolated cellulolytic bacteria, namely *Bacillus* sp., *Acinetobacter* sp., *Kochuria* sp., *Stenotrophomanas maltophilia, Clostridium colinum*. Rakotonirainy *et al*. (2007) managed to isolate from *foxing* stains species of fungi belonging to the following genera: *Aspergillus*, *Bjerkandera*, *Chaetomium*, *Gloeotinia*, *Penicillium*, *Polyporus*, *Saccharicola*, *Trichoderma* and *Ulocladium*. Some species were detected only in 1-2 spots, while others (i.e. *Penicillium minioluteum*, *Gloeotinia tenulenta*) were very frequently encountered. Species of *Myrothecium verrucaria*, *Aspergillus* sp.and *Penicillium* sp. can grow also at a relative humidity of 62-65%. Most often, the first affected are book bindings since they take up air humidity. Some filamentous fungi frequently associated with paper degradation are able to hydrolyze cellulose fibers owing to their cellulolytic enzymatic system. Moreover, they can discolour ink by means of the tannases or produce paper-staining pigments and organic acids, which may induce discolouration and damages to the material. The stains may have different colours (e.g. red, purple, yellow, brown, black) due to the secretion of pigments and metabolic products. Among the pigment-producing species the following can be indicated: *Penicillium notatum*, which exudes a yellow pigment; *Alternaria solani*, which exudes a black pigment; *Fusarium oxysporum*, which exudes a pink pigment (i.e. *fusarubin*) penetrating paper; *Chaetomium globosum*, which exudes a yellow pigment that subsequently turns brown (Szczepanowska and Lovett, 1992). Biodeterioration can be diminished or inhibited by means of ecological mechanisms acting, in particular, in the abiotic environment.

The course of action for using eco-friendly mechanisms to prevent and stop biodeterioration includes the alteration, denaturation or intoxication of the living environment of the biodeterioration agents (Oprea, 2006). The usual techniques applied for treating paper to the purpose of inactivating fungi are based on the use of disinfectant chemical agents or the use of various types of radiations, which frequently have a negative impact on paper. The current research studies on the preservation of paper items focus on the finding of methods that are ecological, less damaging, efficient and can be widely applied (Ioanid *et al*., 2010).

The purpose of this study was the identification of the microbiota and the assessment of the extent of contamination of two old paper supports with the aim of applying effective, eco-friendly and non-invasive decontamination treatments.

**Materials and methods**

In the biological investigation of heritage assets, the analytical tests aim at the following: detection of a real process of biodeterioration and establishing whether such process is active (viable), passive (latent) or inactive (extinct); identification of the causing agent or group of agents involved in the biodegradation process examined; determination or description of the effects of the biodeterioration agents on the items subjected to examination and assessment of their extent; ascertainment and determination of the real and justified need for biocide treatments (i.e. disinfection, pest control), finding alternative ways to prevent biodeterioration or to inactivate and destroy biodeterioration agents; testing of the resistance to biodeterioration of materials and substances used for restoration and contingently, identification of means to enhance biological resistance to the degradation factors; prevention of the outset of any deterioration process during the preservation and restoration stages (Oprea, 2006). The samples to be examined were collected from two types of old paper supports part of a private collection from North Moldavia (Romania), namely a Church book from the end of the 19th century for which the paper was obtained manually from textile fibres, with red and black printing ink, and a book from 1870 with paper from cellulose pulp derived from wood and black printing ink.

*Sample collection*

One of the main difficulties in reaching the biological diagnosis for items of heritage importance is the non-invasive collection of samples, which must be performed in a manner that does not change the items in question, especially when such items are small. A sample collected inadequately or placed in inappropriate, uncontrolled conditions can easily and rapidly modify its microbial composition (Oprea, 2006). The method used in this study was the impression one, consisting in pressing sterile 1 $cm^2$ test specimens of filter paper (impregnated with sterile distilled water) onto the attacked parts, so that the spores and mycelial fragments would adhere to the surface of the test specimens, which were subsequently transferred to culture media.

*Examination and identification of bacteria*

The incubation of the samples inoculated on the nutrient agar medium at $37^0$C for 24 hours was followed by the examination of the growth of bacterial cultures on/around the test specimens was examined. After the preparation of pure cultures (containing a single species or microbial strain), the bacteria were identified based on a complex set of features, namely colony and cell morphology, mobility, sporulation, tinctorial properties, type of respiration, growth particularities (i.e. growth and development in liquid culture media and on solid media). The microscopic evaluation involved the examination of bacterial smears fixed and stained according to Gram's method (Dunca *et al*., 2007). The evaluation can be carried out in numerous ways, from visual inspection with the naked eye or using a magnifying glass to the use of an optical microscope or electron microscope (TEM, SEM) (Oprea, 2006). The

bacterial examination of the smears was carried out using an optical microscope (Olympus) with a 100x immersion objective.

*Identification of fungi*

The identification of the fungi responsible for paper destruction provides the opportunity to study the species involved in the process, in terms of their degradation mechanisms, mechanism of action, growth conditions and factors that may aid to their eradication (Rakotonirainy *et al*., 2007). The same technique was used for isolating the fungi from the paper supports examined as for isolating the bacteria; however, the culture media was different (i.e. Sabouraud agar). The incubation was carried out at $28^0$C for 7 days (Michaelsen *et al*., 2010). On the selected nutrient medium, a strain can generate mono-spore colonies (i.e. formed by the germination of a single spore) or multi-spore colonies (i.e. formed by the growth of multiple associated spores) or from a hyphal fragment, a colony forming unit (CFU).

*Determination of fungal load*

To determine the fungal load, the colonies grown on the Sabouraud culture medium in the Petri dishes inoculated with the samples collected from the supports under investigation were examined. Thus, for each sample, the number of colonies grown on the culture medium was recorded and the fungal load of the supports was estimated. The fungi to be examined were isolated in pure cultures and stored by refrigeration at 4 °C in tubes containing agar media.

*Microscopic examination*

The microscopic examination of the fungal cultures included the following steps: material sampling and placing the sample on a slide in a water drop or in a liquid confining the specimen to be examined; fixation using a fixative which immediately suspends all life processes killing the cells; staining the hyaline structures; mounting the sample in different mounting media. The fungal preparations were examined using a Nikon trinocular microscope.

*Decontamination with high-frequency cold plasma*

The high-frequency cold plasma used, rich in accelerated electrons, molecules, free radicals, electromagnetic radiations and with increased reactive properties makes the medium effective in fighting biodeterioration (Popescu, 1981). The decontamination treatments use only high-frequency cold plasmas with temperatures not exceeding $50^0$ C.

The samples collected from the two paper supports were treated with high-frequency cold plasma in nitrogen atmosphere and gas mixture of nitrogen, argon, and oxygen for different amounts of time (i.e. 60 s, 5 and 7.5 min); the level of contamination was determined taking into consideration the extent of growth of bacterial cultures in the samples transferred onto the media following incubation at 37º for 24 hours. For the electron microscopic examination using a SEM TESCAN VEGA II SBH microscope, the samples were allowed to dry naturally followed by drying under vacuum, and then Au-coated with a 15 nm layer by cathode sputtering.

## Results and discussions

### *Evaluation of the level of microbial contamination of the old paper supports examined*

To the purpose of determining the level of microbial contamination of the paper supports under investigation (to which conventional symbols indicated in Table 1 were assigned), the samples were sampled by impression and grown on culture media specific for bacteria and fungi. The growth of colonies around the specimens placed on the media surface was monitored and the results were indicated in Figure 1 and Figure 2. The results showed differences in terms of growth and development on the culture media among both the

organisms investigated: i.e. bacteria (Figure 3 and Figure 4) and fungi (Figure 5 and Figure 6) and the samples examined.

*Morphology of the bacterial cultures*

When cultivated on solid media, bacteria grow as individual or merging colonies on the surface of or within the media. Their formation on solid media can be explained by the lack of Brownian motion and reduced bacterial motility, which allows the cells resulted from repeated division aggregate in a limited area.

Table 1. Assignment of conventional symbols to the samples examined

| No. | Symbol assigned | Name of sample examined | Sampling points |
|---|---|---|---|
| 1. | $I_A$ | Church book | left page, bottom left corner |
| 2. | $I_B$ | Church book | left page, bottom right corner |
| 3. | Ic | Church book | right page, bottom left corner |
| 4. | $I_D$ | Church book | right page, top right corner |
| 5. | $II_A$ | Church book | middle of fascicle: upper part |
| 6. | $II_B$ | Church book | middle of fascicle: median part |
| 7. | $II_C$ | Church book | middle of fascicle: lower part |
| 8. | $II_D$ | Church book | bottom right corner |
| 9. | $III_A$ | Church book | outside left margin: grey-purple stains |
| 10. | $III_B$ | Church book | middle of fascicle: top |
| 11. | $III_C$ | Church book | middle of fascicle: bottom |
| 12. | $III_D$ | Church book | right page, bottom right corner |
| 13. | $IV_A$ | Church book | book binding string |
| 14. | $IV_B$ | Church book | book binding – middle – piece of string |
| 15. | $IV_C$ | Church book | colour imprint (1/2 upper) |
| 16. | $IV_D$ | Church book | colour imprint (1/2 lower) |
| 17. | $V_A$ | Church book | bottom left (purple stains) |
| 18. | $V_B$ | Church book | left page, top right side |
| 19. | $V_C$ | Church book | top right |
| 20. | $V_D$ | Church book | bottom right |
| 21. | $VI_A$ | Book from 1870 (printed in Paris) | page 112 – top left corner (brown stain) |
| 22. | $VI_B$ | Book from 1870 (printed in Paris) | page 112 – top right |
| 23. | $VI_C$ | Book from 1870 (printed in Paris) | page 112 – bottom left (stains) |
| 24. | $VI_D$ | Book from 1870 (printed in Paris) | page 113 – inside margin, median part |
| 25. | $VII_A$ | Book from 1870 (printed in Paris) | foxing – page 288 – top left |
| 26. | $VII_B$ | Book from 1870 (printed in Paris) | foxing – page 288 – top right |
| 27. | $VII_C$ | Book from 1870 (printed in Paris) | foxing – page 288 - bottom left |
| 28. | $VII_D$ | Book from 1870 (printed in Paris) | foxing – page 288 - bottom right |

The structure and appearance of the colonies are determined by the morphology of the constituting cells, their grouping, and the characteristics of the culture medium under the influence of physicochemical factors. The bacterial cultures (visible to the naked eye for most bacteria after 18 to 24 hours) differ in terms of size (small, medium, large), margins (irregular, lobate, rhizoid etc.), surface (shiny, dull, granular etc.), opacity (opaque, translucent, transparent), elevation (flat, raised, umbonate etc.), colour (pigment-containing, lack of pigment), consistency (viscid, mucoid etc.), adhesion (adherent, non-adherent).

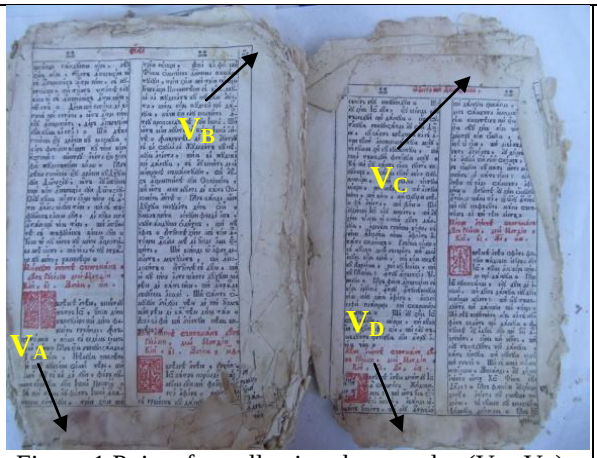


Figure 1.Points for collecting the samples ($V_A$ -$V_D$) from the Church book (19th century)

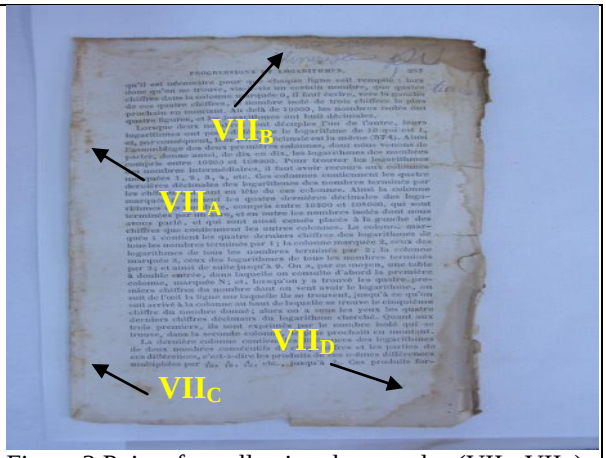


Figure 2.Points for collecting the samples ($VII_A$-$VII_D$) from the book printed in Paris (1870)

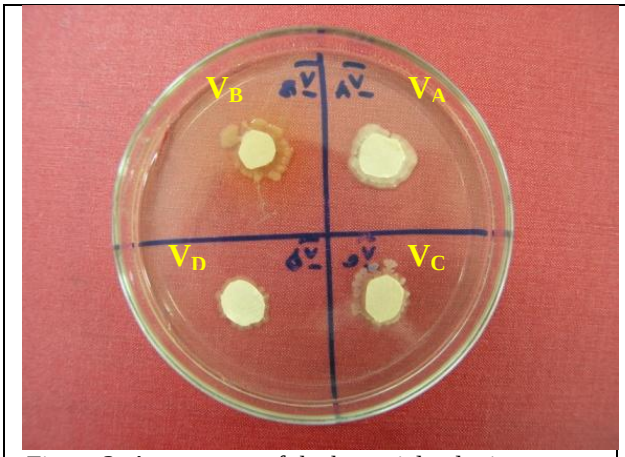


Figure 3. Appearance of the bacterial colonies grown on nutrient agar: samples $V_A$, $V_B$, $V_C$, $V_D$ (the Church book)

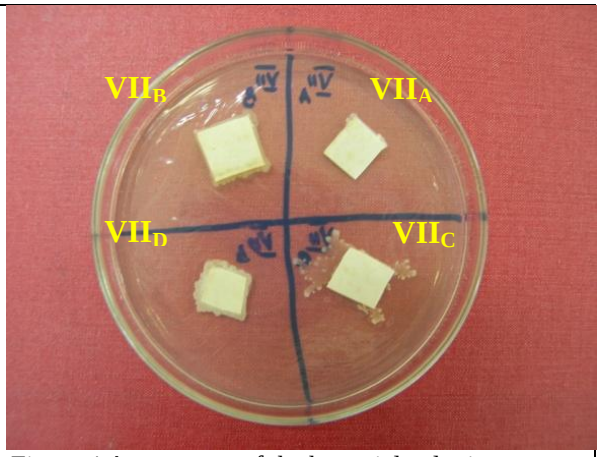


Figure 4.Appearance of the bacterial colonies grown on nutrient agar: samples $VII_A$, $VII_B$, $VII_C$, $VII_D$ (the book from 1870)

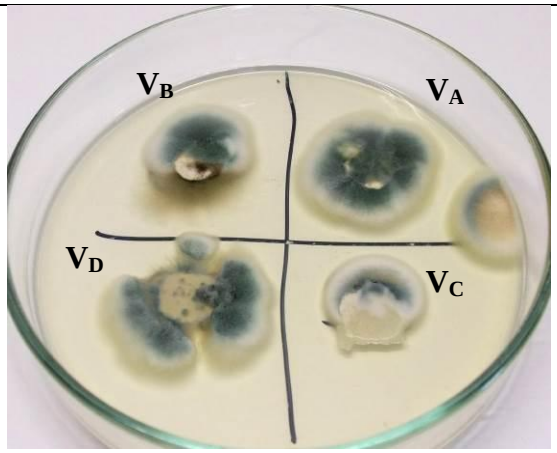


Figure 5. Appearance of the fungal colonies grown on Sabouraud agar: samples $V_A$, $V_B$, $V_C$, $V_D$ (the Church book)

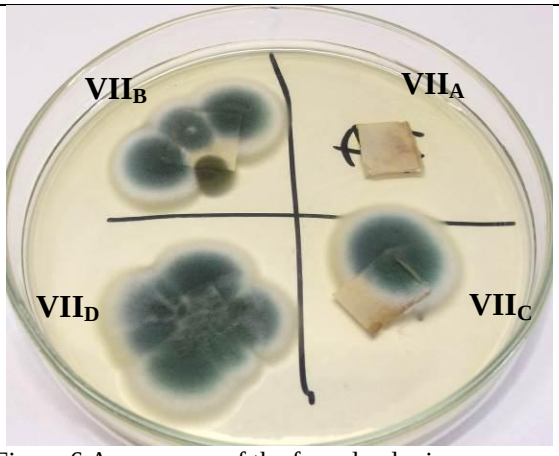


Figure 6.Appearance of the fungal colonies grown on Sabouraud agar: samples $VII_A$, $VII_B$, $VII_C$, $VII_D$ (the book from 1870)

The microbial contamination of the paper supports examined is shown concisely in Table 2.

Table 2.Level of microbial load in the samples examined

| No. | Sample | Sample description | No. of fungal colonies | No. of bacterial colonies |
|---|---|---|---|---|
| 1. | I | – imprints A-D | 7 | 5 |
| 2. | II | – imprints A-D | 2 | 6 |
| 3. | III | – imprints A-D | 4 | 4 |
| 4. | IV | – imprints A-D | 6 | 4 |
| 5. | V | – imprints A-D | 7 | 4 |
| 6. | VI | – imprints A-D | 1 | 0 |
| 7. | VII | – imprints A-D | 9 | 4 |

*The microscopic examination of the organisms isolated* in pure cultures resulted in the identification of the following bacterial cellular morphologies (Figure 7 – Figure 10):

- Gram-negative rods, single or in pairs (as diplo), sporulating (with central and subterminal non-deforming spores): strains$I_B$, $I_C$, $I_D$, $IV_{D2}$;
- Gram-positive rods single, sporulating (with central and subterminal deforming spores): strains $V_A$, $V_C$, $V_D$, $VII_D$;
- Gram-positive rods, single, sporulating (with central and subterminal non-deforming spores): strain $II_A$;
- Gram-positive rods, single, sporulating (with central and subterminal non-deforming spores and mass of spores): strains$II_B$, $II_C$, $III_A$, $III_B$, $III_C$, $IV_A$, $IV_B$, $IV_C$;
- Gram-positive rods single, sporulating (with central, subterminal and terminal non-deforming spores and mass of spores): strain $III_D$;
- Gram-positive rods, single, sporulating (with subterminal and terminal deforming spores and mass of spores): strain$VII_C$;
- Gram-positive rods, single, sporulating (with subterminal and terminal non-deforming spores): strain $II_D$;
- Gram-negative rods, single or grouped in short chains, non-sporulating: strains $I_A$, $IV_{D1}$;
- Gram-negative rods, single, in pairs and short chains, non-sporulating: strain $VII_B$;
- Gram-positive cocci, single and in pairs, non-sporulating: strain $VII_A$
- absence of growth: strains $VI_A$, $VI_B$, $VI_C$, $VI_D$.

*Identification of the main genera of bacteria*

The examination of the macromorphological and micromorphological features led to the identification of the following prevailing genera in the items examined (Church book – 19th century and printed book -1870): *Bacillus* (62 % of the isolated strains), *Clostridium* (21 %), *Pseudomonas* (13 %)and *Micrococcus*(4 %). The taxonomic classification of the bacteria identified is shown in Table 3.

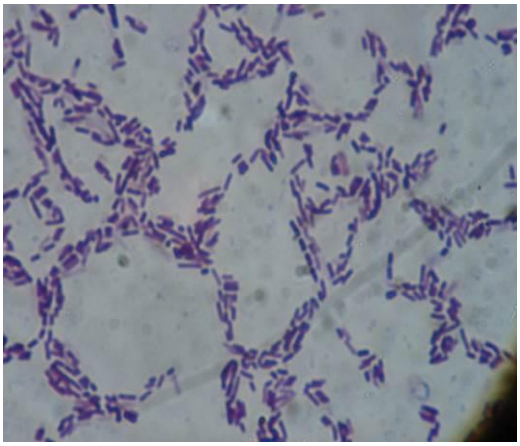

Figure 7. Microscopic appearance of the *Bacillus* sp. strain (1000x)

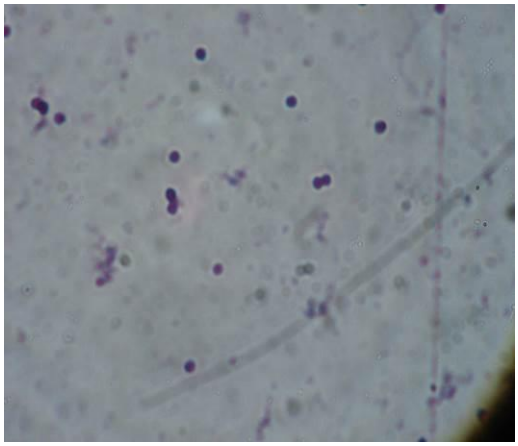

Figure 8. Microscopic appearance of the *Micrococcus* sp. strain (1000x)

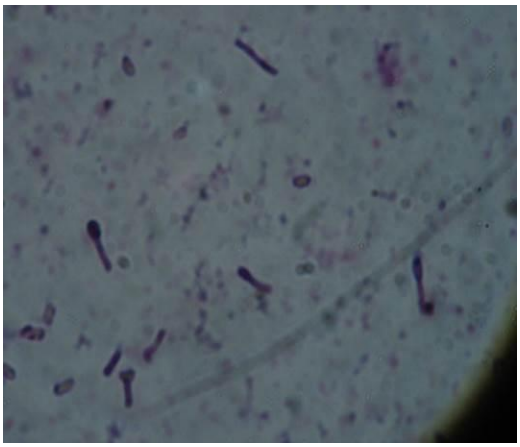

Figure 9. Microscopic appearance of the *Clostridium* sp. strain (1000x)

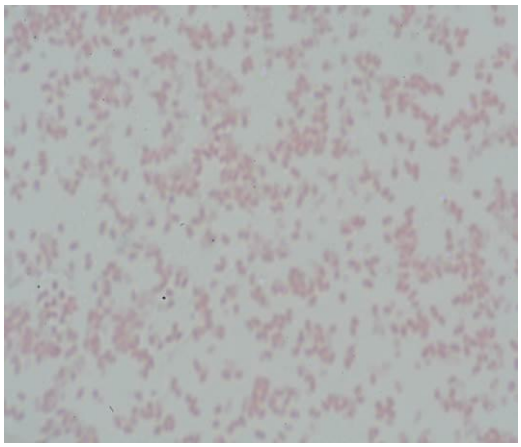

Figure 10.Microscopic appearance of the *Pseudomonas* sp. strain (1000x)

Table 3.Taxonomic classification of the bacterial strains isolated from the paper supports examined

| Strain | Kingdom | Division | Section | Family | Genus |
|---|---|---|---|---|---|
| IB,IC, ID,IIA, IIB,IIC, IID,IIIA, IIIB,IIIC, IIID,IVA, IVB,IVC, $IVD_2$ | *Prokaryotae* | *II. Firmicutes* | 13 – Endospore forming Gram - Positive Rods and Cocci | *Bacillaceae* | *Bacillus* |
| VA,VC, VD,VIIC, VIID | *Prokaryotae* | *II. Firmicutes* | 13 – Endospore forming Gram - Positive Rods and Cocci | *Bacillaceae* | *Clostridium* |
| VIIA, | *Prokaryotae* | *II.Firmicutes* | 12 – Gram - Positive Cocci | *Micrococcaceae* | *Micrococcus* |
| IA, $IVD_1$, VIIB | *Prokaryotae* | *I. Gracilicutes* | 4- Gram- Negative Aerobic Rods and Cocci | *Pseudomonadaceae* | *Pseudomonas* |

Identification of the main genera of fungi

To the purpose of identifying the main genera of fungi found on the supports investigated, the morphology of the colonies grown on the culture medium was examined; morphology examination provides important aspects for the subsequent determination based also on microscopy. The investigation of the different microscopic structures (i.e. hyphae, conidiophores, conidia etc.) by microscopy led to the identification of the principal fungal genera(Table 4).

**Table 4**. Systematic classification of the genera of fungi identified on the paper supports examined

| Kingdom | Phylum | Class | Order | Family | Genus | Strain |
|---|---|---|---|---|---|---|
| **FUNGI** | Ascomycota | Eurotiomycetes | Eurotiales | Trichocomaceae | *Penicillium* | $I_A$, $I_D$, $II_A$, $III_B$, $IV_D$, $V_D$, $VI_A$, $VII_c$ |
| | | Dothideomycetes | Pleosporales | Pleosporaceae | *Alternaria* | $V_B$ |

د ***Penicillium*Link**, *Magazin Ges. naturf. Freunde, Berlin***3**: 16 (1809)
د ***Alternaria* Nees**, *Syst. Pilze*: 72 (1816)

***Penicillium*Link**, *Magazin Ges. naturf. Freunde, Berlin***3**: 16 (1809)

***Penicillium* sp. 1** – strains $I_A$, $I_D$, $II_A$, $III_B$, $IV_D$, $V_D$(Figure 11 and Figure 12).The colony attained 3.7 cm in diameter 6 days after inoculation on Sabouraud medium at 25 °C. The surface of the colony was initially white, velvety, then turned pulverulent, yellow in the centre, subsequently green and finally bluish-green towards the margins. The colony margins were fimbriate. The reverse was yellow and smooth in the centre after 6 days. No secretion of exudates was observed. The conidiophores were branched, bi and ter-verticillate with 5-8 x 2.5-3 μm cylindrical or fusiform phialides. The phialospores were spherical, smooth, slightly yellow, of 3.5-4 μm.

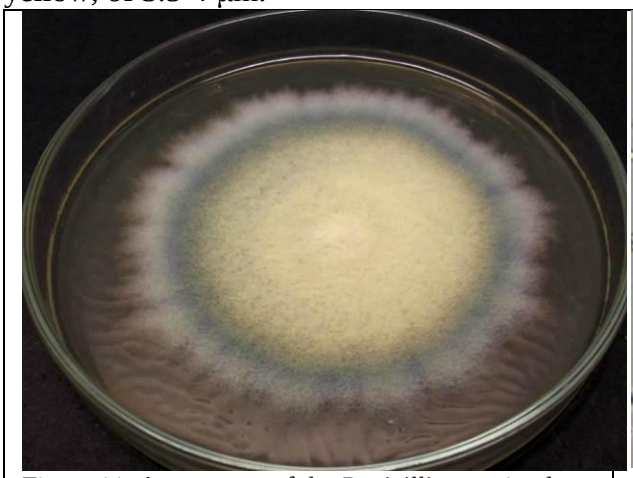

Figure 11. Appearance of the *Penicillium* sp.1 culture

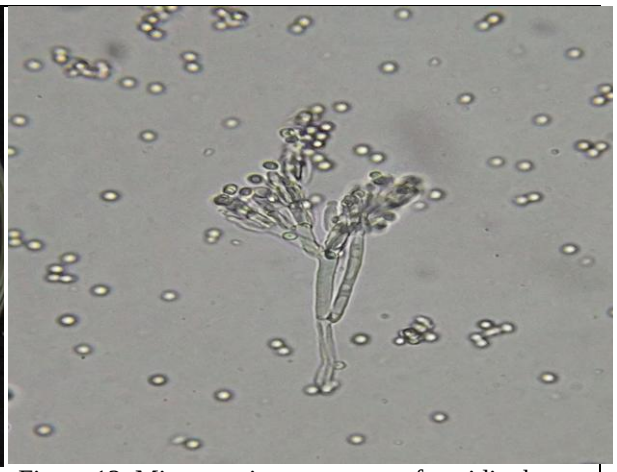

Figure 12. Microscopic appearance of conidiophore and conidia in *Penicillium* sp.1 (400x)

***Penicillium* sp. 2.** – strains $VI_A$, $VII_C$ (Figure 13 and Figure 14).The colony grew moderately reaching 4 cm in diameter 6 days after inoculation on Sabouraud medium at 25 °C. The surface of the colony was initially white, velvety, then turned powdery, uniform – smooth, bluish-green, and finally green in the centre with white margins. The margins of the colony were relatively smooth. The reverse was yellowish-white, smooth. It showed no exudates. The conidiophores were branched, of 230-290 μm in size, bi- verticillate. The phialides were cylindrical and 5-6 x 2.5 μm in size. They formed chains of 2.2-2.8 μm spherical, hyaline, and smooth-surfaced phialospores.

Figure 13. Appearance of the *Penicillium* sp.2 culture

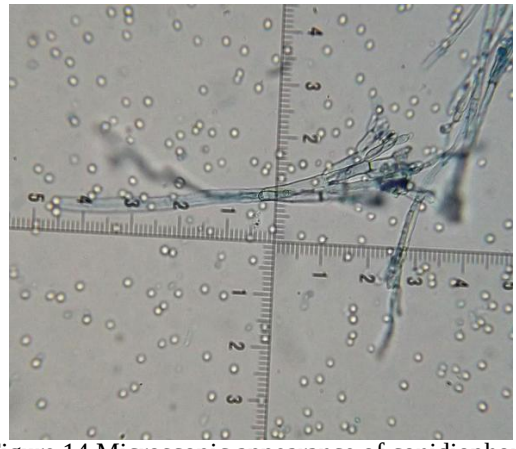

Figure 14.Microscopic appearance of conidiophore and conidia in *Penicillium* sp.2 (400x)

***Alternaria* Nees**, *Syst. Pilze*: 72 (1816)

***Alternaria* sp.,** strain $V_B$(Figure 15 and Figure 16).The colony reached 7.5 cm in diameter in 6 days on Sabouraud medium, at 25 °C. The surface of the colony was initially downy, white, then turned brown in the middle, with irregular areas of different shades of brown towards the margins. The margins of the colony were fibriate, irregular. After 6 days, the reverse was pigmented, with concentric circles of different hues from cream-coloured to dark brown, and smooth. The colony was covered by colourless exudates early on. In submerged conditions, the mycelium exhibited hyphae with numerous chamydospores forming chains, slightly pigmented, refractive, thick-walled. In addition, highly pigmented, reddish brown or dark brown hyphae were found; such hyphae break and produce arthrospores. The aerial mycelium exhibited short, septate, often geniculate conidiophores with a length of up to 62 μm. Sometimes, several conidiophores arose on the same hypha; they were slightly pigmented and moderately thick-walled. The conidia were arranged in chains and were usually knob-like, pear-shaped, oval to linear-fusiform, septate, and frequently muriform. The conidia were pigmented and thick-walled (15-55 x 12-25 μm).

The diversity of the bacteria and fungi isolated from the old paper supports areshown inFigure 17 and Figure 18. The findings show that paper degradation may come in different forms, from the appearance of small stains and yellowing to heavy staining, printed picture discolouration or even paper embrittlement and disintegration. The most commonly seen form is *foxing*, when small, circular, reddish-brown-yellow stains appear on the paper, disseminated throughout its surface.

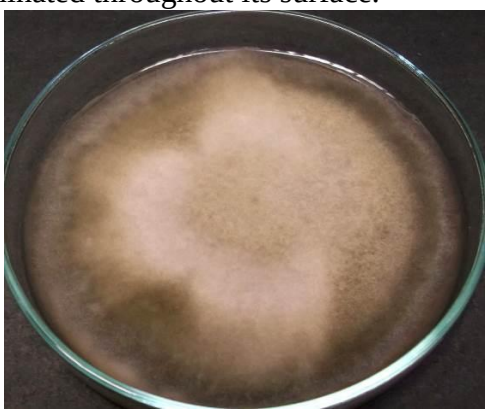

Figure 15. Appearance of the *Alternaria* sp.culture

Figura 16.Microscopic appearance of conidiophore and conidia in *Alternaria* sp. (400x)

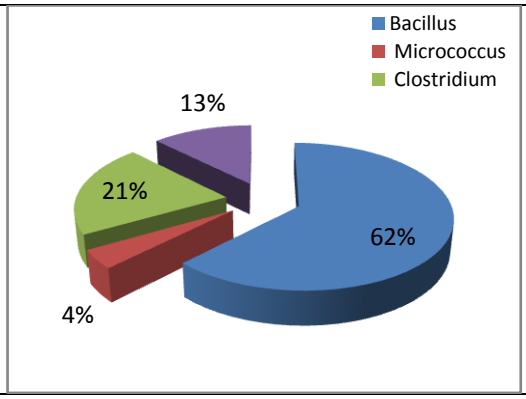


Figure 17. Bacterial genera identified in the samples examined

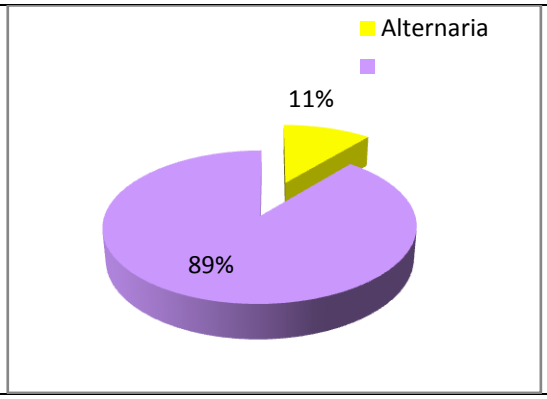


Figure 18. Fungi genera identified in the samples examined

Some authors correlate foxing with chemical processes that take place as a result of cellulose oxidation due to the catalytic action of the Fe, Cu and Co containing compounds, the reaction increasing in speed at high levels of. Many authors consider that *foxing* is caused by biological agents since different species of fungi – inexistent in other areas – have been isolated from such stains (Rakotonirainy *et al*., 2007). Using traditional methods of organism identification, Valentin (2003) found bacteria belonging to the *Bacillus, Micrococcus* and *Streptomyces* genera on the paper samples. Moreover, the bacteria of the *Pseudomonas* genus were frequently seen. Kavkler *et al*. (2011) claim the fungi are the main cause for the degradation of cellulose and cellulose-containing items. Fungi apparently attack first the cuticle and then penetrate the lumen of the fibre degrading it from the inside out. Similarly, Mesquito *et al*. (2009) showed evidence of many types of fungi on parchment, paper, books, most frequently being identified the following species: *Cladosporium sp., Penicillium* sp., *Aspergillus* sp.The only species found in all the supports were *Cladosporium cladosporioides* and *Penicillium chrysogenum*.

***Microbiologic evaluation of the decontamination treatments using high-frequency cold plasma***

In the field of movable cultural heritage preservation, high-frequency cold plasma can be used to decontaminate items made of organic materials (Baklanov *et al*., 2001, Ioanid *et al*., 2010). An environment rich in oxygen, hydrogen peroxide, nitrogen, argon gives the plasma oxidising, reducing and biocide qualities, enhancing its effectiveness. The purpose of the study conducted was the evaluation of the extent of bacterial contamination in the samples collected from the two paper supports (Figure 19 and Figure 21) as well as the assessment of the bacterial decontamination of such supports following the application of two types of high-frequency cold plasma treatments, namely the treatment of the samples for 60 seconds, respectively 5 minutes in nitrogen atmosphere, and the treatment of the samples for 5 minutes and respectively 7.5 minutes in an atmosphere of nitrogen and gas mixture of argon/oxygen (90 % Ar/10% $O_2$) - Figure 20 and Figure 22.

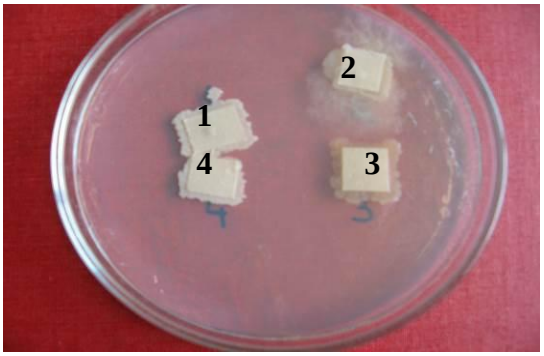


Figure 19. Bacterial culture appearance prior to the treatment with plasma (1-4: samples collected from the 1870 printed book)

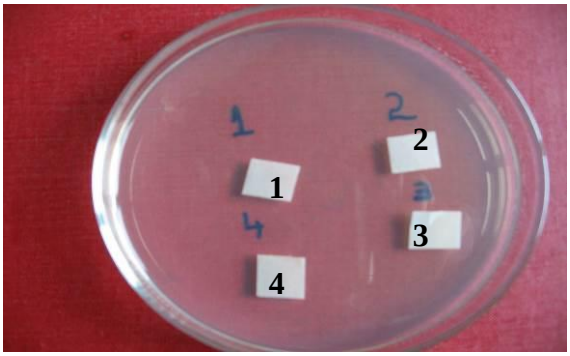


Figure 20. Bacterial culture absence following the treatment with plasma in nitrogen atmosphere and gas mixture of argon/oxygen for 5 min. (1), 7,5 min.(2), in nitrogen atmosphere 5 min. (3) and 60 sec. (4) - samples treated were collected from the 1870 printed book

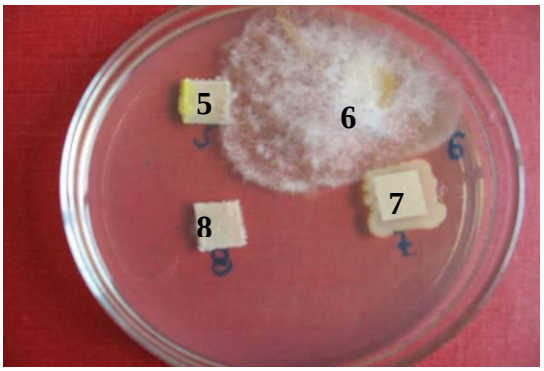


Figure 21. Bacterial culture appearance prior to the treatment with plasma (5-8: samples collected from the Church book, 19th century)

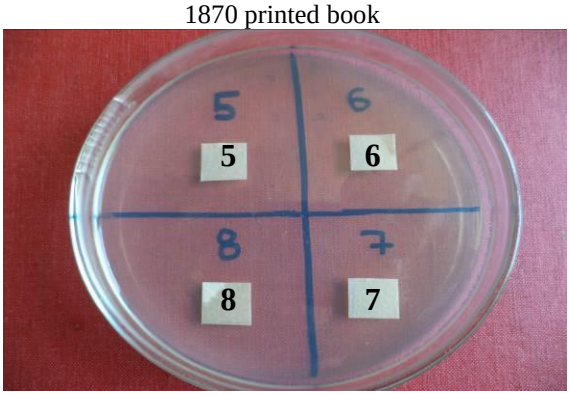


Figure 22. Bacterial culture absence following the treatment with plasma in nitrogen atmosphere and gas mixture of argon/oxygen for 5 min. (1), 7,5 min.(2), in nitrogen atmosphere 5 min. (3) and 60 sec. (4) – samples treated were collected from the Church book

The results showed the inhibiting effect of these types of treatment on the bacterial growth in both paper supports (Figure 23 – Figure 26) right after the samples were exposed to the nitrogen atmosphere for 60 seconds, respectively 5 minutes or to the gas mixture of $N_2$ and $Ar/O_2$ (5 minutes, respectively 7.5 minutes) – Table 5.

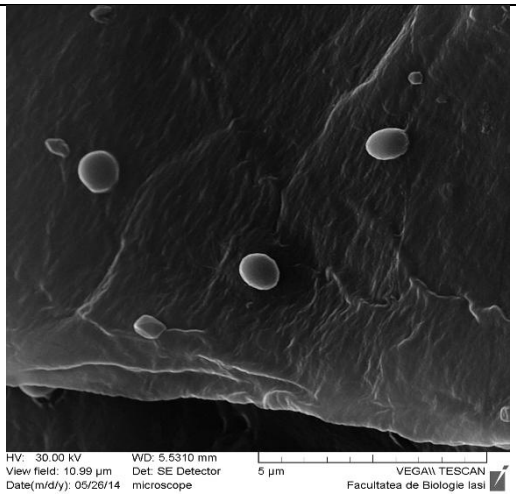


Figure 23. Scanning electron micrograph (SEM) showing microbial load of the paper support (the 1870 printed book) prior to the treatment with plasma

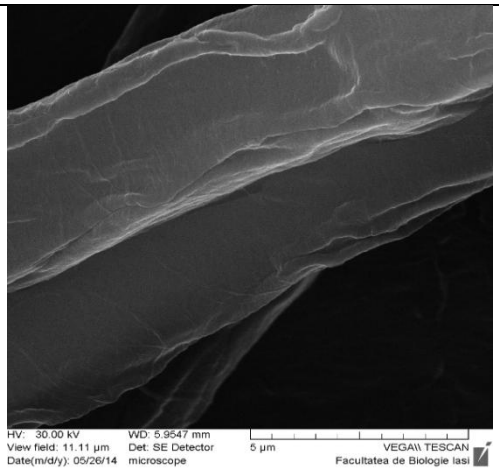


Figure 24. Scanning electron micrograph (SEM) showing absence of microbial load of the paper support (the 1870 printed book) following the treatment with plasma ($N_2$ atmosphere,60 seconds)

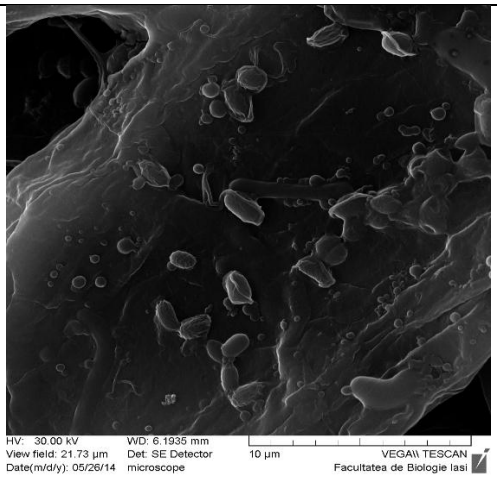


Figure 25. Scanning electron micrograph (SEM) showing microbial load of the paper support (19th century Church book) prior to the treatment with plasma

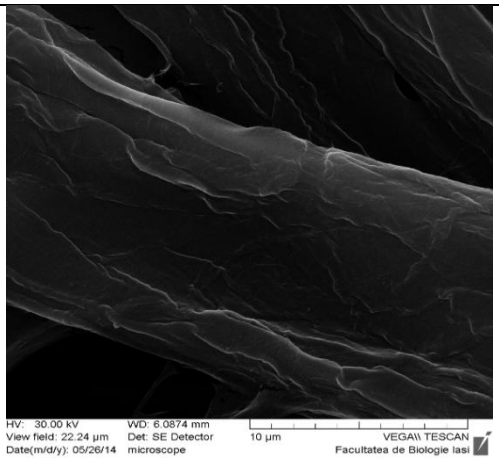


Figure 26. Scanning electron micrograph (SEM) showing absence of microbial load of the paper support (19th century Church book) following the treatment with plasma ($N_2$ atmosphere,60 seconds)

Table 5. Level of bacterial contamination in the old paper supports before and after the treatment with plasma

| Sample | Growth of bacterial cultures prior to treatment | Type of treatment | Growth of bacterial cultures following treatment |
|---|---|---|---|
| 1. | + + - | $N_2$ (5 minute) + $Ar/O_2$ (5 minute) | - - - |
| 2. | + + + | $N_2$ (7,5 minute) + $Ar/O_2$ (7,5 minute) | - - - |
| 3. | + + - | $N_2$ (5minute) | - - - |
| 4. | + + - | $N_2$ (60 seconds) | - - - |
| 5. | + - - | $N_2$ (5 minute) + $Ar/O_2$ (5 minute) | - - - |
| 6. | + + + | $N_2$ (7,5 minute) + $Ar/O_2$ (7,5 minute) | - - - |
| 7. | + + - | $N_2$ (5minute) | - - - |
| 8. | + - - | $N_2$ (60 seconds) | - - - |

*Legend*: 1-4: samples collected from the printed book (1870), 5-8: samples collected from the Church book (19th century), +++: very well growth; ++-: well growth; +--: weak growth; ---: absence of growth.

**Conclusions**

1. Degradation caused by the presence of bacteria manifests by chromatic changes, i.e. stains in a wide range of colours (e.g. purple, yellow, brown, black, red, etc.) and structural modifications to the main components due to enzymes (cellulases, proteases etc.), which induce paper embrittlement or even its partial destruction.
2. A number of 24 bacterial strains belonging to the following genera were isolated to pure cultures: *Bacillus* (15 strains), *Clostridium* (5 strains), *Pseudomonas* (3 strains)and *Micrococcus*(1 strain).
3. Bacterial contamination was found in most samples collected from the Church book (19th century) and the printed book (Paris, 1870), particularly in the foxing spots, the level of contamination varying according to the type of microorganism and the conditions in which such microorganism is active.
4. Among biological factors involved in paper degradation, the most significant are the cellulolytic fungi, which destroy paper by secreting extracellular cellulases, organic acids and pigments.
5. A considerable fungal load was found in all the specimens examined, the identified fungi pertaining mainly to the *Penicillium* and *Alternaria* genera.
6. The wide diversity of the fungi isolated from the paper supports is the result of the ability of the paper support to readily attract and retain water (being a highly hygroscopic material), which stimulates fungal growth and development.
7. The comparative assessment of the two types of treatment with high-frequency cold plasma (in $N_2$ atmosphere or in gas mixture of $N_2$ and $Ar/O_2$) shows the effectiveness of both in bacterial decontamination, their inhibitory effect being easily perceived even after the short 60-second application of the $N_2$ treatment.

**Acknowledgements**

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**References:**

Baklanov, M.R., Shamiryan, D.G., Tökei, Zs., Beyer, G.P., Conard, T., Vanhaelemeersch, S., and Maex, K. Characterization of Cu surface cleaning by hydrogen plasma. *J. Vac. Sci. Technol*., B 19.4: 1201-1211, 2001.

De Paolis, M. R., and Lippim, D. Use of metabolic and molecular methods for the identification of a *Bacillus* strain isolated from paper affected by foxing. *Microbiological Research*, 163:121-131, 2008.

Dunca, S., Ailiesei, O., Nimitan, E., and Stefan, M. Microbiologie aplicată, *Casa Editorială Demiurg*, Iasi, 74-76, 2007.

Kavkler, K., Smit, Z., Jezersek, D., Eichert, D., and Demsar, A. Investigation of biodeteriorated historical textiles by conventional and synchrotron radiation FTIR spectroscopy. *Polymer Degradation and Stability*, 96: 1081-1086, 2011.

Konkol, N., McNamara, C.J., and Mitchell, R. Fluorometric detection and estimation of fungal biomass on cultural heritage materials. *Journal of Microbiological Methods*, 80: 178–182, 2009.

Mesquito, N., Portugal, A., Videira, S., Rodríguez-Echeverría, S., Bandeira, A.M.L., Santos, M.J.A., and Freitas, H. Fungal diversity in ancient documents. A case study on the Archive of the University of Coimbra. *International Biodeterioration & Biodegradation*, 63: 626–629, 2009.

Michaelsen, A., Piñar, G., and Pinzari, F.A. Molecular and Microscopical Investigation of the Microflora Inhabiting a Deteriorated Italian Manuscript Dated from the Thirteenth Century. *Microb. Ecol.*, 60: 69–80, 2010.

Oprea, F., Biologie pentru conservarea şi restaurarea patrimoniului cultural, *Ed. Maiko*, Bucureşti, 418-428, 2006.

Pasquariello, G., Velenti, P., Maggi, O., and Persiani, A. M.. Paper, In *Plant Biology for Cultural Heritage: Biodeterioration and Conservation* (Ed. Giulia Caneva, Maria Pia Nuggari, Ornella Salvadori), 108-110, 2005.

Popescu, I., Iancu, I., and Toader, E. Fizica plasmei si aplicatii. *Ed. Stiintifica si Enciclopedica*, Bucuresti, 75-94, 1981.

Rakotonirainy, M.S., Heude, E., and Lavédrine, B. Isolation and attempts of biomolecular characterization of fungal strains associated to foxing on a 19th century book. *Journal of Cultural Heritage*, 8(2): 126–133, 2007.

Strzelczyk, A.B. Observations on aesthetic and structural changes induced in Polish historic objects by microorganisms. *International Biodeterioration & Biodegradation*, 53: 151 – 156, 2004.

Szczepanowska, H., and Lovett, C.M. A study of the removal and prevention of fungal stains on paper. *Journal of American Institute of Conservation*, 31, 2: 147-160, 1992.

Valentin, N.. Microbial contamination in museum collections: Organic materials, In *Molecular Biology and Cultural Heritage* (Ed. Saiz-Jimenez), Swets & Zeitlinger, Lisse, 85-87, 2003.