COLONIZAREA MORTARELOR DE CĂTRE BACTERII ŞI FUNGI
THE COLONIZATION OF MORTARS BY BACTERIA AND FUNGI

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Microbial colonization and biodeterioration of mural paintings in historical monuments have as result structural and aesthetic damages due to degradation of organic binders, biomineralization, colored biofilms and discoloration.The original and infilling mortar from refectory of Hurezi monastic complex-Romania was analyzed from structural point of view as well as from microbiological point of view. Visual inspection performed in situ on the northern and western walls revealed: cracks, small and large pink areas on the pictorial layer, original and infilling mortar, small black areas on the infilling mortar and white and pink efflorescences appearing mostly on the infilling mortar. Microscopical observation performed both in situ and in laboratory (Optical Microscope and Scanning Electron Microscope) revealed a pink biofilm and small black colonies. Microbiological analysis confirmed the bacterial origin of the pink biofilm and fungal origin of black colonies. The characteristics of the mortars sustain adhesion of the bacteria and fungi and organic deposits favor their multiplication which is reflected in colonization of the substrate.

Colonizarea microbiană şi biodeteriorarea picturilor murale din monumete istorice au ca efect deteriorări estetice şi structurale datorită degradării lianţilor organici, biomineralizării, biofilmelor colorate şi a modificărilor de culoare. Mortarul original şi cel de reparaţie din trapeza Complexului Monastic Hurezi-România au fost analizate din punct de vedere structural şi microbiologic. Analiza directă efectuată in situ pe peretele de nord şi vest a pus în evidenţă: fisuri, zone de culoare roz de dimensiuni reduse şi mari la nivelul picturii murale, a mortarului original şi de reparaţie; zone mici, de culoare neagră pe mortarul de reparaţie; eflorescenţe de culoare roz sau alb în principal pe mortarul de reparaţie. Observaţiile microscopice efectuate atât in situ cât şi în laborator (microscopie optică şi microscopie electronică de baleiaj) au identificat biofilmul de culoare roz şi colonii mici de culoare neagră. Caracteristicile mortarelor sunt favorabile pentru aderarea şi multiplicarea bacteriilor şi fungilor.

Keywords: *microbial colonization, pink biofilm, black biopigmentation, mortar, pictorial layer*

1. Introduction

Microbial colonization and biodeterioration of mural paintings in historical monuments have as result structural and aesthetic damages due to degradation of organic binders, biomineralization, colored biofilms and discoloration. It is a complex process that illustrates the interaction of microorganisms with the substratum and environment, having a major impact for establishing prevention and restoration strategies. The microorganisms producing patina belong to cyanobacteria, bacteria, algae, fungi and lichens. Cyanobacteria produce green-black stains on the hypogean wall paintings and surfaces [1]. Heterotrophic bacteria and particularly actinobacteria are associated with different types of patina like crusts and biofilms, depending on the species and type of substrate [2, 3]. Aira et al [4] found on the walls of the Cathedral of Santiago de Compostela (Spain) brownish – grey patina produced by *Trichoderma viride,* as well as blackish powdery patina produced by *Cunninghamella elegans*. Raimondi et al. [5] performed remote detection of laser induced autofluorescence on pure cultures of fungal and bacterial strains and their analysis with multivariante techniques. Lichens develop crusts on the volcanic rock hewn churches of Lalibela, Northern Ethiopia [6] or are distributed through the fissures [7].

Colonization of the historical monuments is correlated with the availability of nutrients, mineralogical composition and water permeability [8].

The pink discoloration was identified on stone, wall paintings, building and burial-related material from monuments in central and south Europe [9-11] exposed to different climatic conditions with constructional problems that enable water infiltration. The water brings into walls salts coming from the soil. The crystallization of salts takes place on the surface or inside of the pores in correlation with temperature and water evaporation and produce additional pressure, leading to cracking and detachment of the materials [12]. Salt efflorescences and organic deposits are specific

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environment for halophilic and halotolerant microorganisms. On the surfaces covered by efflorescences, microbiota is not diverse. In the initial stage of colonization, the growth of microorganisms on the surface of mural painting causes aesthetic damages though changes in the paint layer are not revealed. Subsequently, cells penetrate deeply and the following changes become more evident: partial detachment, cracking, loss of the pictorial layer. Microbial metabolites produce biochemical and aesthetic damages as well as changes of the original color; sometimes mural painting is completely covered by biofilm, becoming visible the yellow orange color which quickly (depending on the rate of multiplication of biodeteriogens) becomes pink or red [11, 13-17].

The pink pigmentation becomes visible due to ability of microorganisms to produce carotenoids [18]. In case of mural painting, the original color of pictorial layer is changed and on the mortar, pink spots are visible. Imperi et al. [17] found widespread change in the color of superficial layer of frescoes that turned pink, consequent to particularly dry and hot summer season. The black pigmentation is a consequence of the ability of microorganisms to synthesize melanine.

The aim of the present work is to study the bacterial and fungal colonization of mural painting from the refectory of the Hurezi monastic complex-Romania through analytical techniques. We found that pink biofilm, pink efflorescence salts and black colonies lead to aesthetical changes, weakening and enhanced material loss. Changes in the morphology of mortar and pictorial layer are due to physical and biological alteration. The characteristics of the mortars sustain adhesion of the microorganisms and their multiplication reflected in colonization of the substrate.

2. Materials and Methods

2.1. The refectory of the Hurezi monastic complex

The Monastery of Hurezi was built in the 17th century. The refectory was built in the western side of its axis and in 1705 it was decorated with frescoes following Byzantine tradition. During the time, successive lime washes were applied on part of the wall paintings, on the upper side of the walls and on the northern, western and southern walls. The last restoration of the refectory took place in the years ‘70s when part of the plaster of the facade has been replaced with a new one, while on the inside, large areas of lacunae at the lower part of the walls have been treated by mortar infilling. These extended fields of lime based mortar infillings have been kept in their white natural colour, with no further pictorial reintegration. On the northern, western and southern walls of the refectory, an irregular pink discoloration and black areas are visible on the surface of the infillings going up to the original frescoes (Fig. 1a and Fig. 1b). The extension of the colonization is connected with the amount of organic deposits and the environmental conditions, mainly humidity and temperature. HOBO LCD data loggers revealed values of temperature between 8-16^{0}C and relative humidity between 70-90% in October and November 2015.

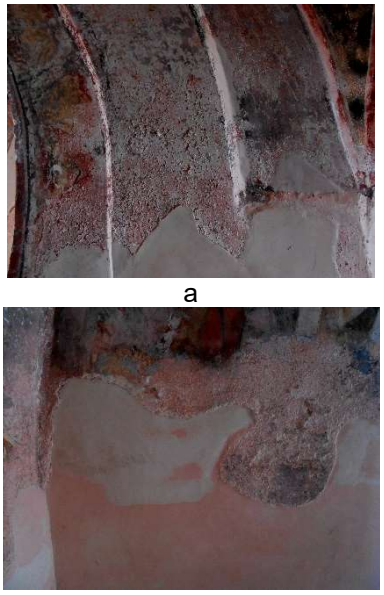

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Fig.1 – Microbial pigmentation on original mortar and pictorial layer (a) as well as on the infilling mortar (b). / *Pigmentația de origine microbiană pe mortar și stratul pictural (a) dar și pe mortarul de reparație (b).*

2.2. Sampling procedure

The sampling areas were established after their historical importance and visible pink discoloration. The conservation status of the northern wall was poor especially on its lower part. The walls were sampled at 150 cm above the ground. The infilling mortar samples were taken using a little hammer and a chisel. Samples for microbiological analysis were collected under aseptic conditions into sterile boxes and stored at 4^{o}C until processing, but no more than 24 hours.

2.3 Grain size distribution analysis

Grain size distribution of siliceous aggregate was established after its separation of the binder. The aggregate separation was performed through wet chemical separation [19]. Then, the aggregate was washed with distilled water and dried. Finally, it was screened on meshes with mesh sieve of 0,55 mm, 0,71 mm and 1 mm. The remained material on each sieve was weighed and the material percentage of each size class was calculated.

2.4. X ray diffraction analysis

X-ray diffraction analysis was performed using a Shimadzu XRD 6000 diffractometer, with

Ni-filtered CuKα radiation (λ=1.5406 Å), with scan step of 0.02°. The total amount of sample used was 1 g powder.

2.5. Cross-section analysis

The samples have been embedded in polyester resin and cross-sectioned with IsoMet Low Speed Saw, submitted to wet polishing and glossing using the instrument Phoenix Beta Grinder / Polishe. The sections have been observed at optical microscope Carl Zeiss AXIO IMAGER A1m endowed with soft and video camera. The analysis was performed on polished sections and thin sections, in polarized light, with cross Nicols and parallel Nicols. The dimension of the field visualized in figures is of 700x520 µm. The sections have been analyzed to define color and thickness of pink biofilm, the structure of the mortar as well as the distribution of different types of aggregates.

2.6. Microscopy analysis in situ and in laboratory

In situ analysis has been performed on the infilling and original mortars as well as on the pictorial layer using Dino-Lite Digital microscope.

A Nikon AZ100 microscope was used to obtain microphotographs of the pink discoloration samples. Analysis has been performed both on the whole sample and after extraction of a round sample with a driller.

Microscopic images of the samples were also obtained with Scanning Electron Microscope (SEM) JEOL JSPM 5200 (Japan), in order to identify bacteria and their types of arrangements. For the image acquisition, samples were coated with gold. Images were acquired at high vacuum employing an acceleration voltage of 30kV.

2.7. Microbiological analysis

The samples were weighed (g) and the decimal dilutions were inoculated on solid media supplemented with 10% respectively 20% NaCl [20] and incubated at 28^0C for 35 days. Pure cultures were obtained using depletion loop technique.

3. Results and Discussion

3.1. Technical analysis of frescoes and recent mortar infilling

The frescoes of Hurezi refectory were executed in the Byzantine tradition of `true fresco`: the support of the painting is composed of two distinct layers of mortar (*arriccio* and *intonaco*) on which paint layers were applied while the *intonaco* was still fresh (during carbonatation process). The same structure of the plaster was used by the restorers for the mortar infillings of large areas of lacunae at the bottom of the walls. As in the case of the original plaster, the infillings have a two-layer structure, a thicker and coarser layer of *arriccio* and a finer upper layer of *intonaco*.

The *arriccio* layer is applied on brick masonry and consists of calcium carbonate (calcite) as binder and river sand (quartz, potassic feldspar, muscovite) in proportion of about 65 % as mineral aggregate. The *intonaco* layer consists of calcium carbonate as binder, 6-5 % river sand as mineral aggregate and short cut fibers of hemp as vegetal aggregate. In both layers, the calcium carbonate originates from carbonated lime. The addition of vegetal fibers in the upper layer of plaster is consistent with a local general practice in fresco technique in which a mineral aggregate (most often river sand) is mixed with vegetal fibers for reinforcement of the mortars.

Light microscopy performed on cross sections and thin sections in direct and polarized light indicates a porous mass of pink colour in the *intonaco* layer of the infillings as well as a thick layer of *arriccio* (Fig. 2a). On section edge it might be microscopically remarked a zone which is colored in pink represented from microscopic point of view by a mass of micro-crystalline calcite in which small crystals of quartz are embedded (Fig. 2b). There are sub/angular-rounded fragments of quartz, having the diameter of 50 µm up to 250 µm, sub-angular-rounded fragments of quartzites, having the dimensions up to 500 µm and sub-angular fragments of feldspars having the diameter up to 250 µm.

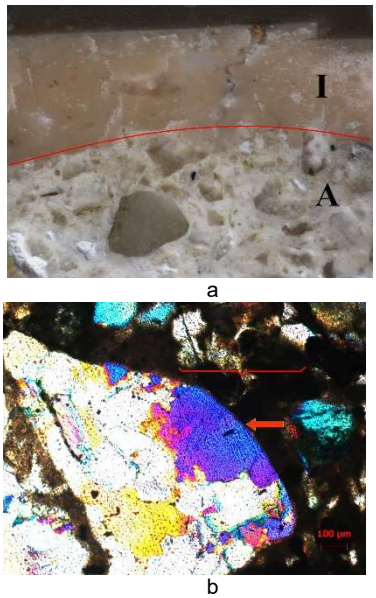

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Fig.2 - Micrographs representing: a) the mortar infilling structure (I=*intonaco*; A=*arriccio*); b) the morphology of granules present in mortar. / *Microfotografii reprezentând: a) structura mortarului de reparație (I=intonaco; A=arriccio); b) morfologia granulelor din mortar.*

Analysis of diffraction with X-rays performed on samples taken from northern wall (compact samples and chippings) showed for *arriccio* layer compounds of binder – lime (calcite) and of aggregate – river sand (quartz, potassic feldspar, muscovite). In the samples of chippings (for example 6NH) was put into evidence the presence

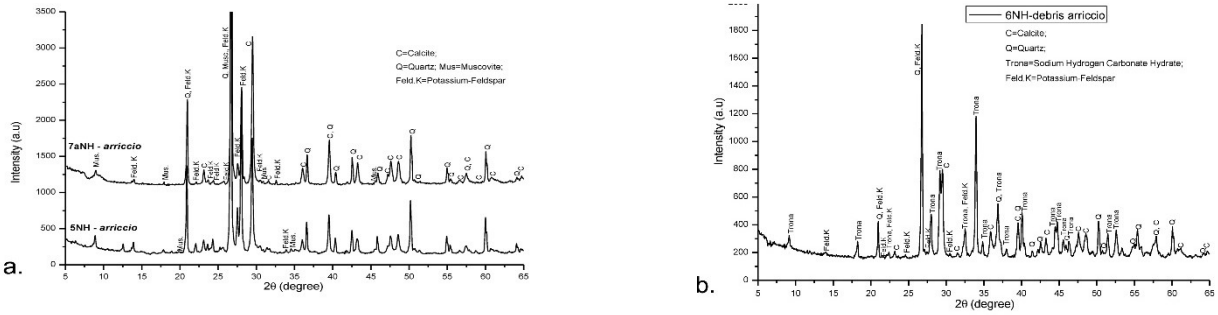

Fig.3 – XRD diagrams of infilling mortar: a) *arriccio* layer code 5NH and 7NH; b) *arriccio* layer code 6NH. / *Diagrame XRD pe mortarul de reparație: a) stratul arriccio, cod 5NH și 7NH; b) stratul arriccio, cod 6NH.*

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of a salt of sodium carbonate ($Na_3H(CO_3)2 \cdot 2H_2O$ - throne), which is possibly a product of degradation (Fig.3a and Fig.3b).

The mass of fine micro-crystalline calcite has small crystals of quartz with diameter varying from 50μm up to 250 μm embedded into it. In both layers, mineral aggregate has a granulation lower than 2mm, with a weight of 41-68% of fine fraction below 0,5 mm.

In the Figure 5 is presented the grain size repartition on grain size classes of the river sand. On some compact samples from *arriccio* layer, the apparent density (determined through immersion in water), absorption of water (in accordance with EN 1015-10) and apparent porosity (in accordance with EN ISO 10545-3) were determined. The values of these characteristics fluctuated in relatively closed limits: apparent density: 1.53-1.78 g/cm^3; absorption of water: 13.5-16.97 %; apparent porosity: 24.13-27.75 %.

3.2. Identification of pink discoloration (in situ and in laboratory)

Analysis performed *in situ* revealed morphological changes on the pictorial layer and on the infilling mortar. Cracks were identified both on the pictorial layer, original mortar as well as on the infilling mortar (Fig. 4a and Fig.4b). Small and large pink areas, black spots and white and pink efflorescences can be seen on the pictorial layer, original and infilling mortar.

The real color of pictorial layer cannot be seen because of pink biofilm. Micrographs showed that the original and infilling mortars were colonized by bacteria and fungi. The new and old pink efflorescences can be seen near black colonies of *Aspergillus niger* (Fig.5a and Fig.5b). These observations clearly show that the process started in the past and in present is under the progress, in connection with the environmental conditions. The migration of salts is a continuous process covering the old pink salts. The pink biofilm was developed under the recently detached layer showing that bacteria could have an important role in the detachment of the mortar. In samples containing already detached vegetable debris, under them as well as in cracks, pink efflorescences were

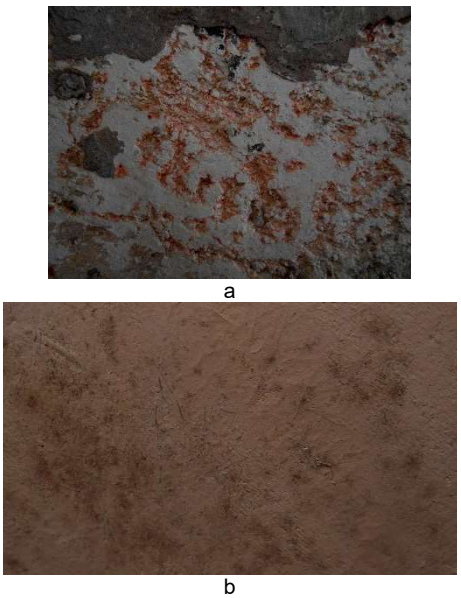


Fig.4 - Microbial pigmentation on original mortar (a) and on the infilling mortar (b). /*Pigmentația de origine microbiană pe mortarul original (a) și pe mortarul de reparație (b).*

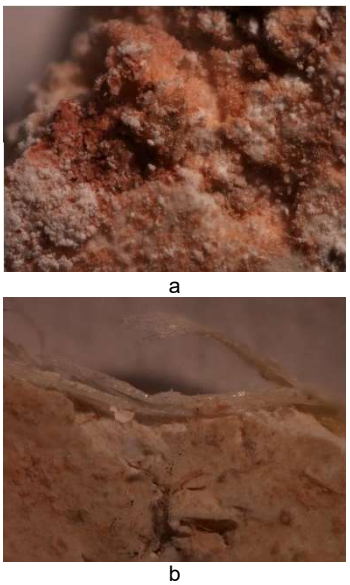


Fig.5 – Micrography of pink bacterial (a) and black fungal colonization of mortar (b). / *Colonizarea mortarului de către „bacterii roz" (a) și „fungi negri" (b).*

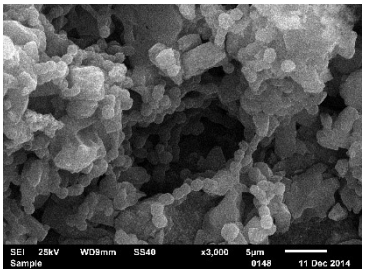
observed. Similar morphology was found after examination of the La Galea Fortress, but the monument was located in the cliff about 80 m above sea level where humidity and day light encouraged the growth of alga from genus *Trentepohlia* [21].

Laiz et al. [11] in three different sampling sites (Sevilia and Postumio tombs in the Roman Necropolis of Carmona, Spain) and Imperi et al. [17] in the Crypt of Original Sin, found the bacterial aetiology of rosy discoloration. Our results have been obtained in a monument with different environment. The refectory is situated on the ground and indoor, with the door open during the day. Thus, relative humidity and temperature are different during the day and night. Convective currents could contribute to the dissemination of biodeteriogens inside the monument. The main sources of bacteria producing pink discoloration are: water, outdoor microbiota and already contaminated areas. The most contaminated was the northern wall, but we identified that the colonization of the western wall began as a consequence of the extended growth of biofilm developed on the northern wall (yet unpublished results). The refectory is used on the occasion of major religious events when the personnel and pilgrims gather at the site. More, the door is open so visitors could come during the day to visit the refectory, bringing more biodeteriogens or contributing by touching contaminated area to the spread of microorganisms. The study of intradiurnal levels carried out in the nave of the Cathedral of Santiago de Compostela (Spain) revealed grater microbial concentrations at 13,00 h when the influx of visitors was massive [4].

SEM micrographs revealed that some samples are partially or completely covered by bacteria. Bacterial cells were put in evidence on the surface of the mortar, in fissures, into the pores and on the vegetable debris (Fig.6a and Fig.6b). Some samples were colonized by two different coccoid shaped bacteria arranged in groups and in chains. Thick layers of bacteria developed around and into enlarged pores (Fig.6a) lead to the alteration of substrate pore size and changes of moisture circulation. Extracellular polymeric substances (EPS) were detected around the cells forming biofilm.

Our results revealed that water is a good vehicle not only for salts but also for bacteria producing carotenoid pigments. Biogenic pigments are stable on the materials although bacteria are already dead, which is in accordance with Piñar's et al. [9] results.

When dried biofilm and salts is detached from the mortar, mineral grains are also removed (SEM5). Mineral components mixed with cells were observed in the fissure. Laiz et al. [11] reported similar results for *Rubrobacter* cells found into porous mineral matrix. Also they mentioned the formation of crystals in intimate contact with the *Rubrobacter* film and the detachment of mineral grains upon biofilm retraction. The genus *Rubrobacter* was found to be responsible by rosy discoloration of Byzantine frescoes in the Crypt of the Original Sin (Matera, Italy) by Imperi et al. [17], in the tomb of Roman Necropolis of Carmona Spain [13]. The authors concluded there is a relationship between heavy contaminations by *Rubrobacter* related bacterioruberin-producing bacteria and rosy discoloration on ancient wall paintings. In the present study we demonstrate that three morphotypes of moderately halophilic and halotolerant bacteria grow on the investigated area of 20 µm. The major impact of environment could explain the results. Laiz et al. [11] suggested that *Rubrobacter* is a widespread bacterial group present in a variety of habitats like desert, arid soil and monuments, playing an important role in the biogeochemical cycle of elements and mineral precipitation.



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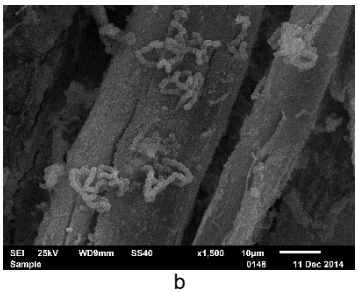

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Fig.6 – SEM micrographs of bacteria on the mortar (a) and vegetable debris (b). / *Imagini SEM ale bacteriilor pe mortar și fibre vegetale.*

The strong mineral-bacterial interactions have been found in most of the samples having as result pink discoloration as well as detachment. X - ray diffraction showed that in infilling mortar, mainly in *arriccio* layer, calcite and river sand could be easily embedded in pink biofilm. From optical microscopy images of thin sections is observed that the mortar is porous with pore diameter sizes from a few microns to 100µ. The mass of mortar is traversed also by micro-cracks sized up to 50µ, some of which are filled with calcite. Under these circumstances it is obvious that bacteria adhere to rough substrate through EPS and can move together with saline solutions inside of pores.

Our results clearly reveal that pink biofilm is produced by halotolerant coccoid shaped bacteria arranged in groups having the role of first colonizers of the substrate and most probably enriching the substrate with organic compounds.

Also black spots are produced by black fungi which are the second colonizers of the mortars. On the mural painting and mortars from the refectory of Hurezi monastery we found a heterogenous microbial community containing three morphotypes. They are moderately halophilic and halotolerant microorganisms producing carotenoid pigments excepting the filamentous one (yet unpublished results). The bacterial morphotypes colonizing the mural painting and mortars and the relationships they have with the abiotic environment can be considered as part of the microecosystem from a microecological perspective applied to conservation and restoration of historical monuments. The most colonised was the northern wall, but bacteria were spread also on the west wall.

3.3. Detection of biodeteriogens

The inventory of the microorganisms associated with damage to the mural painting and infilling mortar is a compulsory requirement for including biodeterioration as the process whose effects need to be removed during restoration. Colonies of bacteria were isolated from counting plates on the basis of their colony morphology, purified by streaking on the same growth medium and differentiated by assessing the morphology on SEM as previously mentioned. Microbiological analysis of original and infilling mortar samples as well as pink efflorescence revealed a consortium containing pink, yellow and white colonies. The work of cultures purification showed that a red colony was made from red and white cells. Three types of bacterial colonies were isolated: smooth, opaque, reddish that develop slowly on nutrient agar; opaque and whitish to yellowish that develop quickly on nutrient and white, rough with fungus-like branched networks of hyphae but being bacteria with fungal type morphology. White efflorescence did not contain microorganisms.

When cells are together, in 3-4 days of incubation a well grown culture is obtained. Else, whitish-yellowish colony grows in 5 days and the reddish colony in 7-10 days. They are halotolerant and moderately halophilic strains. The white rough colony needs more than 30 days of incubation, being represented by a halophilic strain. SEM images reveal that all morphotypes grow together in specific microenvironments.

4. Conclusions

The colonization of mortars in the refectory of Hurezi monastic complex is the consequence of the environmental conditions, the presence of salts (efflorescences) and microbial cells. Pink and black pigmentation can be localized by visual inspection but only the multianalytical techniques are able to identify their microbial origin.

The multianalytical techniques highlight for the first time the major role of communities of halotolerant and moderately halophilic bacteria and fungi in the aesthetical damage of mural painting and mortars from refectory of Hurezi monastic complex from Romania.

Direct and microscopy examination allowed establishing the pink biofilm and distribution of bacteria on the surface, on and under the vegetable fibers, deep into the fissures and into the pores. With SEM observations it was possible to observe a heterogeneous microbial community containing three morphotypes: coccoid shaped bacteria arranged in groups and chains as well as filamentous bacteria. Clearly 3-6 layers of bacteria or a mixture of salt crystals and bacteria have been found.

Polarized microscopy, the study of the cross-sections under microscope and X ray diffraction pointed out characteristics of materials such as the percentage of aggregates, specific minerals, pores and grain size and a better analysis of composition, technology and deterioration produced by halophilic bacteria producing carotenoids. The infilling mortar has been applied in two layers: the first one, named *arricio*, is composed of lime and river sand in percentage of about 65%; the second one, named *intonaco* contains lime mixed with river sand in less percentage (5-6%) and vegetable fibers. Bacteria sized between 1,0-1,3 μm can move through pores and adhere to their walls, starting colonization and pink discoloration.

The study of cross sections under microscope revealed a thin biofilm (less than 1 μm) on the surface and into the fissures. It has an important role in water absorption and retention, enhancing microbiological and salt weathering mechanisms.

Microbiological analysis of mortar samples revealed a consortium containing pink, yellow and white colonies. Pure cultures have been obtained in order to study cultural and physiological characteristics for molecular identification.

The present study points the main steps and analytical methods for identification of pink aesthetical damage of mural painting and for discrimination between chemical and biological origin. Without the multianalytical techniques applied, the pink discoloration could be misinterpreted as chemical damage.

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