



Application of imaging spectroscopy, SEM-EDS and DNA analysis for monitoring the preservation status of a manuscript of the first decade of 19th century

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Abstract This paper presents an interdisciplinary approach on the preservation status of a manuscript made in the early nineteenth century on paper support. This approach was based on the analysis of the pigments and inks, as well as the damage of the manuscript, which was provoked by biological attack. This analysis was accomplished with the application of innovating techniques such as imaging spectroscopy using high resolution cameras and DNA analysis. The use of new technologies from diverse science can provide useful information on the inks and pigments as well as the type of biological agents found in the manuscript. Furthermore, apart from the knowledge about the inks and pigments, important elements can be retrieved on their preservation status. The aim of this research is the combined use of the detected information on the types of inks and pigments with the biological investigation in order to trace damage caused by biological attack and to investigate the causative agent of this attack. For this reason, imaging techniques, SEM/EDS analysis were applied to the inks and pigments as well as a microbiologic and molecular analysis of the fungi found on the pigments of manuscript.

1 Introduction

This paper describes a study on a nineteenth century manuscript using multispectral imaging, SEM/EDS and biological analysis in order to examine the inks and pigments of the manuscript as well as the presence of biological agent that could cause damage to them. Up to now many investigation methods have been applied to the pigments and inks characterization using spectroscopy and SEM/EDS analysis [1–8], as well as to address biological attacks in libraries and in microbial investigation using DNA analysis which is considered as the most innovative and sensitive method to successfully discriminate the type of biological agents.

The imaging techniques have been often used in manuscript analysis such as spectral imaging of the pigments that have been applied using multispectral and hyperspectral imaging, giving valuable conclusions on the characterization of pigments. In most cases, the imaging techniques have been complemented with other physicochemical methods, such as SEM/EDS analysis. Bonifazi et al. [9] used the Hyperspectral Imaging to trace the changes of painting samples and they succeeded to monitor the ageing features of pigment and watercolor samples based on a multivariable approach. Strojnik and Bravo-Medina [10] examined pigments used in Mexico in the past and they compared their transparency through different regions of spectrum. Deborah et al. [11] discriminated pigments used by Munch in his painting “The Scream” and created a mapping of these pigments for the entire work of art. Jovanović et al. [12] identified pigments and ground materials of a degraded work of art painted by Lazar Vozarević in 1961, titled “Untitled”, applying among others the SEM/EDS analysis. Pronti et al. [13] proposed a mapping for the identification of white pigments using multispectral and UV-fluorescence imaging. Polak et al. [14] created a database based on the combination of the hyperspectral

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imaging and signal processing techniques, retrieving features in order to classify pigments and validated the results using the SEM/EDS analysis among other used physicochemical techniques. The SEM/EDS analysis can provide information on the elements of the materials, tracing any examined pigment or ink. Titubante et al. [15] analyzed inks and papers of Arabic-Christian manuscripts in order to find similarities among these materials using physicochemical analysis, in which the infrared reflectography helped to discriminate inks into categories. Lizun [16] applied multispectral techniques and SEM/EDS in order to study the Liu Kang's palette, investigating simultaneously the brushstrokes of the artist. Michaelsen et al. [17] examined different treatments to inhibit fungal attack and compared their results using molecular analyses. Sequeira et al. [18] present a review of chemical and physical methods used to avoid fungal paper biodeterioration and some contemporary approaches. Pinzari et al. [19] present innovative methods applied to microbiological research for book and they showed that unsuspected microbial consortia are involved in the discoloration and biodeterioration of books and archival materials. Mesquita et al. [20] identified infecting fungi from documents on the Archive of the University of Coimbra using DNA analysis and morphological micro and macro traits. Zyska [21] isolated many species of fungi from library material and considered it very difficult to deal with the growth of biological agents in libraries.

The aim of this study was to apply a combination of analysis techniques in order to study the materials used in illuminations and texts of manuscript and the biological agent found in a pigment of illuminations of manuscript. Based on this aspect, multispectral imaging was applied using cameras with high resolutions. High resolution cameras give a completely different picture and allow to capture sharp, detailed images faster, ensuring minimal distortion on images, with the best level of quality and precision. Thus, macroscopic images were taken from the entire page of the manuscript in different regions of the electromagnetic spectrum: ultraviolet, region between ultraviolet and visible, visible, region between visible and infrared and infrared. Imaging techniques have been mainly used in order to recognize differences and similarities of the optical behavior of pigments and manuscript inks as well as on their preservation status. Taking image from the entire area of a painting or a page of manuscript can offer an overview of all pigments and inks as well as their preservation status simultaneously. This proved very useful in the case of this research, because using multispectral imaging, difference between regions of the same pigment was detected. Multispectral imaging was supplemented with microscopic imaging (ultraviolet, ultraviolet fluorescence, visible and infrared) in different regions of electromagnetic spectrum. Thus, the characteristic texture of a biological agent appeared in the microscopic imaging, which was further studied. Although the imaging spectroscopy and the SEM/EDS analysis can provide significant information on the traces of the application process, in the elements constituting the pigments and inks and their preservation status, the biological attack can be more thoroughly examined by biological methods such as cultivation of the biological material and DNA analysis that will provide information about the viability of the microorganism and its identification. Increased awareness of the factors that have favored the presence of the biological attack is necessary in order to apply measures and treatments that would prevent its further development. Following the observation of the presence of the biological agent which resembled a fungus-type microorganism that has contaminated specific pigmented spots of the manuscript, this study has attempted to obtain a species-level identification of this unknown microorganism. This purpose was achieved by culturing the microorganism in laboratory conditions and then applying a DNA identification analysis using the Internal Transcribed Spacer (ITS) region which is a fungal phylogenetic marker [22–25]. The DNA analysis was used to investigate the biological agents found in the pigments and inks of the manuscript. Finally, the SEM/EDS analysis was applied to pigments and inks used in the illuminations and the texts of manuscript in order to recognize the elements that consist of.

2 Materials and methods

2.1 Description of the studied manuscript and the environmental condition of its storage

The studied manuscript is an ecclesiastic book belonging to the Pezaros' Collection of Historical and Paleographical Archive, Cultural Foundation of the National Bank (Greece), with catalogue number 2. It is a *Παρρησια*,¹ written by Priest Margaritis Sekkelidou in Tyrnavos on 28 May 1807, according to the note on page 3 verso and 9 verso. The text is written in one column on the paper support with black and red inks. The paper support is made of handmade paper that was made with rags, so it is made of pure cellulose. In most cases the names of the manuscript were written in uppercase letters using red inks and in lowercase letters using black ink. The manuscript is carrying two half-page illuminations (pg. 4 and pg. 10) and two full-page rectangular decorations (pg. 3 and pg. 9) that contain texts. In both cases the texts are entitled *Ιερα Προθεσις*.² In the illuminations and decorations, the black ink of the synthesis drawing and four or five pigments have been used at least. For the last twenty years, the manuscript has been kept in a renovated building and in a specific room, where all the manuscripts of the Paleographic and Historical Archives are kept. The renovation of the building lasted about 5 years. The manuscript storage room has a conservation system in accordance with international conservation standards. The prevailing conditions are 19 °C with a maximum variation of 1 °C per 24 h and a relative humidity of 55% with a maximum variation of 5% per 24 h. The manuscript is kept in complete darkness in closed cabinets and the space is equipped with a system that has filters to remove harmful particles and gases from the air.

¹ *Παρρησια* is the book containing the names of the believers, living or dead, which are mentioned in the sequences of a monastery.

² *Ιερα Προθεσις* in the Orthodox Church is the place where the offered gifts were preparing in order to be performed the sacrament of Holy Communion.

2.2 Multispectral imaging

For each studied page of the manuscript, four images were taken in different electromagnetic regions, such as in the visible, in the ultraviolet, in the region between visible and infrared and in the infrared region. Finally, in the chosen points of the letters, the decorations and the illuminations, microscopic images were taken in the visible, in the ultraviolet, in the region between ultraviolet and visible, where the fluorescence phenomenon happens, and in the infrared, as it is described below.

2.2.1 Macroscopic images

Through the macroscopic images the entire page is recorded on where the inks and pigments of interest exist. Images were taken using the cameras Nikon D 5200 for the images in the visible region and Fujifilm XT-10 Mirrorless, Full Spectrum Camera for the images in the non-visible regions. Nikon D 5200 camera with the lens Nikon Nikkor Af-S DX 18–55 f/3.5–5.6 has 24.0MP and the spectral response is approximately between 400 nm and 780 nm. The Fujifilm XT-10 Mirrorless, Full Spectrum camera with the lens Nikon Nikkor 50 f/1.8 has 16.0MP and the spectral response of its CMOS sensor is approximately between 360 nm and 1100 nm [26]. Using the Nikon D 5200 camera, images in the visible region were taken. Using Fujifilm XT-10 Mirrorless, Full Spectrum camera, images in the infrared, ultraviolet region of electromagnetic spectrum were caught. Except for the cameras, the set up is composed of a couple of lights, a color scale,³ a grey scale and different optical filters on the camera lens, as described below for each capturing in the different regions of spectrum:

1. Images in the visible region: The optical filter of 486⁴ B & W was placed to the front of camera lens and the couple of halogen lights was diffused in the visible and the infrared region [27, 28].
2. Images in the ultraviolet region: The lighting derived from a couple of UV black lights [29], while in front of the camera lens the optical filter of 403⁵ B & W was used.
3. Images in the region between visible and infrared region: The couple of lights emitted in the visible and the infrared, as above, and in front of the camera lens the optical filter 099⁶ B & W was placed.
4. Images in the infrared region: The lights emitted in the visible and the infrared as above, and in front of the camera lens the optical filter 093⁷ B & W was placed.

2.2.2 Microscopic images

The microscopic images were taken in the visible, ultraviolet, infrared region and the region between ultraviolet and visible using two digital microscopes (Dino Lite Microscopes). These microscopes have magnifications between X20 and X220. The microscopic images are the recording of a specific point, where the inks and the pigments are of interest.

- The first one⁸ (AM4115T-CFVW) presents radiation regions of interest approximately between 400–780 nm in the visible. Furthermore, it is viewing fluorescent objects by using 400nm near UV excitation LEDs and it has a long-pass type 430nm emission filter.
- The second one (AD4113T-I2V) is taken images in the ultraviolet region and the infrared region. The radiation regions of interest are 395nm in the ultraviolet and 940nm in the infrared.

Firstly, the areas of interest were detected and then from these locations, images were taken in different regions of the electromagnetic spectrum. The images in magnifications X70 and X180 in different spectral regions were selected because they led to better results for the characterization of the inks and the pigments.

2.3 SEM/EDS analysis

The used electronic scanning electron microscope (SEM) was JEOL JSM-6510LV connected to the energy-dispersive X-ray spectrometer of Oxford X-act. The analysis was accomplished using micro-sampling⁹ after taking some particles of the material on small cotton swabs. The micro-samples were placed in a BDL-TEC CED 030 carbon evaporator for 10 min. The micro-samples were then examined in high vacuum conditions using an accelerating voltage of 20 kV and a working distance of 10 mm. All micro-samples

³ The use of the two scales, gray-level and color, is an essential tool for ensuring the accuracy and consistency of image colors, making them more comparable and easier to analyze.

⁴ Handbook, B+W FILTERS, Optical Perfection World-Wide, p.21.

⁵ Handbook, B+W FILTERS, Optical Perfection World-Wide, p.21.

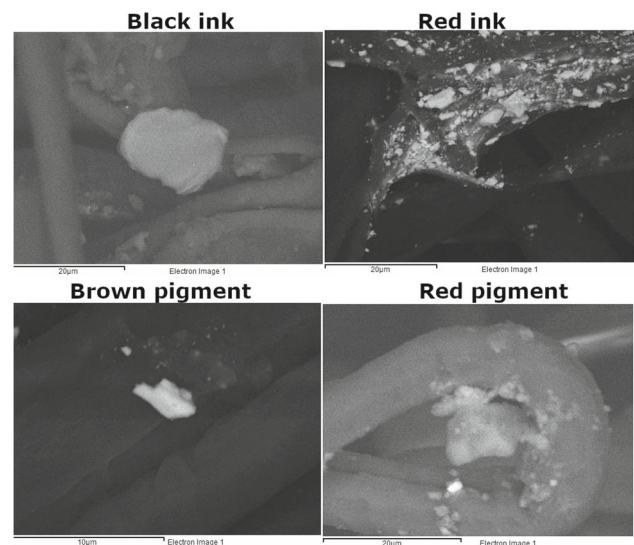
⁶ Handbook, B+W FILTERS, Optical Perfection World-Wide, p.19.

⁷ Handbook, B+W FILTERS, Optical Perfection World-Wide, p.19.

⁸ <https://docs.rs-online.com/a1f4/0900766b814406e5.pdf>.

⁹ R. KLOCKENKAMPER and A. VON BOHLEN, Analytical characterization of artists' pigments used in old and modern paintings by total-reflection X-ray fluorescence, *Spectrochimica Acta*, 48B (2): 239–246 (1993).

Fig. 1 Scanning Electron images of inks and pigment



were investigated using BSED (Backscattered Electron Detector), which contributed to their examination. The SEM images of the inks and the pigments were in magnification X1800–X2700, apart from the images of the brown pigment, which presented very small granules and a small number of them, hence its magnification was X6000 (Fig. 1).

2.4 Microbiologic analysis

Samples of the unknown microorganism (presumably a fungus due to the evident presence of mycelium) were collected from two different spots of the manuscript with a sterile swab and were spread immediately on the surface of sterile petri dishes with Sabouraud Dextrose Agar (SDA), a medium selective for fungal growth. The petri dishes were sealed with parafilm and were transferred the same day to the Food Microbiology Lab for incubation in 22 °C for 5 days. Following the growth of fungal mycelium, observation with optical microscope with Lactophenol Blue stain (x400) and Scanning Electron Microscope (SEM, JSM-6510LV) was performed.

The samples of the fungal colony taking from the petri dish were cut to a size of approximately 3 × 3 mm. To observe the microanatomical morphology and their structure in the electron microscope, chemical fixation of the samples was performed, which included placing them in 2% formaldehyde (HCHO) aqueous solution 0.1M for 30 min, 2% potassium dichromate (K₂Cr₂O₇) aqueous solution 0,1 M for 30 min. Dehydration was then performed in increasing concentrations of alcohol (20%, 40%, 60%, 80%, 100%) for 5 min at 25 °C. The samples were the gold-coated with cobalt gold alloy using the sublimator (Bal-Tec CED030), in argon (Ar) 10–2 mbar for 2 min. Other operating parameters were: vacuum 1×10^{-9} Torr, electron acc

eleration voltage 20 kV, working distance (WD) of the sample surface from the detector 15 mm with a possible deviation of ±1 mm. The observation was made with secondary electrons (SEI).

2.4.1 Molecular identification analysis

The Fungal Internal Transcribed Spacer region which is a DNA sequence located between the fungal nuclear genes that code for ribosomal RNA (the small subunit ribosomal RNA or SSU-18 S and the large subunit RNA or LSU-28 S) was used for DNA analysis. The ITS region is characteristic in all organisms belonging to the kingdom of Fungi but because it exhibits high genetic variation among different fungi species, it is considered suitable for molecular phylogenetic sequence identification of fungi [21–24]. The database used in this study was the International Sequence Database (INSD: GenBank at the National Center for Biotechnology Information NCBI, US National Library of Medicine). GenBank can identify approximately 15,500 fungal species [24].

DNA extraction of the two samples collected and a positive control (the filamentous fungus *Aspergillus niger*) was performed with the NucleoSpin Food kit (Macherey-Nagel, GmbH & Co. KG, Germany) following the instructions of the manufacturer with some modifications in the prelysis step in order to improve the degradation of the cell walls of the fungi. Initially, a loop of the mycelium of the fungal culture from the petri dishes was placed into 200 µL peptone water and then 550 µL Lysis buffer and 20 µL proteinase K were added. Subsequently the tubes were placed in an ultrasonic water bath for 2 h at 65 °C and then were left for overnight incubation at 65 °C. After lysis, the binding, washing and elution steps were followed according to the manufacturer's instructions to elute highly pure DNA. DNA quality and concentration of samples was determined spectrophotometrically (nanospectrophotometer Biotek, Epoch) by measuring the OD₂₆₀/OD₂₈₀ ratio which was between 1.7 and 1.9, a ratio which renders the extracted DNA suitable for PCR analysis. DNA concentration was measured 9.877 ng/mL and 17.405 ng/mL for each of the samples analyzed. The primers ITS1 and ITS2 were selected for PCR amplification of the ITS region of the fungus (Table 1). PCR Reactions were performed in a 25 µL final volume, containing 12.5 µL of Master Mix (KAPA SYBR GREEN Fast qPCR, KAPA

Table 1 Primers used for PCR targeting the ITS region of the fungus

Primer	Size (bp)	Sequence (5–3)	Target gene	Gene Location (bp)	Reference
ITS1 (F)	290bp	TCCGTAGGTGACCTGCGG	Fungi Internal	1761–1779	White et al 1990
ITS2 (R)		GCTGCGTTCTTCATCGATGC	transcribed Spacer region	2024–2053	

BIOSYSTEMS), 1 μM of each primer, 5.5 μL of eluted DNA and 6 μL distilled sterile H₂O to add up to 25 μL . The amplification procedure consisted of a 10 min initial denaturation step at 95 °C, followed by 40 cycles of 60 sec denaturation at 95 °C, 60 sec annealing at 62 °C, extension at 72 °C for another 60sec and a final extension step at 72 °C for 7min. PCR products were visualized by DNA electrophoresis on 2% agar stained by ethidium bromide.

PCR amplicons were sequenced by Sanger Sequencing, (Applied Biosystems ABI3730xl by Cemsa SA) and electropherograms were viewed using the program Chromas.¹⁰ Sequences were submitted in Basic Local Alignment Search Tool (BLAST) which finds regions of similarity between biological sequences and is provided freely by the National Center for Biotechnology Information, US National Library of Medicine.¹¹ The program compares the nucleotide sequences submitted to sequence databases and calculates the statistical significance between the submitted sequence and the sequences in the database named “Nucleotide collection (nr/nt)” using the program BLASTN 2.10.0 (which optimizes for somewhat similar sequences) [30, 31].

3 Results-discussion

3.1 Multispectral imaging

In visible region of the spectrum, we can see the real image of the page with the real color of the paper, the inks and the pigments. This region is the only one region of the spectrum in which a full range of colors exists, while in the infrared and ultraviolet regions a variety of colors doesn't exist because of their monochromatic nature. However, based on the different absorption of the inks and the pigments in these regions of spectrum, they have different reflective ability that can give significant information on the nature of these materials. The ultraviolet radiation is a radiation that can provide information on the surface of the inks and the pigments, such as the varnish existence on them. In the ultraviolet region of the electromagnetic spectrum, we can ascertain the preservation state of the paper, the inks and the pigments. Usually, in the infrared radiation, different absorption of the inks and the pigment can be recorded due to the different nature of them. Sometimes, this material absorption can complete information about the characterisation of the materials.

3.1.1 Macroscopic imaging

Multispectral imaging gave satisfactory results about the optical behavior of the inks and the pigments used in the texts, decorations and illuminations of the manuscript, allowing the study of the inks and the pigments of manuscript. Figure 2 shows the illumination on page 4 that is belonging to the manuscript no. 2 of the Pesaros' Collection in four different regions of the electromagnetic spectrum. The inks and the pigments according their nature absorb different or similar electromagnetic radiations. Using multispectral imaging, two red colors were detected, one of them presented similar optical features with the red ink, especially in the infrared region, and the other one presented different optical features from the red ink, especially in the infrared region. This becomes distinct in the illumination depicting the Virgin, especially in the Virgin's red garments and Her Halo, where a different absorption of ultraviolet region is observed.

Similar, in the illumination on page 3 verso, at the same color was detected different absorption of ultraviolet radiation (Fig. 3), difference that is limited only in the red pigment without any extension on any other pigment around it.

Using macroscopic imaging techniques significant conclusions on the inks and pigments were taken (Fig. 4). All black inks of texts displayed the same absorption in the visible, ultraviolet, infrared and in the region between visible and infrared. Similarly, all red inks of texts presented the same absorption of each studied corresponding radiation. The similar absorptions indicate that all texts have been written with the same type of black and red inks [47, 48].

Similarly, the decorations and illuminations have been painted using the same corresponding pigments, such as the same blue pigment used in all decorations and illuminations of the manuscript (Fig. 5). In some cases, the pigments on the decorations and the illuminations present more than one density. These colors present typical brushstrokes of watercolors, hence, it is about semi-transparent materials [32, 33].

¹⁰ www.technelysium.com.au.

¹¹ <https://blast.ncbi.nlm.nih.gov/Blast.cgi>.

Fig. 2 Illumination of manuscript on the page 4 recto in different regions of electromagnetic spectrum (Red arrows indicate the fungi, blue arrows indicate the one red pigment and the green arrows indicate the second pigment)

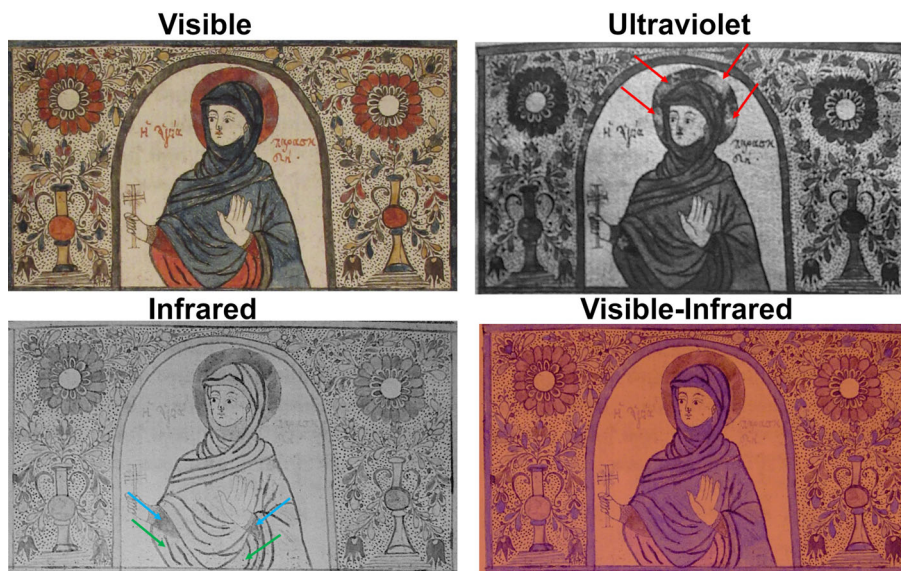
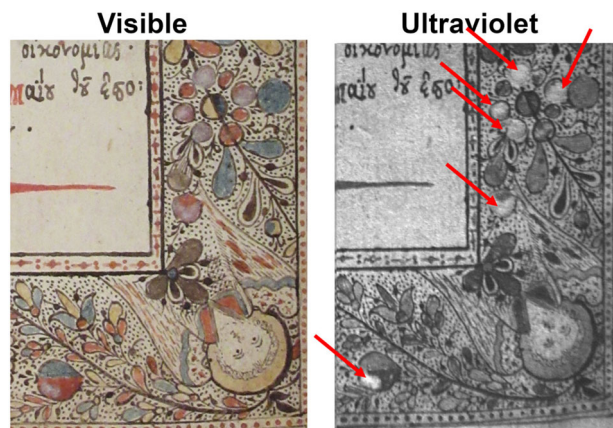


Fig. 3 The right bottom part of page 3, in visible and ultraviolet region of spectrum. Red arrows show the different absorption of ultraviolet radiation in the areas with the same colour



3.1.2 Microscopic imaging

Through the microscopic images certain features could distinguish that were not legible or clearly visible in the macroscopic images. This data allowed us to derive accurate and precise results from the macroscopic images. Studying the microscopic images in the locations where a different absorption of the same color had been observed, a biological agent was ascertained. The biological agents is distinguished on page 3 with the red color as in Fig. 6 is presented.

Most observations of the macroscopic imaging were verified through the microscopic imaging as shown in Fig. 7 about the inks of the manuscript.

As mentioned above, there was a difference in the absorption of the same red color. Using microscopic imaging, the deviation proved to be caused by the biological attack (Fig. 6). The type of damage and the affected sites (extent and boundaries) can now be clearly distinguished. In addition, a significant multiplication of the biological attack was observed, mainly in the red color and especially in areas where the red color has different characteristics from the red ink. For this reason, the SEM / EDS analysis was decided to be applied to both red pigments and other pigments and inks in order to look for the relationship between the two red colors and the materials used in the texts, decorations and illuminations.

3.2 SEM/EDS analysis

From the macroscopic and the microscopic images in different regions of the electromagnetic spectrum, it was proved that some pigments and inks with the same color had different optical behavior; therefore, the analysis of micro-sampling of specific inks and pigments using Scanning Electron Microscope (SEM) and the energy-dispersive x-ray spectrometer (EDS) was considered useful. Except for the pigments and the inks with same color but different absorption, analysis was applied to more pigments found around the presence of the biological attack. The micro-sampling was focused on targeted locations found through the imaging spectroscopy, especially the microscopic images. In this way, the micro-sampling was precise taking a very small amount (some

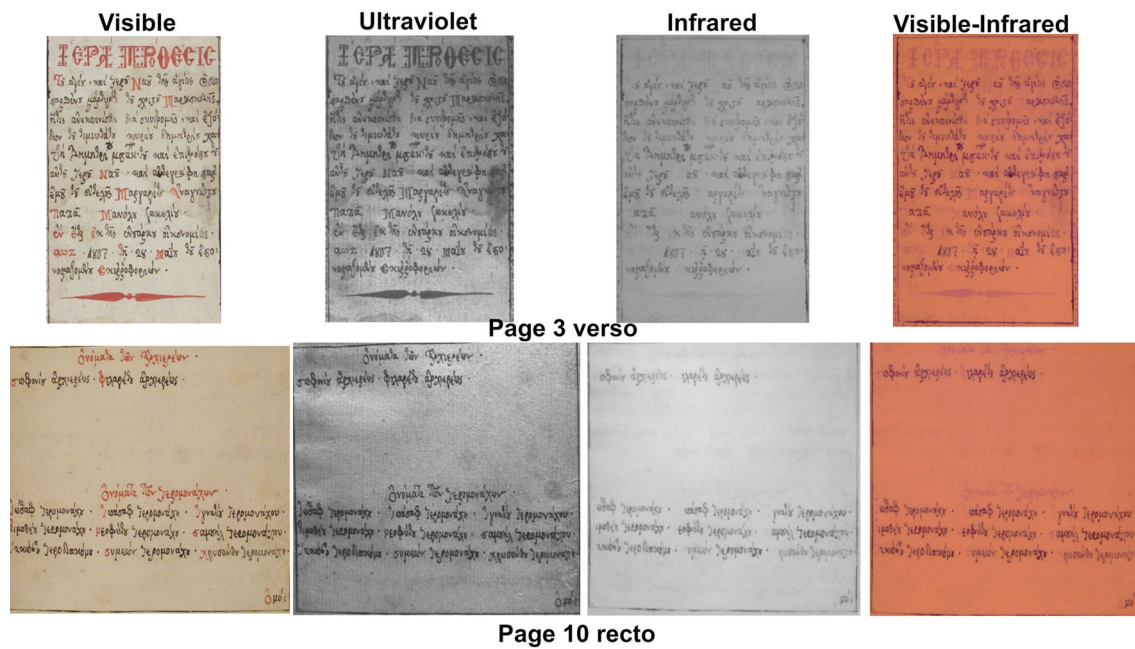
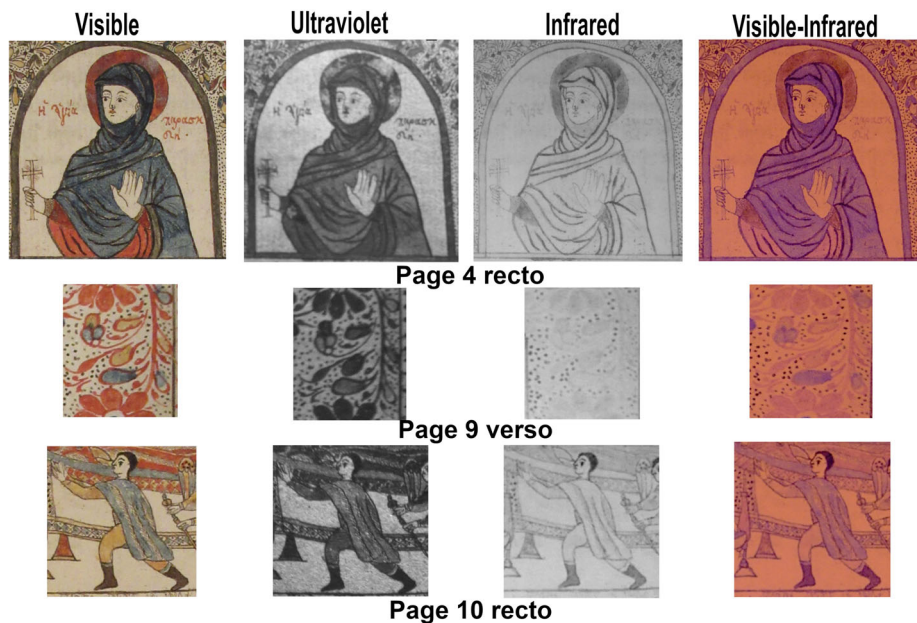


Fig. 4 Examples of texts in different regions of spectrum)

Fig. 5 Image examples from areas of the manuscript in which details containing red, blue and yellow pigment in different regions of spectrum are depicted (Yellow arrows indicate different densities)



granules) of the materials. Subsequent to the SEM/EDS particles analysis, in Fig. 8 a typical example of the spectrum of the yellow pigment, deriving from the decoration on page 3, is presented. This spectrum shows significant peak of silicon and oxygen and a slightly smaller peak of aluminum and carbon, following smaller peaks of sodium and some traces of iron, magnesium, calcium and potassium. Based on the detected elements, it indicates that the yellow pigment is probably a yellow earth, a yellow ochre that could come from biotite [34, 35].

The SEM/EDS analysis was applied on one microsample of the all types of inks and pigments used into the texts and decorations of manuscript. Materials microsamples were taken using a sterile cotton swab [36]. In all cases of microsamples were determined carbon (C) and (O) oxygen due to the presence of the cellulose of cotton swabs. In the black ink microsample appears the presence of (Fe) iron in high amount. Although the high amount of the (Fe) iron elements could indicate an iron-gall ink, the absent of sulfur doesn't allow us this definition. In the SEM/EDS analysis of the red ink was determined high amount of (Hg) mercury with the simultaneously existence of (S) sulfur particles, which indicates that the red ink derives from the cinnabar pigment used as a writing ink. Through the imaging spectroscopy the existence of two different pigments used in the illuminations and the decorations was

Fig. 6 Microscopic images of the location where red pigment exists on page 3 in magnification X70

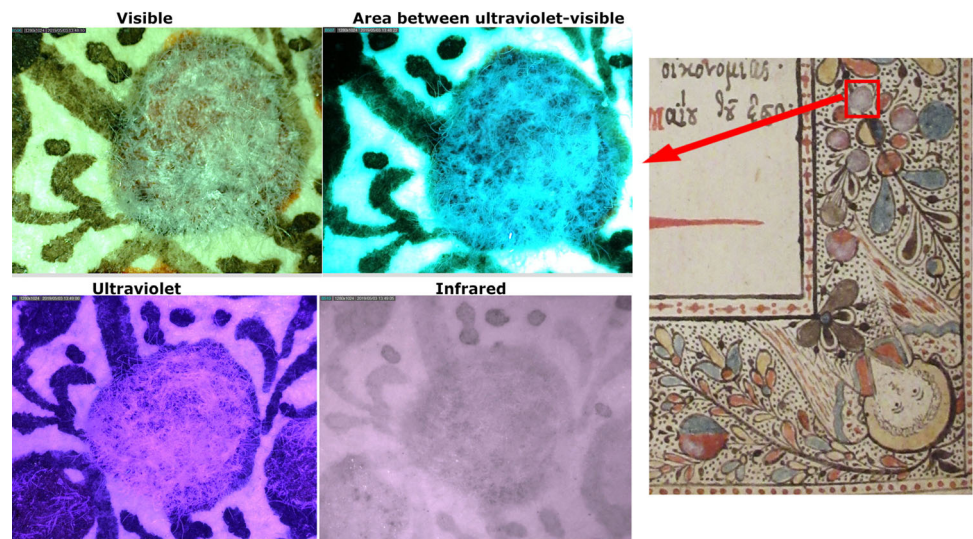
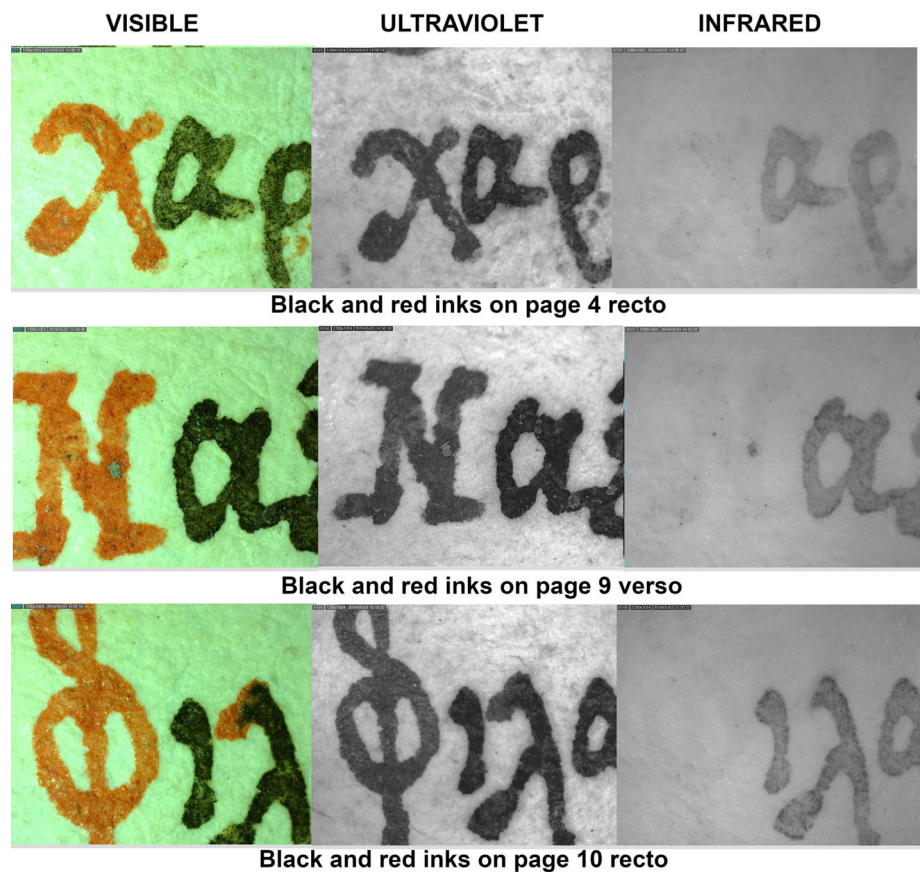


Fig. 7 The microscopic images of black and red inks of the manuscript



indicated (Fig. 8). One of them has similar optical features with the red ink, but these features do not appear in the other red pigment. In the SEM/EDS analysis of the red pigment with different features from the red ink, the detected elements (Table 2) show possibly an earth pigment, such as red ochre, because except for the existence of (Fe) iron elements, (Si) silicon, (Al) aluminum elements and other typical traces of the earth pigments were detected. Similarly, concerning the blue pigment, during the SEM/EDS analysis, the detected elements (Table 2) indicate probably natural ultramarine¹² but sulfur (S) was not detected and on another hand detected

¹² Natural ultramarine is known in ancient times. Necas R., Vsiansky D.. Ultramarine - not just a pigment of traditional folk architecture plasters, Elsevier, *Procedia Engineering* 151 (2016) 114–118. Synthetic ultramarine was invented in 1826. Lotut Z. Blue in Eighteenth-Century England: Pigments and Usages, *Revue de la Societe d'etudes anglo-americaines des XII et XVIII siecles*, (2018).

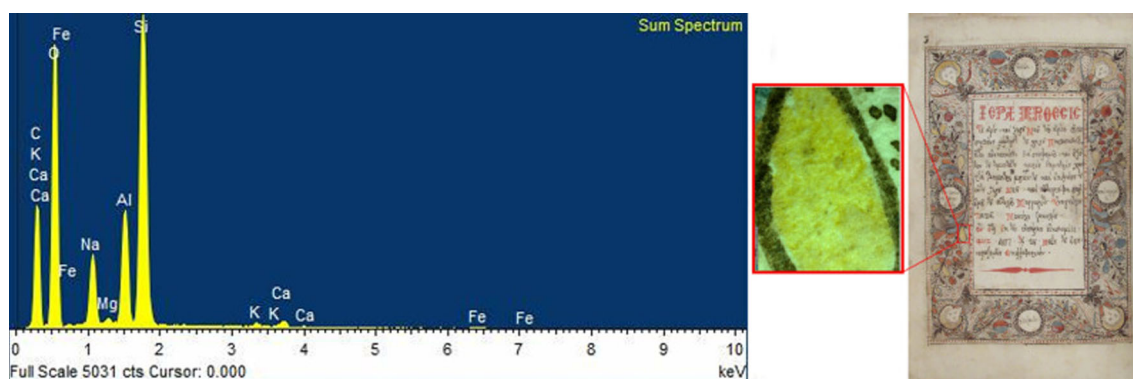


Fig. 8 SEM/EDS analysis of the yellow pigment used into the manuscript decoration

Table 2 The SEM/EDS analysis of the inks and pigments of the manuscript

Color	Elements (Weight%)
Black ink	C (43.74), O (36.82), Fe (12.47), Si (3.11), Al (1.81), Mg (1.48), Ca(0.39), K (0.18)
Red ink	C (41.19), O (27.47), Hg (25.24), S (4.46), Ca (1.35), Si (0.29)
Black pigment	C, O
Red pigment	C (56.67), O (37.64), Fe (2.49), Si (1.89), Na (0.48), Al (0.43), Ca (0.32), Mg (0.08)
Red pigment	C, O
Blue pigment	C (48.24), O (41.71), Si (5.42), Al (2.25), Na (0.91), Ca (0.88), Fe (0.23), K (0.22) Mg (0.07), Cl (0.06)
Brown pigment	C, O
Black pigment	C (60.95), O (38.51), Ca (0.29), S (0.16), Si (0.09)
Yellow pigment	C (37.10), O (46.20), Si (9.47), Na (3.23), Al (3.22), Ca (0.30), Fe (0.20), Mg (0.16) K (0.12)

iron (Fe) that corresponds to the iron blue pigments such as Prussian blue¹³. Probably this blue color is a mixture of both pigments. Similar elements were detected in the yellow pigment as depicted in Table 2. Although the presence of iron is significantly dwindled, the distribution of all elements indicates a typical yellow earth derived probably from biotite, as it was mentioned above. Finally, regarding the brown pigment, it showed consistently very small particles, mainly consisting of (C) carbon and (O) oxygen. The extremely high content of carbon and oxygen makes us speculate the probable use of an organic color.

3.3 Microbiologic analysis

Macroscopic observation of the colonies formed in SDA medium showed the formation of white hyphae typical of filamentous fungi (Fig. 9a) that was similar for both samples. Microscopic observation with lactophenol blue stain (Fig. 9b) and Scanning Electron Microscopy (Fig. 9c, d) revealed long tubular branching structures. However, no distinct morphological characteristics such as spore-producing structures (eg. conidiophores) that could aid the identification of the species of the microorganism could be observed.

DNA electrophoresis of the two samples and the positive control (*Aspergillus niger*) showed clear DNA zones that had a length of 290 base pairs (bp) which is the typical length of the DNA sequence of the ITS region that was amplified by PCR with the specific primers used (Fig. 10). This result confirms that the DNA extracted from the samples had an appropriate quantity and quality and more importantly that the unknown samples tested are both fungi since they test positive for the ITS sequence.

The PCR samples were sequenced by Sanger Sequencing and the electropherograms viewed by the program Chromas (Fig. 11a) were submitted in Basic Local Alignment Search Tool (BLAST). Results (Fig. 11b) showed 100% similarity with the sequences of the fungus *Chaetomium globosum* (GenBank accession numbers SUB7245160 20200312CS1P2 MT279537). This result confirms that the fungus investigated belongs to this species (if the similarity of ITS sequence is above 97% then it is considered the same species [23]).

Chaetomium globosum, (genus belonging to phylum Ascomycota) is ubiquitous, occurring on a wide variety of substrates, in air and in marine environments. This species is recognised as a cellulolytic and/or endophytic fungus and produces secondary metabolites and mycotoxins [37, 38]. *Chaetomium globosum* has also been reported as an air contaminant causing adverse health

¹³ Prussian blue was synthesized around in 1706. Kraft A. Notitia Coerulei Berolinensis linensis nuper inventi on the 300th anniversary of the first publication on Prussian blue Bulletin for the History of Chemistry. 36 (1) 3–9 (2011).

Fig. 9 Observation of the mycelium of the unknown fungus **a** in SDA culture, **b** under the photonic microscope (x400) with lactophenol blue staining, and in the scanning electron microscope at x1,200 (**c**) and x1,800 (**d**)

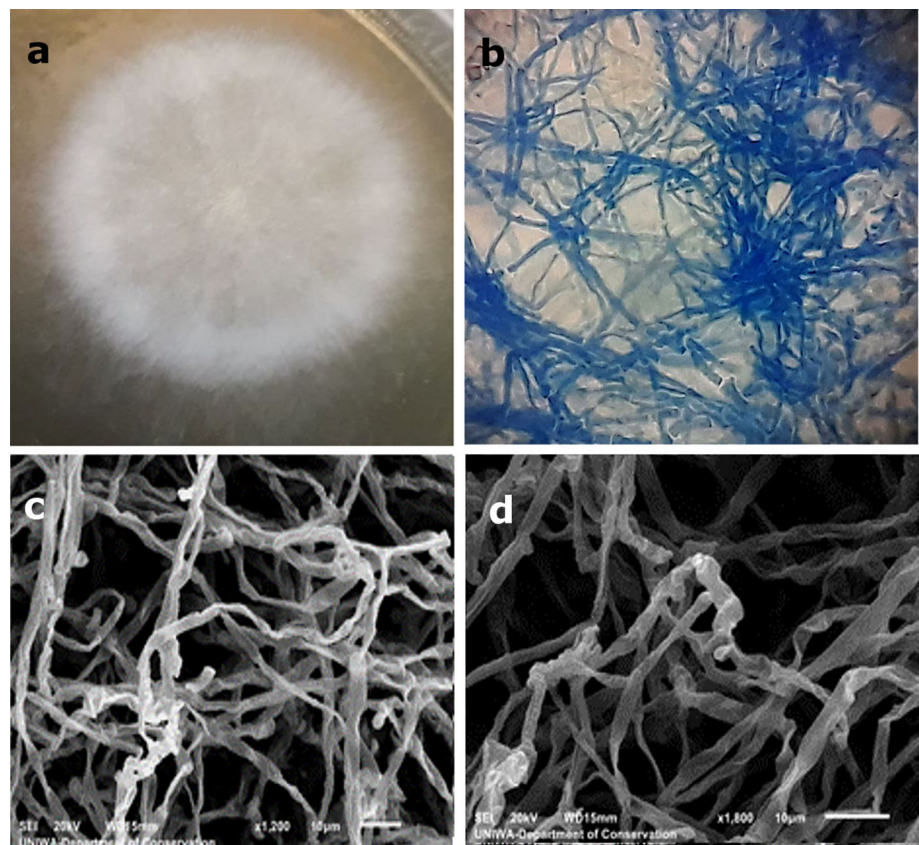
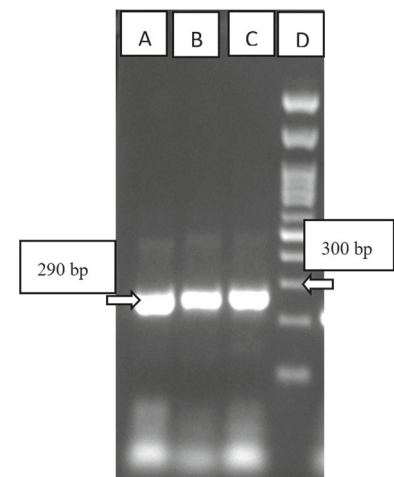


Fig. 10 DNA electrophoresis in 2% agar visualized under UV light. All samples (**a**, **b** and **c**) formed characteristic DNA bands that had a length of 290 base pairs (bp) which is the characteristic length of the amplified ITS sequence. This result confirms that both samples (**a** and **b**) of the unknown microorganism are fungi. The positive control **c** (*Aspergillus niger*) also exhibits a DNA zone at 290 bp as expected. Sample D is a DNA ladder with zones of known length used as a standard



effects such as allergies and as causal agent of human fungal infections [39, 40]. These fungi are widespread and commonly live on cellulose, and are found on wood, compost, sheet rock paper, straw [41], and similar materials such as biodeteriorated paint film [42]. They can infest the inside of buildings by growing on a variety of substrates containing cellulose, including document archives, wallpaper, textiles and construction materials [39, 43]. Most *Chaetomium* species grow best between 25 and 35 °C and require a relatively high-water activity (a_w , an indication of the available water in the environment) higher than 0.90 for growth that is why they are commonly found in wet damaged buildings [41]. Spores of *Chaetomium* can be trapped in building materials remaining inactive and may become airborne and active by growing fungal hyphae again during renovations [37, 43]. The presence of the viable fungus in this manuscript could be explained by the renovation of the building that may have resulted in the contamination of the storage environment. Several studies have revealed the presence of *Chaetomium globosum* in ancient documents and library material [21] such as documents from the Archive of the University of Coimbra [20] and documents from the 18th and 19th century in USA [44] and in some cases it had caused severe biodeterioration. The identification of the causative agent of biodeterioration is important in order to assess possible methods of treatment and prevention of biological damage. There is a lack of standardized



Fig. 11 a Electropherogram as produced by the sequencing of the target sequence and b Results from Basic Local Alignment Search Tool (BLAST) which identify *Chaetomium globosum* with 100% similarity (GenBank accession numbers: SUB7245160 20200312CS1P2 MT279537)

effective treatments mainly because the interaction with materials and organisms is highly variable and depends on too many factors to be used routinely [19]. For the treatment of the fungal strains responsible for paper biodeterioration, Fabbri et al. [45] reported that the azole antifungals econazole and miconazole were the most efficient in inhibiting the fungal growth and in another study by Karbowska-Berent et al. [46] disinfection solutions containing quarternary ammonium salts (QAC) were tested in controlling cellulolytic fungi. Further research should be conducted to establish an efficient method for controlling the cellulolytic fungi, without damaging the original material.

4 Conclusion

By applying the macroscopic imaging spectroscopy, the supervision of the entire page of the manuscript was accomplished. Thus, the absorption discrepancies in the various studied regions of the electromagnetic spectrum allowed for the differences or similarities among inks and pigments as well as the damage locations to be located. Yet through the microscopic imaging, some features of the inks and pigments could be clearly distinguished. The microscopic observation of the locations, where the absorption discrepancies on the same pigment exist, allowed the characterization of the damage as biological attack. Moreover, the SEM/EDS analysis gave the elements of the pigments and inks, allowing the further investigation of the reasons of the biological attack. The use of the imaging spectroscopy and SEM/EDS analysis revealed significant results not only on the type of inks and pigments but also their preservation status in the biological attack case, information that is proved useful in making decision about the conditions under which the manuscript should be stored or displayed. Renovated buildings should be screened for biological agents before storing a rare collection of manuscripts. In addition, the necessary conditions in the storage of old manuscripts are described by the

bibliography¹⁴ and guidelines of major museums and organizations dealing with the preservation conditions of museum and library collections¹⁵, such as ICOM, UNESCO, IFLA, ICRROM, etc. On the other hand, the DNA analysis allowed the identification of the biological agents as *Chaetomium globosum*, making easier the choice of the conservation treatments on the manuscript. However, it is important to detect all the contaminant fungi in libraries not only to be able to protect the documents and ensure their safe storage but also to protect the library personnel and the Archive researchers from adverse health effects such as airway infections, mycosis, allergies or skin irritations [20].

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Declarations

Data Availability No data associated in the manuscript.

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