Chapter

Microscopical Methods for the In Situ Investigation of Biodegradation on Cultural Heritage

Verginica Schröder, Daniela Turcanu Carutiu, Adina Honcea and Rodica-Mariana Ion

Abstract

The processes of cultural heritage sites' degradation reveal interactions between the chemical characteristics of the substrates, the underlying substrate penetration, and the microbiota systems. Microorganisms penetrate the stone, causing extensive disaggregation of the materials. This chapter reveals comparative studies between the usual research approaches applied in biodegradation studies, especially optical microscopy, epifluorescence, and electron microscopy (SEM). These in situ microscopy techniques propose some complex analyses for the evaluation of the relationship between the microorganism's cells and the stone surfaces (adherence, interactions), and also for the evaluation of the level of health or balance of the niche complex, from mesoscale to microscale. The stages of the exact monitorization and evaluation of lithotypes and deterioration phenomena are periodical sampling and monument mapping. The aim of this chapter is to identify microscopical methods used in biodegradation studies, especially the facilities provided by these methods. Our in situ analysis (light microscopy, epifluorescence, and scanning electron microscopy) performed for the first time on the painted Matia-Fresco Loggia (Corvin Castle, Romania) highlighted several aspects, such as mixtures of mineral elements with different chromatic appearance and porosity, shredding degradation, depigmented areas, cracked portions, and highly biota activity (bacterial and fungal) on painted surface.

Keywords: microscopy, epifluorescence, biodeterioration, microbiota, stone surfaces, Matia-Fresco Loggia, Corvin Castle

1. Introduction

Most cultural monuments are subject to a degrading phenomenon induced by a number of abiotic and biotic factors: pollution, temperature, and humidity variations; periodic conservation interventions; touristic actions; and the colonization with biota. The microclimate defines the level of colonization, the type of contamination, the complexity of community, and its specific composition [1]. As a result, the degradation process is one that must be understood from an ecological

perspective by considering the abiotic and biotic components and, respectively, their interactions. Studies on the complexity of biotic systems that interfere with the degradation of components of works of art and/or historical monuments represent an important part of the monitoring of processes that can lead to irreversible phenomena.

Knowledge of colonization, bioreceptivity, microbial diversity, and interactions between microorganisms are ways to "diagnose" historical monuments. By understanding the biofilm-induced phenomena from the ecological point of view (abiotic factors, biotic diversity, and biotic-abiotic interactions), the difference could be made between biodegradation and biological colonization without or with a protective effect, and this would allow for a closer approach to the way in which restoration or biocide intervention occurs on the surfaces of artworks or heritage buildings.

The biofilm is present in all types of environments (being ubiquitous), and is one of the most interesting biological systems with a very long history, dated over 2.5 billion years [2]. By definition, it consists in "microbial communities on interfaces" [3]. The interactions between abiotic factors and biotic elements induce changes at both structural and compositional level [4–8].

Cultural and historical monuments (buildings and archeological sites) or some artworks (pictures, tailored, or wooden objects) made from different materials (paper, textiles, glass, and wood) can constitute the habitat for micro- and macroorganisms. The species that have been identified on these materials range from microscopical bacterial cells to higher plants and animals [4]. In a study concerned with the biodegradation and restoration of monuments, it is important to know how microorganisms colonize surfaces.

Also, it is necessary to determine the risk induced by abiotic and biotic factors, and the indication of the biofilm-induced hazard is required as well. The existence of variable interactions or similar effects induced by different causes which act in synergy could yield remarkable findings. Some microorganisms (lichens and bacteria) may have protective effects on historical monuments [9]. For example, lichenic coating reduces the presence of water inside the rock, thus protecting the rock material from physical decay and disintegration [10] and such microorganisms could be positively used for the cleaning of salt crusts otherwise difficult to remove by traditional restoration methods [11].

2. The biofilm and biodegradation (BD)

Biofilm can be considered among the most complex living biological systems. Studies have highlighted numerous interactions and intrinsic mechanisms that reveal this complexity, and which manifest themselves spatially and temporally, resulting in the formation and maintenance of the biofilm.

The mechanisms involved in the complex biofilm formation process are cell-cell interaction [12], the development of mechanical forces and the correlation with the type of substrate, cellular metabolism (growth and energy efficiency) through material exchanges, and the horizontal gene shifts in the biofilm [8].

Microorganisms within the biofilm structure are characterized by the existence of certain surface active compounds (SAC) participating in the interaction interface [3]. These may include extracellular polymeric substances (EPS) of bacterial origin, multimeric cellular appendages, flagella, fimbriae, and pili which act as cell to surface adhesins, cell to cell adhesins [13], proteins of the amyloids and lectin type (Lec A and Lec B), Psi-binding proteins, the *Pseudomonas aeruginosa* model, enzymes, polysaccharides, hydrophobins, biosurfactants (*Bacillus subtilis* model)

[14], microbial surface-active compounds (SACs) such as amphiphilic polymers, and polyphilic polymers [3].

Another component of the matrix is the DNA. Extracellular DNA as a matrix component in biofilm is apparently involved in intercellular links (cell to cell interconnecting compound in many different biofilms) as well as in the horizontal transfer of genes [13, 15].

A single species can produce several different types of matrix components of the biofilm [13]. This ability to produce different matrices helps colonize various niches through different biofilm development pathways. Understanding the mechanisms through which infections occur and the comprehension of structures has been made possible by the development of microscopy analysis techniques [16–18].

Knowing the biological components in the biofilm structure and environmental exchanges finds multiple applications in fields such as building industry [19, 20], biofouling [21, 22], medicine [23], and biodegradation of buildings and/or works of art [5, 24–29].

Between the most interesting mechanisms of colonization are those that affect the hard substrate with different degrees of porosity (stone and building materials) [8, 19, 30, 31]. The studies on these communities and the interactions between micro- and macroorganisms are various [6, 7, 22, 30, 32–34] and they have allowed the identification of amazing diversity according to taxonomic, physiological, and ecological criteria. The microorganisms which are adapted to the strong surfaces of cultural monuments have ecological niches of the endolithic type and are classified as chasmendolithic (fissures and cracks), cryptoendolithic (internal porosities), and euendolithic (forms actively penetrated through the rock) [16].

The way of action on the surfaces and the ecological links between bacteria, algae, lichens, and fungi explains the biodegradation of monuments and works of art. The structure of the communities and the phenomena associated with alteration by biological activity vary and depend on the nature of the material (stone, wood, paper, textiles, metal, leather, glass, and painted surfaces) on which associations of microorganisms are formed. The organisms involved are bacteria (including actinomycetes and cyanobacteria), fungi, archaea, algae, and lichens [35].

The microorganisms that can be investigated can be divided according to metabolism into chemolithoautotrophic and photolithoautotrophic (algae and cyanobacteria). Chemolithoautotrophic bacteria are a specialized group included in the sulfur and nitrogen cycle. This group includes sulfur-oxidizing bacteria *(Thiobacillus* sp.), oxidation-reducing bacteria of nitrogen compounds, especially ammonia substrates *(Nitrosomonas* sp.), and nitric acid (*Nitrobacter* sp.) [32, 36]. Some bacteria from this group can grow mixotrophically, which denotes the assimilation of organic nutrients for the anabolic formation of cell substance (chemolithomixotroph). Chemoorganotrophic (bacteria and fungi) base their metabolism on organic substrate oxidation and are even capable of gaining energy through the oxidation of metal cations such as Fe^{2+} or Mn^{2+} [32]. Chemoorganotrophic bacteria are specialized, having the ability of inducing the appearance of an acidic medium and with causing mineral dissolution on the surface of the stone.

The organisms with photoautotrophic specialization extend to external stone whenever there are favorable conditions of humidity, heat, and light. These organisms are attached to the surface of the structures, with a very large adaptation in this respect, with visible effects both on their color and with morphological changes.

Lichen species have major implications for the pedogenic activity present on lithic substrate. Through their metabolism, they release organic acids with chelating properties containing complex mineral cations. Epi- and endolithic lichens that can be isolated from stones in archeological sites may include *Xanthoria, Caloplaca, Verrucaria, Aspicilia, Lecanora*, and *Protoblastenia* [4].

Fungal species that can be identified include *Cladosporium, Trichoderma, Phoma*, *Penicillium*, and *Fusarium*. Fungi induce the reduction and oxidation of mineral cations with specific activity. The function of fungi in the biodegradation of monuments has for a long time been ignored or undervalued, as they have been considered secondary colonizers compared to other microorganisms (chemolithotroph bacteria, cyanobacteria, and algae) or lichens [37].

All microorganisms which form communities with implications in the degradation of surfaces must be analyzed under special laboratory conditions for identification. A frequently assayed technique is microbiological culture. The use of new, modern automated and more selective culture media to identify new bacterial strains could help to identify in situ diversity [38].

Instrumental techniques which can be applied for the in situ detection of bacterial activity on rock samples collected from monuments or archeological sites include fluorescent antibody technique (FAT), enzymatic methods for hydrocarbon analysis (DHA) [39], alone or together with the determination of the protein content in the filtered solutions of the stone powder samples [40], colorimetric tests [41], bioluminescence tests that quantify ATP content or coupled with differential flow calorimeter determinations, and differential flux calorimeter (DFC) [41].

3. Microscopy techniques for highlighting the in situ BD phenomenon

The degradation process can be studied both by common methods (microscopy and culture of bacterial strains) and by modern techniques (in situ microscopy, molecular analysis, and in situ fluorescence induction).

The microscopic study of surfaces and the highlighting of deposits created over time allow a better understanding and correlation of degradation mechanisms. The most common method for the observation and evaluation of microorganisms on surfaces is light microscopy.

3.1 Light microscopy

This type of microscopy is the most used means in studying the damage caused by biological factors through in situ detection. Using the results of this evaluation, microorganisms can be identified on inorganic components in situ, that is, when the biological components are not separated from the lithic material [16].

The microscopy methods used over time have allowed for more and more remarkable observations, including important details. Thus, classical microscopy, light microscopy, and stereomicroscopy have underpinned the identification of microorganisms and the substrate-microbiota interaction [1, 7, 42]. The advantage with these methods is that the magnitude of the presence of the microbiota and the identification of the taxa can be established.

Large colonies of lichens and the presence of pigment forms such as cyanobacteria and green algae can be identified on surfaces by stereomicroscopy and optical microscopy. In determining these species, the advantage is given by the fact that the biological material does not require preparation and coloring for identification, photographing, and evaluating the images being a simple and sufficient method for the classification of taxa.

As a technique, analysis by direct image evaluation is preferred compared to other quantitative techniques. Such an assessment and the argumentation of the obtained imagistic analyses are made by comparing the cyanobacteria-induced biofilm on the stone surfaces with chlorophyll measurements (chlorophyll a measurement) [33]. Excepting direct observation, highlighting specific stains can be

done through methods such as Gram staining and periodic acid Schiff (PAS), which identify details of the interaction between bacteria/fungi and the substrate. PAS colors fungal hyphae in red through the interaction of the dye with polysaccharides or other cell constituents (glycoproteins and glycolipids).

3.2 Fluorescence microscopy

Lately, new types of fluorescence microscopes that have been developed use sophisticated image capture and processing methods to identify as many fine details of fluorochromes-emitting and fluorescent-emitting cellular components. The most recent super-resolution fluorescence microscopes can push the limit resolution down even further to about 20 nanometers (nm) [43]. This technique has a superior sensitivity as compared to other microscopy analyses. For example, over 100–1000 times more dye being required in bright-field systems to yield the same visual qualities as those of fluorochromes [44].

The direct epifluorescence filter technique (DEFT) is successfully used in determining bacterial activity in various fields such as environment, nutrition [45], safe sterilization [46], as well as in clinical practice [47]. Many of the methods of analysis for the identification of biological activity by epifluorescence use the marking of structural components (cell walls and nucleic acids), by which the characteristics of microorganisms can be defined.

Fluorescence is recommended in evaluating the cell viability of certain microorganisms [44, 48], or for the study of microorganisms on plant surfaces and is ideal for studies of the general distribution of populations of bacteria and yeasts on natural surfaces of all kinds [49]. Some common methods used to identify cellular changes of the type of those present in apoptosis include several procedures of morphological structures' staining such as EB/AO (ethidium bromide and acridine orange), DAPI, Hoechst, annexin V, caspase-3/7 activity, and ssDNA staining [48, 50], SYTOX Green, SYBR green, and PicoGreen staining [32].

Methods of fluorochrome labeling are based on loss of membrane integrity and marking of intracellular structures (DNA, enzymatic systems, and structural proteins). The advantage of these techniques is given by the possibility of establishing the viability of the studied microorganisms [51–53]. The study of viability was also used in the assessment of bioactivity on some historical monuments. Highlighting and assessing the level of biological activity on the surfaces of different monuments or works of art is superior with the fluorescence microscopy technique, which can be used in situ as well.

Of particular relevance is the use of the fluorochromes acridine orange (AO) and DAPI (4; 6-diamidino-2-phenylindole) for details [50]. These fluorochromes can be used directly with epi-illumination, which represents an advantage for in situ analyses. Acridine orange is a fluorochrome that binds to the nucleic acids of bacteria and stains them orange. Also, some details related to structure and nuclear activity are highlighted using DAPI.

3.3 Electron microscopy

Electron microscopy studies include two most commonly used yet different techniques: scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The first, SEM, helps evaluate surfaces, while TEM evaluates subcellular components by highlighting details. Although the biological material is processed in order to be visualized, the benefits in both cases become evident owing to the fact that they allow for very large spatial resolutions. Modern methods have allowed the establishment of a system that performs in situ analyses, scanning

electron microscopy with backscattered electron (SEM-BSE). This technique allows the assessment of the viability of the bacterial biofilm, or of the lichens, on the analyzed surfaces. The advantage is that several square centimeters are analyzed in situ, at a very high resolution, which is comparable to that of the TEM analysis system [16].

The modern methods for the identification and localization of microbes indicate that it is useful to make investigations through correlative light microscopy (LM) and electron microscopy (EM) techniques [54]. This modern approach helps identify both morphological and ecological details by visualizing microorganisms in their environment. Images obtained by optical microscopy techniques and fluorescence microscopy can be combined with images of subcellular structures obtained by electronic microscopy [55] to generate a database and obtain complex results that may reveal certain superior interpretations.

4. Particularities and advantages of modern methods for in situ BD assessment

Researchers use a technique or another according to the specific advantages of each method (**Table 1**), which can be related to faster identification time, better accuracy of details, avoidance of artifacts, small amount of sample being needed for analysis, etc. For instance, optical microscopy, although classical, offers the advantage of in situ observations that quickly establish interactions between organisms and lithic surfaces [16]. In situ techniques are therefore useful, more so because they only require small amounts of material [56].

These observations of details help in establishing more accurate ecological niches for the different microorganisms present in biofilm. The technique of strain selection in culture cannot shed any light on this relationship and cannot show with certainty that the selected species is the one that induced biodegradation.

Modern techniques using electron microscopy bring methodological advantages through the possibility of obtaining microstructure details (**Table 1**). In addition to images with very good resolution (less than a few nanometers), modeling can also be done via 3-D representation [57] of the studied samples.

The identification of bacterial species through modern molecular biology techniques has allowed the accumulation of a wide range of information, but with many unknown variables as regards the implications of this information in understanding the ecology of species on the surfaces of monuments. Quantified molecular differences also raise a number of practical problems (selective DNA extraction, selective PCR amplification, and the lack of a DNA amplification technique in the mixture) [58]. Genetic studies allow the identification of species by developing methods of labeling target proteins. In addition to the identification and classification of microorganisms, marking techniques bring data associated with the particularities of the expression and functioning of the target proteins, aspects related to the specificity of cell adaptation under particular environmental conditions.

These target proteins as well as nonprotein-labeled components (nucleic acids, lipids, and glycans) greatly contribute to obtaining data that increase the applicability of the correlative image method. However, molecular biology methods reveal the diversity of biological communities and can contribute to finding monument rehabilitation solutions based on the knowledge of these details of molecular flexibility (**Table 1**).

Lately, studies treating cultural monument degradation have been improved by successfully applying the laser-induced fluorescence technique, known as light

Technique	The advantages of application	The type of investigations and complexity level
Light microscopy	• Quick observations and establish organisms with lithic surface interactions; • Mark end evaluations of the microbiota communities; • Important details for identifications of the taxa; • Small amounts of material and sample; • Fast evaluations of bioreceptivity; • Limited artifacts	Laboratory analyses; In situ investigations; Direct analyses and samples without difficulty in preparations (stain methods)
Fluorescence microscopy	• High resolutions; • Sensitivity; • The highlight of the cellular components (cell walls, nucleic acids, and proteins); • The changes of microbiota cell viability and predictions of biodegradation; • Allow only observation of the specific structures which have been labeled for fluorescence	Laboratory analyses; In situ investigations; Preliminary sample preparations with medium difficulty (stain methods)
Electron microscopy (SEM and TEM)	• Very high resolutions; • Spatial variability; • Subcellular component evaluations; • Complex details for correlation and interpretations of data	Laboratory analyses; In situ investigations; Preliminary sample preparations with medium and high difficulty
Molecular biology	• High diversity of biological communities identified; • Molecular flexibility used for rehabilitation strategy; • Biota-surface interactions	Laboratory analyses; Preliminary sample preparations with high difficulty

Table 1.
The advantages of the microscopical and molecular techniques used for the evaluation of biodegradation and level of their complexity.

detection and ranging (LIDAR). This technique was originally developed and applied for the study of vegetation and the marine environment and has recently been extended to the field of cultural monuments' preservation [59].

5. Case study research: in situ analyses at the painted Matia-Fresco Loggia, Corvin Castle, Romania

The diagnosis of the degradation stage of different materials such as natural stone, crushed stone, sands and gravels, clay, inorganic binders (lime, dolomite, natural cements, hydraulic lime, and gypsum), mortars, and artistic components (painted surfaces, Matia-Fresco Loggia, stone) from the Corvin Castle (**Figure 1**) have been examined by using advanced investigative techniques according to the recommendations on restoration and preservation operations.

Modern high-fidelity portable and laboratory equipment has been used for analyses: scanning electron microscopy (SEM-EDS), optical microscopy (OM),

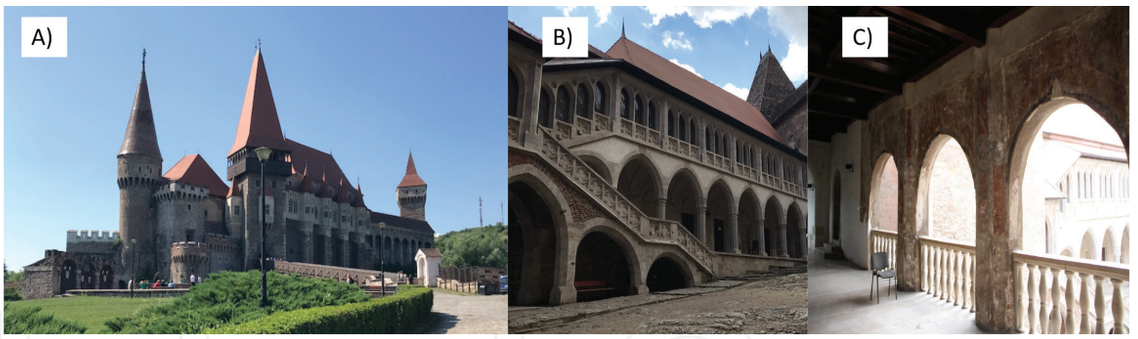

Figure 1.
The locations of sampling points. (A) Corvin Castle—general view; (B) Matia Loggia—outside view; and (C) Matia-Fresco-Loggia—inside, two pillars view.

X-ray diffraction (XRD), Fourier transformed infrared (FT-IR), and Raman spectroscopy as well as polychromic analysis (by chromatic parameters).

Besides the use of all of these, the monitoring and characterization of weathering/decay features and of environmental weathering effects are essential for this monument's preservation [60].

Based on XRD and WDXRF analyses, the Corvin Castle was constructed from dolomite-limestone blocks from local natural resources, crenelated in the upper part. XRD mostly indicates the presence of dolomite, calcite, and quartz, with small amounts of illite, muscovite, paragonite, montmorillonite, wonesite, feldspars, chlorite, and some clayey raw material: whitmoreite, kornelite, micas, and other heavy minerals.

Also, iron silicide is present in most of the samples, as recognized by the used analytical techniques. Incompatibilities between traditional materials and the new ones (wood-mortars and mortars-cements) observed after restorations over time are essential. It is important to focus on their effects on the walls and painted surfaces as well. For the whole area, the pH value is around 5.3.

Besides all of these investigations, it is important to ascertain the humidity migration and circulation inside of the Matia Loggia. The local measurements revealed higher humidity values at the external position (higher air circulation) of the loggia and lower humidity values at the internal positions (**Figure 2a**).

The deterioration of stone usually takes place at higher relative humidity, above 65%, and for external walls, it is more accentuated for porous building materials where it is caused by the excessive moisture content [61]. Changes in temperature induce a thermal gradient (**Figure 2b**) between the surface layer and the inner layer of materials which may result in the degradation of the mechanical properties of the material and can lead to the formation of fine cracks.

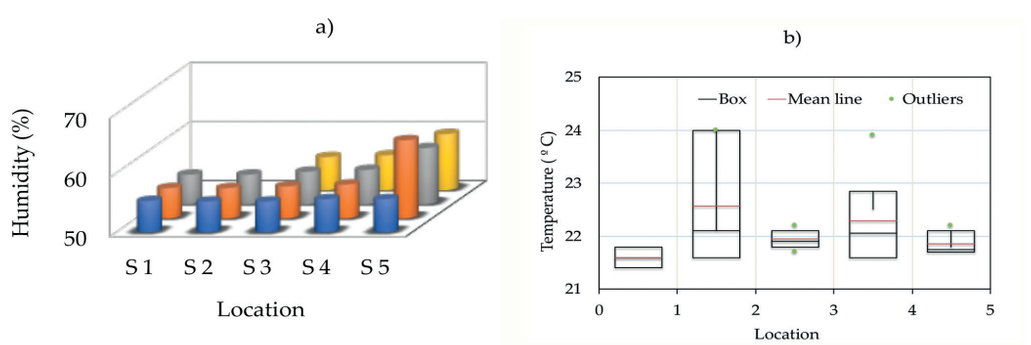

Figure 2.
(a) The changes of the humidity and (b) the variations of temperature with the location: S1—the innermost; S5—the most outward.

Studies on the degradation of fresco areas have been done at macro- and microscopic level. The in situ analysis (22 samples), performed for the first time in the fresco area, highlighted several aspects that concur with the phenomenon of degradation: the existence of materials with high porosity which favor moisture maintenance and increase the potential for biodegradation in the case of restored areas (**Figure 3a**), the presence of extensive deterioration over the entire painted surface, including depigmented areas (**Figure 3b** and **i**) and cracked portions (**Figure 3c** and **d**) and the existence of biogenic pigments green, reddish brown (**Figure 3f** and **g**) or black (**Figure 3h** and **j**).

The harvesting of biological samples on the surfaces indicating a visible degradation phenomenon was done by means of a sterile needle, by the removal from fresco areas of a few millimeters. The harvesting of the samples was done in sterile plastic containers (**Figure 3**). After scraping, the samples were processed and studied in the laboratory.

Microscopic preparations were analyzed by several microscopy techniques (stereomicroscopy, optical microscopy, epifluorescence, and SEM microscopy), both to highlight structural details and to evaluate the biological activity present on surfaces. To identify the morphological details of the microorganisms, methylene blue dyes and fluorochrome acridine orange (AO) were used, while for epifluorescence, the excitation filter of 488 nm and the emission filter of 515 nm were utilized. Small sample fragments were introduced into approximately 5 μL of distilled water and buffer solution (pH 6.8), and the cell suspensions were placed on slides and analyzed with the optical and epifluorescence microscopes.

To exemplify the way that the types of observations were correlated, the details of in situ microscopic analyses are presented for three types of samples collected at different soil levels and distinct degradation characteristics, considered as models of analysis.

Sample 1 was collected at a height of about 1 m to the soil, from painted portions with visible degradation elements (**Figure 4**) on the respective surfaces. Macroscopic and microscopic observations have identified porosity and high brittleness of materials. Dark brown biogenic pigmented areas and depigmented portions that are easy to detach are highlighted (**Figure 4a**). At the morphological level, there are different structural components with (**Figure 4b** and **c**) uneven

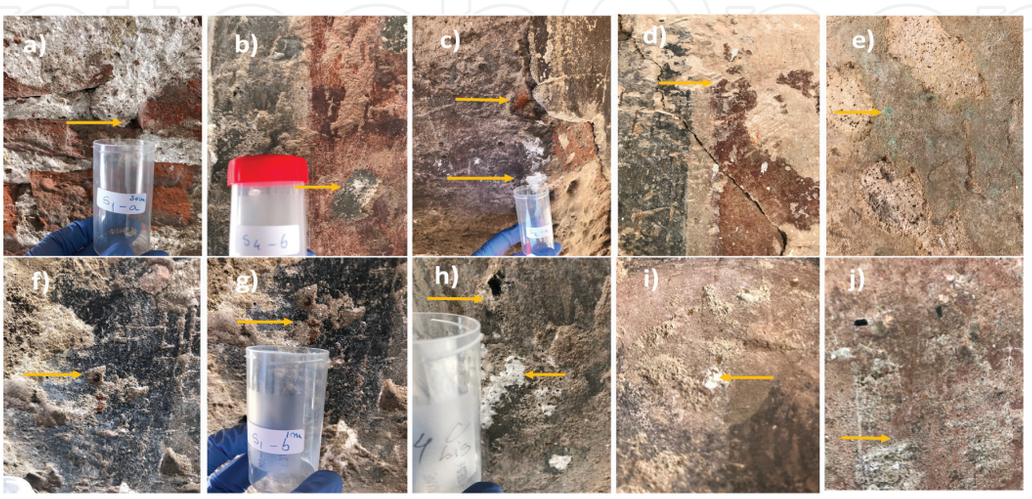

Figure 3.
Sampling and indicating areas of high degradation of the material (yellow arrows): (a) the materials with high porosity in restored areas, (b) depigmented areas, (c) cracked portions, (d) deterioration areas with fissures and cracks, (e) green pigment portions, (f, g) biogenic reddish-brown pigment, (h) black pigment, (i) deterioration with depigmented areas, and (j) black pigment and extensive deteriorations areas.

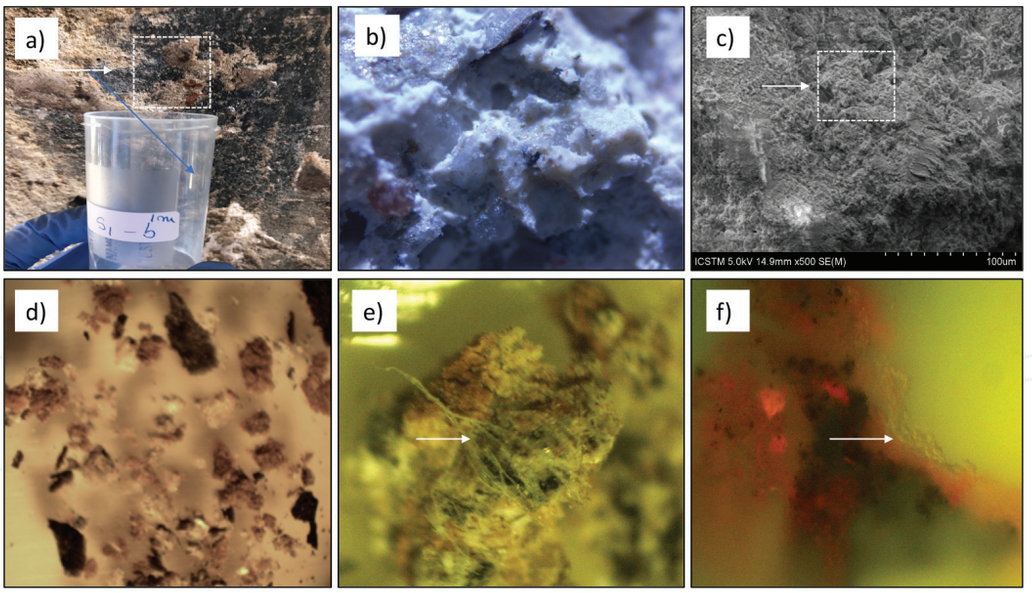

Figure 4.
Evaluation from macroscopic to microscopic level of the degraded surface; (a) direct visual evaluation, surface details light microscopy 400×, (c) electron microscopy (SEM) 7000×, (d) stereomicroscopy 40×, light microscopy 400×, and (f) epifluorescence microscopy 400×.

surfaces (**Figure 4b**), and at the micromorphological level, there is an obvious uniformity of the surface.

At this level, porosity is very fine, with ordered microspheres suggesting biogenic origin. In the sample analyzed with the optical microscope, during the first minutes after harvesting, very intense bacterial activity was revealed. The very large number and mobility of bacteria is evidence of the intense colonization of these microgalleries of the substrate. Extemporaneous samples also surprised fungal filaments (**Figure 4e**) adhering to the substrate and the stratification of the biofilm (**Figure 4f**), aspect suggesting the existence of anoxic conditions at the microhabitat level.

Sample 2 was collected at 1.50 cm to the soil, from an area with fine cracks (**Figure 5**), where superficial layers are easily detached.

Direct observation (**Figure 5a**) of the area of sample collection indicates that the zone was intensely degraded, with excavations likely induced by the shredding of the material and a mosaic appearance as a result of depigmentation. Microscopic analysis reveals fine granulation and mixtures of mineral elements with different chromatic appearances and porosities (**Figure 5b** and **d**). Shredding degradation is also visible in SEM analysis (**Figure 5c**). The study of wet microscopic preparations indicates fungal agglomerations (**Figure 5e**) and bacterial activity evidenced by intense mobility. Several bacterial morphotypes and clusters of the type of hulls found in clusters of irregular or filamentous forms were visible (**Figure 5f**).

Sample 3 was collected at 1.70 cm from the ground, painted with obvious bumps and dents, with a modified chromatic look (**Figure 6a**), possibly paint overlays overlapping previous restoration attempts. Microscopically, we found components that are different in shape and color (**Figure 6b**) arranged in layers with variable porosity and chromatic sequence (**Figure 6d**). From a biological point of view, the surfaces are intensely colonized (**Figure 6e** and **f**) of very large density micrococcus forms.

The analysis with specialized microscopy with epifluorescence highlighted the activity of the biota within the analyzed system. Live bacteria with an emission in

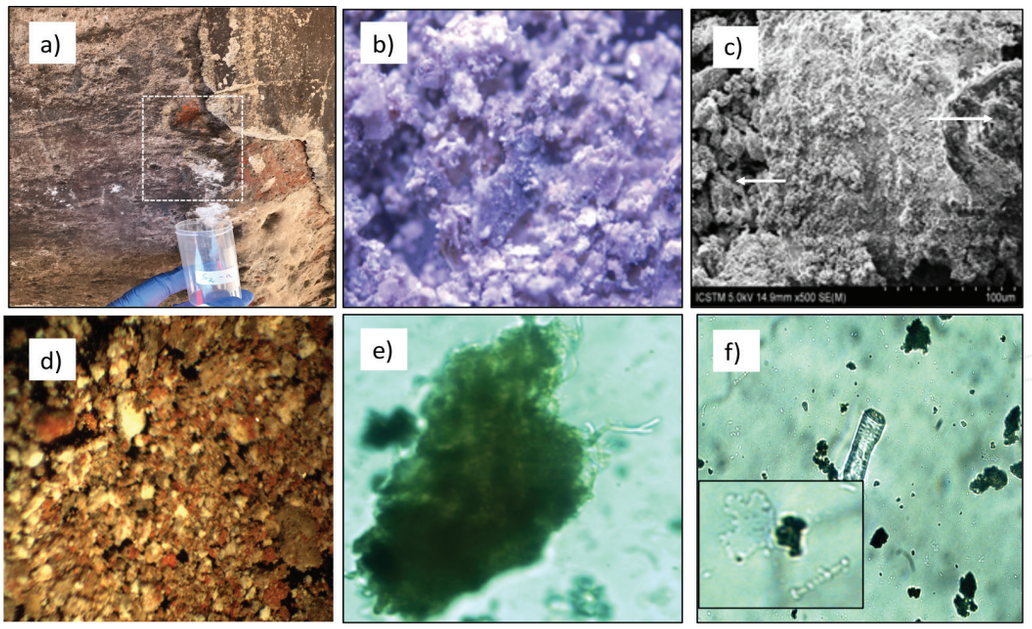

Figure 5.
Evaluation from macroscopic to microscopic level of the degraded surface; (a) direct visual evaluation, (b) light microscope 400×, (c) electron microscopy (SEM) 7000×, (d) stereomicroscopy 40×, (e) light microscopy 400×, and (f) light microscopy 1000×.

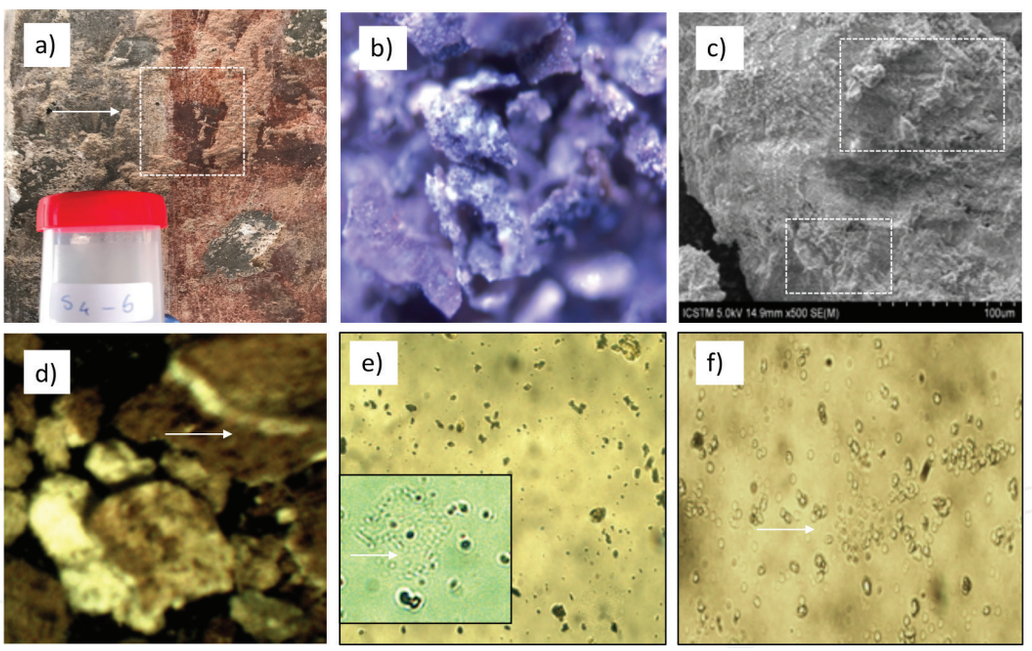

Figure 6.
Evaluation from macroscopic to microscopic level of the degraded surface; (a) direct visual evaluation, (b) optical microscopy 400×, (c) electron microscopy (SEM) 7000×, (d) stereomicroscopy 40×, (e) optical microscopy 1000×, and (f) optical microscopy 1000×.

orange shades have been noted in the presence of fluorochrome AO in an acid pH (**Figure 7a**). On inorganic surfaces, live components such as free-flowing filaments (**Figure 7c**) or filaments attached to the substrate (**Figure 7b, d** and **e**) were identified by this technique.

Metabolic activity is also noted through acidification phenomena marked by red emission on the carrier particles, probably caused by the death of some components of the microbial complex (**Figure 7b**).

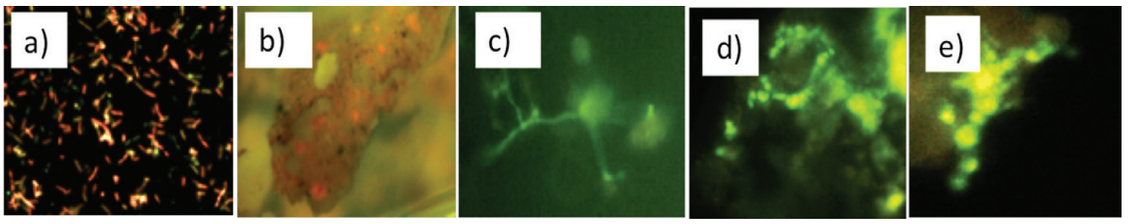

Figure 7.
In situ observations using the epifluorescence microscopy technique and AO stain: (a) bacteria in suspension sample, (b) biofilm at substrate, and (c–e) fungal hyphae, (magnification 400×).

6. Conclusions

The methods and techniques for assessing biofilm characteristic to historical monuments are difficult to standardize because of differences that may interfere (local climate, natural or artificial microclimate, microflora diversity composition, microbial pigment appearance, and the presence of rare or unknown species) and change the results between samples.

Among the easiest to apply and less expensive methods, but with a high degree of relevance, are the microscopy techniques. From the classic optical to the specialized microscope with fluorescence microscopy, to the most advanced microscopy (SEM or SEM-BSE, TEM), all of these have increased the degree of enlargement and the identification of structural details, respectively.

The discovery of morphological types and interactions between microorganisms (symbiosis, attachment, and complexity of matrix synthesis) was a feature of superior electron microscopy analysis systems and contributed to increasing the level of understanding of the ecology of microbiota systems.

On the analyzed Fresco area, Matia Loggia as a case study, in situ techniques applied for biodegradation assessment reveal an intense biological activity on the analyzed lithic systems. Following microscopic analyses, the extemporaneous samples highlighted the colonization of the analyzed surfaces with bacterial and fungal hyphae.

The intense bacterial activity was noted in all the samples taken from the points located at a distance of 1 m from the base of the walls by the in situ technique. The presence of fungus has been noted in interference areas between different types of substrates.

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Conflict of interest

None of the authors have any competing interests in the manuscript.

Contributions

Verginica Schröder, Daniela Carutiu Turcanu, Adina Honcea, and Rodica-Mariana Ion equally designed and performed the research. All authors reviewed the manuscript.

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