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ENVIRONMENTAL MICROBIOLOGY

Melding the Old with the New: Trends in Methods Used to Identify, Monitor, and Control Microorganisms on Cultural Heritage Materials

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Abstract Microbial activity has an important impact on the maintenance of cultural heritage materials, owing to the key role of microorganisms in many deterioration processes. In order to minimize such deleterious effects, there is a need to fine-tune methods that detect and characterize microorganisms. Trends in microbiology indicate that this need can be met by incorporating modern techniques. All of the methods considered in this review paper are employed in the identification, surveillance, and control of microorganisms, and they have two points in common: They are currently used in microbial ecology (only literature from 2009 to 2015 is included), and they are often applied in the cultural heritage sector. More than 75 peer-reviewed journal articles addressing three different approaches were considered: molecular, sensory and morphological, and biocontrol methods. The goal of this review is to highlight the usefulness of the traditional as well as the modern methods. The general theme in the literature cited suggests using an integrated approach.

Keywords Microorganisms · Biofilms · Biodeterioration · Destructive and non-destructive techniques · Microbial growth and survival · DNA · RNA · Fungi

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Introduction

The presence and activity of microbial communities in and on cultural heritage material is a chief cause of aesthetic, physical, and chemical damage [1]. Such damage is described by the term biodeterioration, i.e., any process arising from biological activity that affects the materials [2] (Fig. 1). Cultural heritage (CH) covers a wide diversity of archaeological monuments and sites, as well as historic buildings and objects deemed to be of significance to both local and international communities. Identification and characterization of the microbial communities present in CH is a good starting point for analyzing biodeterioration [3]. This also facilitates an understanding of the role played by both the microorganisms and the materials [3, 4].

Microorganisms occur and/or proliferate on CH for a variety of reasons. For example, in closed environments, such as museums, churches, castles, libraries, and caves, the source of microorganisms could be from the external atmosphere and/or from human activities within the sites. Thus, bacteria and fungi may originate from skin, and respiration-induced alteration could favor further proliferation of microbial communities [5]. Knowledge and control of the aerobiology of buildings that house CH is fundamental to preventing this proliferation and consequent biodeterioration [6]. This is particularly true when the temperature and relative humidity (RH) become optimal for microbial growth, such as in warm environments with temperatures >20 °C and humid sites with RH >65–70 % [7, 8].

Although several methods of analyzing CH materials have been established, very few of these can be used when only small amounts of sample are available. A minimally invasive approach is required in such situations, which are common in the context of CH [9]. This has been considered in laboratory-based studies with stone samples obtained from a quarry, where unlimited numbers of samples can usually be collected

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Fig. 1 Mayan figurehead, dating from between 250 and 600 AD, covered by dark-colored microbial communities (probably lichens and fungi). The figurehead forms part of the "Building of the Five Stories," Edzná (Mexico). Photo: Dr Patricia Sanmartín (January 2014)

[10]. The findings obtained in this type of study can be translated to historic monuments in which the stone under study is a dimension stone. However, such stone is not always available for study, as the original quarry is often unknown, has been mined out, or is no longer accessible [11]. Fine-tuning of methods that can be applied directly to cultural property is therefore a major challenge.

In this review, we will present and discuss the current methods—reported in papers published in the last 6 years (2009–2015)—used to study the microbial ecology of CH materials. Three different approaches are considered: molecular, sensory and morphological, and biocontrol methods. Molecular methods are valuable tools for investigating the diversity and structure of cultivable and non-cultivable organisms. Sensory and morphological methods provide valuable information about the viability and growth of microorganisms and are often not invasive or destructive. Biocontrol methods include the most novel approaches for suppressing microbial growth. An overview of current perspectives and future directions in the use of these three approaches in CH is presented (for definitions of acronyms used, refer to Table 1). A summary of the techniques and findings from some of the key studies addressed in the text is reported in Table 2.

Molecular Methods

The ability to study biological function rapidly in microorganisms via molecular biology led to developments in diverse fields such as medicine and environmental biology. However, until a few years ago, only traditional microbiological methods were commonly used in studying biodeterioration on cultural heritage materials.

The use of DNA and RNA-based methods in biodeterioration of cultural heritage has gained much traction over the past decade [12, 13]. The primary use for these methods has been in the detection and characterization of microorganisms in environments that are notoriously hard to culture from. Recent advancements in metagenomics and high-throughput science have made these assays more accessible for use in multiple environments.

Proteins have historically been analyzed for measurement of microbial activity. For instance, simple assays for measurement of enzymes have been available for many years. However, their use in cultural heritage microbiology has been more limited. Important functional roles of microbes in their environment can be analyzed using enzyme-based methods.

Along with other -omics technologies, progress in metabolomics has increased the breadth of microbial ecology research. Analysis of metabolites produced by microbes in response to their environments can provide useful insight into microbial function. Though these chemical methods are relatively new in the cultural heritage field, they hold great promise for revolutionizing the field.

Nucleic Acid-Based Techniques

All the methods described below under the umbrella of nucleic acid-based techniques employ polymerase chain reaction (PCR)-based amplification of ribosomal RNA (rRNA) or other genes, followed by fingerprinting techniques (such as denaturing gradient gel electrophoresis (DGGE) and/or clone library construction and sequencing. Recent studies have employed functional gene targets either by themselves or in concert with rRNA genes. Shotgun sequencing-based metagenomics have not yet been used in this field, and next-generation sequencing methods are still rare.

In the cultural heritage context, many different types of materials, including wood, stone, and paper, have been sampled and analyzed using these techniques. One of the main challenges while dealing with this range of materials is the means to perform non-destructive sampling. For this reason, many non-invasive approaches have been designed, such as careful swabbing with nitrocellulose membranes [14].

The most widely used molecular method in the identification of microorganisms is based on the ribosomal operon. Since it is evolutionarily well conserved and contains hypervariable regions that allow for distinguishing between taxa, the bacterial and archaeal 16S rRNA gene (or the 18S/28S rRNA gene/intergenic transcribed spacer (ITS) region for fungi) is the gene of choice for identification. Bacterial, archaeal, and fungal ribosomal gene databases have grown to such an extent that it is now possible to identify microbial species.

In a study on the wooden surfaces of a seventeenth-century church, Lupan and colleagues successfully used both culture-based and culture-independent methods to isolate and/or identify bacteria and fungi [15]. Cellulose-degrading bacteria such as *Bacillus* and *Solibacillus*, expected due to the cellulose-rich substrate, were detected using culture-independent methods. However, *Planomicrobium* and *Variovorax*, isolated in culture

Table 1 Acronyms used in this manuscript

Acronym	Expansion
ATP	Adenosine triphosphate
cbh I	Cellobiohydrolase I
cDNA	Complementary DNA
CH	Cultural heritage
CLSM	Confocal laser scanning microscopy
CMC	Carboxymethyl cellulose
D-ALA	Endogenous protochlorophyllide precursor d-aminolevulinic acid
DGGE	Denaturing gradient gel electrophoresis
EDS	Energy dispersive spectroscopy
EIS	Electrochemical impedance spectroscopy
EPS	Exopolysaccharides
ESEM	Environmental scanning electron microscopy
ESR	Electron spin resonance
FESEM	Field emission scanning electron microscopy
FISH	Fluorescent in situ hybridization
FTIR	Fourier transform infrared
GC/MS	Gas chromatography/mass spectrometry
HR-MAS	High-resolution magic angle spinning
LM	Light microscopy
LTSEM	Low-temperature scanning electron microscopy
MALDI-TOF	Matrix-assisted laser desorption ionization/time-of-flight mass spectrometry
MUF-NAG	Fluorogenic 4-methylumbelliferyl and N-acetyl-beta-D-glucosamine
NAGase	N-acetylhexosaminidase
NMR	Nuclear magnetic resonance
PCA	Principal component analysis
PCR	Polymerase chain reaction
PDT	Photodynamic antimicrobial chemotherapy
PLM	Polarization light microscopy
qPCR	Quantitative PCR
RH	Relative humidity
rRNA	Ribosomal RNA
ROS	Reactive oxygen species
SEM	Scanning electron microscopy
SEM-BSE	Scanning electron microscopy in backscattered electron mode
SEM-EDX	Scanning electron microscopy with energy-dispersive X-ray spectroscopy
SEM-SE	SEM with secondary electrons
SRB	Sulfate-reducing bacteria
TEM	Transmission electron microscopy
VP-SEM	Variable pressure SEM
XRD	X-ray diffraction

and identified using the 16S rRNA gene, had not previously been detected on wood. Fungi isolated using culture-based methods were rapidly identified using ribosomal operon-based genes. In contrast, traditional identification methods involve culturing on microbiological media and observation of hyphal and spore morphology, a process that could take days to weeks. A similar study was conducted on historic wooden churches of Chiloé, Chile, built in the eighteenth and nineteenth centuries [16]. The authors detected white, brown, and soft rot fungi on the native wood that the churches are built of. Using PCR-based rRNA gene analysis, many different wood decay fungi were identified, including some that were reported for the first time in Chile. In two more exploratory studies on wooden objects, Rajkowska [17] and Palla and colleagues [18] used rRNA-based molecular identification to study microorganisms in the buildings in the Auschwitz II-Birkenau concentration camp and archaeological wood from an ancient shipwreck, respectively. Use of these rapid techniques enhances the ability to make decisions on preservation within hours rather than days, which is crucial since the wood deterioration process can progress rapidly.

In contrast, biodeterioration of stone objects and structures is not as rapid. However, these artifacts are prone to deterioration and can become brittle due to microbial action over time. Kusumi and colleagues studied biofilm formation on sandstone in the twelfth-century Bayon Temple in Cambodia [19]. The authors used PCR amplification followed by DGGE for analysis of the community composition in these biofilms. This method enables fingerprinting of the communities, i.e., it provides an instant map of the similarities and differences between them. Briefly, the 16S rRNA gene is amplified via PCR, following which the amplicons are electrophoresed on a polyacrylamide gel containing a denaturing chemical on a gradient. This unique feature enables separation of the bands that differ only by slight changes in their sequences. In addition to being a good measure of differences in the members comprising the community, the advantage of this method stems from the fact that the individual bands are easily separated and then identified by constructing clone libraries followed by sequencing of individual clones.

Kusumi and colleagues [19] used DGGE to differentiate biofilms on stone from airborne communities. In addition, using clone libraries, they successfully identified genera that were common to all the biofilm samples. In 2012, Polo and colleagues [20] characterized the sessile ecological communities on lithoid surfaces and the airborne surroundings of the Richini courtyard (University of Milan, Italy). These authors also used DGGE to identify the genera present in both airborne communities and biofilms on mortar and stone. They performed principal component analysis (PCA) to analyze the data derived from DGGE.

In another study in the Bayon Temple, the presence of fungi and their role in the succession of the

Table 2 Characteristics appraised by some representative studies relating microbial ecology to cultural heritage (CH)

Microbial ecology	Material	Occurrence	Technique				Reference
			Molecular	Sensory/ morphological	Biocide	Biocleaning	
A, B, C, F, L	Dolostone rock quarry	Madrid, Spain		●	●		Cámara et al. [10]
C	Frescoes, Luca Signorelli frescoes	St. Brizio Chapel. Orvieto Cathedral, Italy	●	●	●		Cappitelli et al. [27]
A, C	*Neapolitan Yellow Tuff* stones	Naples, Italy	●	●			Cennamo et al. [22]
A, B, C, F	Art Nouveau polychrome ceramic coating tiles	Grande Albergo Ausonia and Hungaria, Venice, Italy	●	●			Giacomucci et al. [48]
B, C	Sandstone rock and air	Bayon temple in Angkor Thom, Cambodia	●				Kusumi et al. [19]
B, F	Wood	Nicula, Romania	●				Lupan et al. [15]
F	Filter paper, high-lignin paper	Laboratory study	●		●		Michaelsen et al. [36]
F	Wood	Chiloé, Chile	●	●			Ortiz et al. [16]
B	Waterlogged wood	Sicilian islands, Italy	●	●			Palla et al. [18]
B, F	Parchment, Archimedes Palimpsest	Walters Art Museum in Baltimore, Maryland, USA	●	●			Pinar et al. [32]
B, F	Paper, Leonardo da Vinci's Atlantic Codex	Biblioteca Ambrosiana. Milan, Italy	●				Principi et al. [14]
A, B, F	Mortar, stone, and air in Milan University's Richini's courtyard	Milan, Italy	●	●			Polo et al. [20]
A, B, C, F	Oolitic limestone rock	Buonconsiglio Castle. Trento, Italy	●	●	●	●	Polo et al. [65]
A, B, C, F, L	Wood and brick	Auschwitz II-Birkenau concentration camp, Poland	●				Rajkowska [17]
A, B, C, F, L	Palaeolithic paintings on limestone rock	Cave of Bats. Zuheros, Spain	●	●			Urzì et al. [46]
F	Color cinematographic films	Cuban Institute for Cinematographic Industry and Arts (ICAIC), Havana, Cuba	●	●			Vivar et al. [8]

A algae, *B* bacteria, *C* cyanobacteria, *F* fungi, *L* lichens

microbial communities was investigated [21]. Previous studies had indicated that complex communities exist on these walls. In this study, the authors isolated fungal mycelia and identified the fungal cultures by PCR amplification and sequencing of both the ITS region as well as beta-tubulin genes. The use of two separate genes made the identification more robust [21]. Another important reason non-ribosomal genes are used is due to their more reliable copy numbers, as compared to the variable copy number of rRNA genes.

Cennamo and colleagues [22], who studied the phototrophic encrusting on *Neapolitan Yellow Tuff* stones of walls in historical sites of Naples, Italy, also used DGGE to characterize biofilm growth. Their goal was to study biofilms before and after cleaning to study the efficacy of the cleaning methods [22]. After DGGE analysis, clone libraries were constructed and specific members identified by sequencing. The authors used both microscopy and molecular techniques, citing that the latter were useful and better at accurate identification.

In another study in Southern Italy, microorganisms on deteriorating medieval paintings in churches [23] were identified using rRNA-based sequencing methods. Nitrocellulose membranes were found to be useful for non-invasive sampling. Pure cultures of bacteria and fungi were obtained and identified using 16S and ITS regions of the rRNA gene.

In a recent study of painted surfaces of the Mogao Grottoes, Ma and colleagues [24] conducted a comprehensive study of

bacterial and fungal communities, in an effort to characterize the cause of biodeterioration of these surfaces. Five different caves were sampled, using both culture-dependent and culture-independent methods. Cultivable communities were grown on a range of different pH, salinity, and temperatures, to isolate a wide variety of microorganisms. Using PCA and Pearson's correlation to statistically compare the microbial communities from various samples, the authors concluded that fungal communities were significantly correlated with the age of the caves. This finding was possible because of the large number of clones screened. Data derived from conventional methods are usually not available on this scale.

Though automation of many processes makes it easier to screen large numbers of clones, the volume of data obtained is still small compared to that available from next-generation sequencing methods. Vasanthakumar and colleagues [25] used next-generation sequencing to study bacterial and fungal communities on the walls of King Tutankhamun's tomb. Due to inhibition by melanoid pigment in the samples, the authors used a nested PCR approach to amplify ribosomal RNA genes. These amplicons were then sequenced by 454 pyrosequencing methods. This method enabled ~1000–3000 sequence reads to be derived from each area sampled. This large volume of data was then analyzed using appropriate sequence analysis pipelines, which included statistical analyses. In this study, due to low sample availability, amplicons had to be constructed prior to 454 sequencing, a process that possibly introduced bias. However, the study utilized other approaches to complement data from the sequencing approach [25]. Though next-generation sequencing is still new in the CH field, it is an attractive option for microbial community analysis.

In a study on painted surfaces in an Etruscan tomb, bacteria from the order Rhizobiales were discovered to be the primary colonizers [26]. The authors constructed ribosomal operon-based clone libraries using both DNA and mRNA, the latter to detect metabolically active members of the community. Analysis of the clones suggested a prevalence of Rhizobiales, which the authors attributed to the presence of many roots in the tomb, since Rhizobia are commonly associated with plant roots. The use of both DNA and RNA was useful in distinguishing between the total membership and the metabolically active members in this community.

Cappitelli and colleagues [27] tested the efficacy of two cleaning methods on the frescoes in the Orvieto Cathedral. A rosy discoloration that partially obscures the frescoes has long been associated with microorganisms. The authors conclusively identified cyanobacteria using microscopy, cultivation, chemical, and molecular methods. PCR amplification of the cyanobacterial 16S rRNA gene, followed by DGGE, was the definitive method of identification. This study illustrates the ease of using the rRNA gene in a rapid test for efficacy of cleaning methods.

The studies described so far have been on wood and painted surfaces on various types of stone. Biodeterioration can be a significant problem on other archival materials as well. Historic library materials, including paper and parchment, are extremely susceptible to microbial attack [28]. Cellulosic materials, such as ancient books, can deteriorate rapidly if microbial growth is left unchecked (Fig. 2). Paper is particularly susceptible to fungal growth, especially at high relative humidity and temperature. Cappitelli and colleagues noted that conservation could be improved by using more advanced microbiological approaches to slow down the rate of paper deterioration [28]. Microbial communities on the surface of the stained regions of Da Vinci's Atlantic Codex were characterized by Principi and colleagues [14], who used non-invasive sampling and molecular methods. As in many recent studies, the authors used a combination of DGGE/PCA and cloning/sequencing to identify potential stain-forming microorganisms. The use of advanced statistical tests in concert with molecular tools that generate large amounts of data is gaining favor because it allows for hypothesis testing. The authors were able to conclude that potential cellulolytic microorganisms—detected in the study—could grow under the right conditions and consequently pose a threat to the ancient manuscript.

Fig. 2 Stained and deteriorating pages of an ancient book. The paper is brittle, and there are black stains spread over the pages, due to fungal growth and activity. Photo: Dr Nick Konkol, Laboratory of Applied Microbiology, Harvard University (2009)

Cellulose-degrading fungi were the subjects of research at an old library in the Medina of Fez, one of the oldest cities in Morocco, where ancient manuscripts were analyzed for fungal growth [29]. Pure cultures were obtained and identified using the ITS region of the rRNA operon. In addition, the authors studied enzyme activity relating to cellulose degradation.

In yet another study on ancient paper, Michaelsen and colleagues [30] investigated a deteriorating sixteenth-century book, "Le Stanze del Bandello" by Matteo Bandello, stored in the Braidense Library in Milan, Italy, using conventional and molecular techniques. Biodeterioration of paper can affect its strength, integrity, and aesthetic qualities. Therefore, it is crucial to detect and characterize the microorganisms causing the damage so that appropriate measures can be taken. Using multiple non-invasive sampling techniques such as swabbing, lifting with adhesive tape, and nitrocellulose membrane sampling, Michaelsen and colleagues obtained fungal material in culture, as well as total fungal DNA for further investigation. PCR-amplified ITS regions were separated by DGGE and bands of interest studied further using clone libraries and sequencing. Using this combination of methods, the authors discovered eight different fungal species on the ancient book. These findings enabled a targeted restoration process.

In a 2010 study, Michaelsen and colleagues investigated another ancient manuscript dating back to 1293 [31]. This thirteenth-century manuscript had been written on paper made of linen and had a binding consisting of parchment. The authors sought to culture the fungi that they observed using SEM but were unsuccessful. Therefore, they used PCR-DGGE as well as clone libraries to characterize the microbial communities. This study provides yet another instance of the importance of molecular methods in studying biodeterioration of CH.

Many ancient manuscripts are made of parchment, which consists primarily of collagen and is susceptible to degradation by gelatinolytic microorganisms. Piñar and colleagues [32] used molecular techniques to identify and characterize microbial contamination on the Archimedes Palimpsest, an ancient parchment manuscript. Bacterial and fungal structures were visualized with electron microscopy. Using PCR/DGGE/clone library/sequencing, microorganisms similar to members of the human skin microbiome were identified. The authors speculated that these potential collagen degraders are responsible for the deterioration of the parchment. In another similar study on parchment, Piñar and colleagues [33] used molecular methods and scanning electron microscopy with energy-dispersive X-ray spectroscopy (SEM-EDX). Based on their results, they suggested two possibilities: *Saccharopolyspora* and related salt-loving microorganisms could produce rhodopsin, a pigment that causes the characteristic purple discoloration. Also, actinobacteria and fungi such as *Aspergillus versicolor* were strongly associated with the deteriorated sections, suggesting a role for them in the biodeterioration of parchment.

Montanari and colleagues [34] cite the use of molecular techniques, in combination with microscopy and culturing, as key to identifying the fungal species on library materials stored in Compactus shelving. *Eurotium halophilicum*, a fungus with high tolerance to water stress, was associated closely with Compactus library materials and could pose a risk to these materials.

All the studies reviewed above are based on standard PCR amplification, which provides only qualitative results. Advancements in PCR technology allow for quantification by quantitative PCR (qPCR). This assay enables measurement of concentration of template based on standard curves. Ettenauer and colleagues [35] inoculated five different insulation materials with fungi and placed them in a historical building under conditions conducive for fungal growth for up to 6 months. Samples were removed and tested for using qPCR of the beta-actin gene. Results ranged from 5 to 5000 copy numbers/ng extracted DNA from the test materials, and the detection limit was ~30 copy numbers/ng DNA. The ability to detect low numbers makes this a highly sensitive assay. The authors recommended that this is a useful technique for detection of biodeterioration caused by fungi, especially in cases where limited samples are available. In the investigation of the Archimedes Palimpsest, Piñar and colleagues [32] also used qPCR on the beta-actin gene and concluded that fungi were more closely associated with deteriorated areas of the parchment than healthy areas. The variable copy number of rRNA genes does not make it a good choice for qPCR; therefore, housekeeping genes, such as those that code for actin and tubulin, are used.

Though the studies described so far were based on genomic DNA, the same methods could also be applied to mRNA, which can then be reverse transcribed to obtain the complementary DNA (cDNA), which can be amplified by PCR. This change in template enables an estimate of the metabolically active microorganisms in the environment. Michaelsen and colleagues [36] analyzed three different cleaning techniques on fungal-infested paper. To assess the efficacy and longevity of their cleaning techniques, they used both DNA and RNA methods. The 18S rRNA region was reverse-transcribed and amplified by PCR, after which samples were analyzed by DGGE. RNA appeared to be a good estimate of metabolic viability of the fungal material remaining on the paper [36]. In addition to using RNA as the template, Villa and colleagues used functional genes to study sulfur cycling in polluted and unpolluted areas [37]. The authors investigated both rRNA genes as well as those in the sulfur oxidation and sulfur reduction pathways. Metabolic capabilities were characterized and enabled comparison of microbial community function (i.e., sulfur cycling) in polluted versus unpolluted areas. This study demonstrates that microbial functionality can be understood by investigating metabolically active microorganisms [37]. However, this capability is dependent on the time course of sampling and might require multiple samples collected over

an extended period of time. Krakova and colleagues [38] also used functional genes for characterization of fungi on museum artifacts such as wood and paper. Primers based on a gene predictive of cellulolytic activity, viz. cellobiohydrolase I (*cbh I*), were designed. The use of both *cbhI* and ITS regions helped to identify the isolated fungal strains. The authors suggested that these functional genes are useful in characterizing the potential of microbial activity on CH.

Based on a recent study on Roman catacombs, Krakova and colleagues [39] strongly recommended the use of multiple approaches for studying microorganisms. Molecular methods, on one hand, rapidly yield reproducible data on community diversity and membership. However, without a cultured representative, it is difficult to study the chemical processes underlying deterioration. The authors, therefore, recommended the use of culture-based and molecular methods in tandem [39].

Nucleic acid-based methods in cultural heritage microbiology provide many advantages. They bypass the need for culturing, thereby saving time; access a larger group of microorganisms; are more sensitive in detecting diverse microorganisms; and enable community-level comparison and characterization. However, they also have limitations: Using solely DNA, one cannot make conclusions about metabolic potential. Therefore, genomic analysis cannot be used as the sole approach for detection or characterization. Messenger RNA-based methods provide improvement, in that they are able to predict the expression of the genes.

As the sections below briefly outline, recent methods in protein analysis and metabolomics are being modified and optimized for use in various environments. It is likely that metabolomics will soon become a useful method for studying microbial community dynamics in cultural heritage.

Enzyme Assays

Almost all the research studies reviewed in the previous section included cultivation as well as molecular techniques for microbial identification and characterization. Another theme that has emerged is the use of enzymes that are predictive of microbial chemical ecology. For instance, on paper materials, cellulose-degrading microorganisms are usually present and metabolically active. Bergadi and colleagues [29] examined the cellulase activity and filter paper degradative ability of fungal strains isolated from ancient manuscripts in Medina. Using carboxymethyl cellulose (CMC) containing media, fungal cellulolytic activity was screened in a semi-quantitative manner. The filter paper assay was also employed to quantify enzymatic activity. This activity, named FPU, i.e., filter paper unit activity, was calculated by measuring the amount of reducing sugars released when the fungal cultures act on the filter paper under the appropriate reaction conditions. Cellulases are widely produced by fungi and are commonly expressed on cellulose substrates. Hu and colleagues [21] found that *Aspergillus* strains isolated from sandstone in the Bayon Temple also demonstrated high cellulolytic activity in vitro. Cellobiohydrolases, galactosidases, and other cellulases were assayed for in this study. Interestingly, fungal growth on top of previously formed biofilms appeared to help remove previously existing biological growth, possibly by enzyme activity.

Other enzymes that can be studied easily in vitro are those that are responsible for nitrogen or sulfur cycling. Many pathways are characterized for these microbial processes. For instance, in a follow-up to the study by Villa and colleagues, enrichment cultures could be made and enzymes responsible for sulfur metabolism, especially the ones whose genes were transcribed at higher levels, could be assayed.

In a recent study on Chinchorro mummies, DeAraujo and colleagues studied collagenolytic and gelatinolytic activity in bacteria and fungi isolated from degrading mummy skin (DeAraujo, personal communication). The results of the enzyme assay were unexpected in that the activity of one isolate was much higher than that of any of the others. This suggested to the authors that this bacterial isolate could play an important role in the degradation of the skin. Similar assays would be extremely useful for characterization of microorganisms on parchment, which is also composed of collagen.

Microbial physiological characterization assays are now commercially available in high-throughput form (for instance, from BioLog Inc., Hayward, CA). Many of these could be customized for detection of enzymes. Enzyme profiling is a useful tool in the microbial ecologist's repertoire. Advances in proteomics have also added to the ease of studying enzyme profiles. Chromatography and mass spectrometry-based techniques provide exquisite sensitivity. With large databases being available, these superior methods are becoming more attractive.

Metabolite Profiling

By-products of microbial metabolism can provide useful insights into microbial ecology. Microbial secondary metabolites—biochemicals that are by-products of secondary metabolism pathways—have historically been of interest for their varied properties. For instance, bacteria such as *Streptomyces* and fungi such as *Penicillium* produce antimicrobial compounds to defend against other microorganisms [40]. Another class of secondary metabolites includes pigments such as phenazine (produced by *Pseudomonas* spp. and *Streptomyces* spp.), which have been shown to play important roles in microbial survival and fitness [41].

Detection of microbial metabolites on CH might be a less daunting task than detecting the microorganisms themselves, especially if the latter exist in low abundance. Once metabolites such as pigments, melanins, or organic acids are detected, it would also be simpler to design specific culture conditions

for microbial isolation. Metabolite profiling via methods such as gas chromatography/mass spectrometry (GC/MS) and matrix-assisted laser desorption ionization/time-of-flight mass spectrometry (MALDI-TOF) offers enormous potential. Kirby and colleagues [42] used peptide mass fingerprinting, a small molecule analysis method, to identify collagen-based materials on CH. The ability to use this method on artworks such as paintings, pottery, and sculpture indicates that it is easily modifiable for microbial detection and characterization.

Vasanthakumar and colleagues studied the possible microbial origin of inactive brown spots on the walls of King Tutankhamun's tomb in Upper Egypt [25]. A survey of the samples using pyrolysis-GC/MS revealed that malic acid, a common microbial metabolite, was associated with the brown spots but not with areas around it. The identity of the brown spots, unknown for now, could also provide a clue into the microbial involvement in the formation of these spots. Chromatography and mass spectrometry, in addition to being sensitive, are in a rapid growth phase and form the basis for many enhanced proteomics and metabolomics methods. These approaches have become mainstream in microbiological studies and, therefore, are certain to soon become useful methods in cultural heritage as well.

Corsaro and colleagues [43] used a proton high-resolution magic angle spinning (HR-MAS) nuclear magnetic resonance (NMR) technique to detect metabolites in the degradation of cellulose in both ancient and artificially aged manuscripts [43]. The authors were able to detect low molecular mass compounds in the cellulose fibers of the manuscripts. A major goal of the study was to demonstrate the value of this technique on objects of cultural heritage. These studies highlight recent developments in metabolite profiling methods, which promise to provide useful data on microbial ecology of cultural heritage.

Sensory and Morphological Methods

In the context of this review, sensory and morphological methods refer to those methods involving visual aspects and the physical appearance of the microorganisms under study. Most authors have stressed that combination of these methods provides an efficient way of determining the presence and viability of microbial communities.

Optical, Electron, and Scanning Microscopy

In 2009, Cappitelli and colleagues carried out a comprehensive study of a rosy discoloration of biological origin, which partly masked the Luca Signorelli frescoes in St. Brizio Chapel (Orvieto Cathedral, Italy) [27]. Pink powder samples were examined by optical epifluorescence microscopy and SEM to estimate the presence, size, and form of microorganisms that formed them. In the same year, Santos and colleagues used SEM-EDX, environmental scanning electron microscopy (ESEM), and FISH to examine the oil-on-wood painting entitled "Cristo con la Cruz a Cuestas," belonging to the 1600s Spanish School and at present found at the Archbishopric of Toledo, Spain [44]. The analyses revealed that bacterial-fungal biofilm structures were closely attached to the painted surface. More specifically, SEM-EDX analysis enabled characterization of the pigments present and correlation of these with the communities of microorganisms associated with them. Thus, for example, bacteria were the main contaminants in copper-based pigments and appeared in low abundance in vermilion pigments, whereas fungi were present in low abundance in both types of pigments.

De los Ríos and colleagues studied the biodeterioration caused by lichen and fungi (lichenized and non-lichenized) on carbonate building stone (dolostone and limestone) in four Romanesque churches in Segovia (Spain): La Vera Cruz, San Lorenzo, San Millán, and San Martín (declared by UNESCO as World Heritage sites) [45]. These authors used images taken on TEM, SEM-EDX, SEM-SE, SEM in backscattered electron mode (SEM-BSE), and low-temperature SEM (LTSEM) to analyze the presence of microorganisms, and their possible role in the biodeterioration, in samples of stone. Fungi appeared to be predominant on the areas showing maximum deterioration. Furthermore, fungi were related to mineral crystals, identified by LTSEM as calcium oxalate dihydrate, which were possibly formed as a result of biomineralization. The images suggested that epilithic lichen caused mechanical damage on the stone.

Michaelsen and colleagues [30] studied fungi in old paper, specifically paper from a copy of "Le Stanze del Bandello" by Matteo Bandello, printed at the end of 1600s in Italy and kept in the Braidense Library in Milan (Italy). These authors used a stereoscopic (dissecting) microscope to examine the book before the sampling procedure. Thus, they examine stained and deteriorated areas of the book under a stereoscopic microscope equipped with low-temperature fiber optic lighting. Finally, they used VP-SEM combined with EDS for detailed characterization of the grade of paper and the presence of fungi (by means of visual definition of the structure of fibers in very small (<4 mm) samples). This method is non-destructive and does not require sample metallization. The same authors later performed another case study, using SEM to examine a manuscript from Italy, dating from 1293 [31]. Use of SEM enabled visualization of fungal spores and hyphae attached to cellulose fibers. In the field of speleology (defined as the scientific study of caves), Urzì and colleagues [46] used light and epifluorescence microscopy and TEM to characterize the diversity of phototrophic microbial communities in paintings dating from the Paleolithic era preserved in the Cave of Bats, Zuheros (Spain).

In a study mentioned in the introduction, Cámara and colleagues [10] characterized the environmental microbiology of a dolostone quarry in a region north of Madrid (Spain) to determine the mechanisms of biodeterioration. They then translated the observations made on quarry stones to the historic monuments in which similar dolostone was used as building material. They used SEM-BSE to study and evaluate the extent of colonization of the different quarry fronts under study.

Metals such as bronze/copper, silver, and iron are the main metal substrates in CH artwork and are often actively corroded. Within the framework of the EU-ARTECH and BAHAMAS projects, Joseph and colleagues [47] undertook a bioremediation study of the transformation of metal compounds into metal oxalates, by using different species of fungi such as *Aspergillus niger* and *Beauveria bassiana*. They used X-ray diffraction (XRD), Fourier transform infrared (FTIR) microscopy, Raman microscopy, ESEM, SEM-EDS, EIS, and colorimetry to monitor the transformation process. ESEM observations have enabled characterization of the crystals with different aggregation shapes and FTIR microscopy when conversion of the first layer of corroded surface was complete.

Studies on the microbial communities on artistic tiles (generally ceramic plaques glazed on one side) are scarce. Nevertheless, two studies of substantial value from an ecological point of view have been published recently. Giacomucci and colleagues [48] investigated the biodeterioration of Art Nouveau polychrome ceramic coating tiles, dating from 1914, from the façade of the Grande Albergo Ausonia and Hungaria, in Venice (Italy). The biodeterioration had become apparent after cleaning and restoration work was carried out in 2007. The authors used stereo, optical, and electron (ESEM) microscopic techniques with the aim of confirming the microbial deterioration and characterizing the microbial community that caused it. Stereomicroscopic examination of samples revealed color alterations at the interface glaze pottery and pottery layer. Optical and electron microscope (ESEM) images confirmed the presence of cryptoendolithic niches formed by phototrophic microorganisms. Coutinho and colleagues [4] published a study of glazed ceramic tiles dating from the second half of 1900s from a passageway, called the Triton tunnel, in the Pena National Palace (Sintra, Portugal). The artistic tiles were heavily colonized by photosynthetic microorganisms and showed flaking in the glazed parts. The authors proceeded to identify the microorganisms responsible for the black and green patinas and their role in the biodeterioration process. Use of light microscopy (LM) enabled identification of some of the microorganisms involved: two green algae, one dematiaceous fungus, and some not well-developed lichens. Use of the same technique revealed the first stages of the lichenization process. These authors also used field emission scanning electron microscopy (FESEM) to determine the surface topography, microorganism morphology, and the distribution of microorganisms and their relationship with the substrate material. The FESEM images revealed thick biofilms over the glazed surface, as well as inside the material, and strong adhesion of the filamentous organisms by EPS to the glaze. Use of confocal laser scanning microscopy (CLSM) facilitated characterization of organisms forming biofilm on the tiles. The microbial ecology community mainly consisted of bacteria, cyanobacteria, microalgae, and some lichenized fungi, confirming the observations made using LM. Fluorescence-mode CLSM revealed photosynthetic pigments and small spots of EPS identified with Con-A. Furthermore, in some cases, the 3D images obtained by CLSM were used to determine the biofilm architecture and to measure its thickness.

Polo and colleagues [20] used epifluorescence microscopy and molecular methods to study lithobiontic (defined as "on and within hard rock substrates") communities in the Richini courtyard, Milan University (Italy). Use of epifluorescence microscopy showed a diversity of airborne microbiota and also enabled detection of endolithic species. Results of CLSM, SEM, and culture-independent molecular methods also revealed that algae and/or fungi predominated over bacteria in the samples. Specifically, green algae and black fungi were the main harmful microorganisms. SEM images revealed the mature biofilm (showing a complex 3D structure) attached to the lithic substrate (viz. mortar) causing aesthetic and physical damage. FTIR and XRD analyses, however, did not show any chemical degradation. Furthermore, FTIR analysis revealed information about the tertiary bioreceptivity of mortar, which had been restored with synthetic polymers. The tertiary bioreceptivity indicates the colonization potential of materials submitted to conservation treatments. In this case, the bioreceptivity increased, which, according to the authors, is possibly because the components of polymers that act as nutrients for the biodeteriogens (viz. fungi) are more abundant in this area.

Similarly, Scrano and colleagues [49] studied the microbial community colonizing calcarenite stones in a private historical building located near the archaeological site of Lavello, in the region of Basilicata (Italy). Use of SEM confirmed fungal populations on calcarenite rock and provided information about their morphological and molecular characteristics and their role in deterioration of the historical building.

Biodeterioration of wood has been studied to lesser extent than the biodeterioration of stone. Bacteria and fungi are the main biodeteriogens of wood. Fungi cause aesthetic deterioration (associated with staining in white and brown rot) and the wood rot process. Bacteria degrade wood differently, forming tunnels and erosion caves. In a mini-review paper, Singh [50] analyzed the degradation, particularly that caused by bacteria, in wooden CH objects recovered from buried and waterlogged environments. Singh notes three possible causes

for the bacterial decay of wood: tunneling bacteria, erosion bacteria (principal mode of degradation), and soft rot. According to the author, TEM and SEM images clearly show the deterioration caused by bacteria, also by light microscopy, especially in the early stages. Long and deep erosion channels are well recognized by SEM and PLM. The paper also shows a collection of TEM micrographs of bacterial degradation of wood. In another recent study, Ortiz and colleagues [16] investigated fungal-derived deterioration of eight historic wooden churches, built in the late 1700s and 1800s in Chile and considered since 2000 by UNESCO as World Heritage Sites. They examined samples of the wood by SEM to identify the decay fungi present in each church and to determine the different types and the diverse stages of decay.

Celluloid, the material used to make cinematographic films, is vulnerable to both chemical and biological deterioration. In the early 1970s, film archivists became aware of the serious decomposition of film material from the Cuban Institute for Cinematographic Industry and Arts (ICAIC), considered to be part of the Cuban CH. In 2013, Vivar and colleagues [8] published a study about the fungal deterioration of several color cinematographic film from this archive, dating from between 1980 and 1991. Optical microscopy images revealed the biodeterioration of film, showing the presence of fungi and to a lesser extent, pollen grains and mites. It presented varying degrees of aging in the studied film, with the loss of many film images as well as entire reels of film. Furthermore, to assess the type and extent of biodeterioration and the microorganisms involved, various ICAIC films were studied by SEM and ESEM. Results revealed the presence of fungi, although at different levels on the different films. In most cases, active fungi (as revealed by epifluorescence microscopy) indicated a future risk for the collection.

Rosado and colleagues [51] successfully combined SEM and enzymatic dehydrogenase measurement (molecular method) to identify the microbial growth causing the biodeterioration of the fresco paintings, dating from 1531, in the Santo Aleixo Church (Portugal). Similarly, Cennamo and colleagues [22] used optical and electron (SEM) microscopy to determinate the phototrophic biofilm formed by cyanobacteria and, to a lesser extent, algae growing on *Neapolitan Yellow Tuff* stones of walls in historical sites of Naples (Italy). SEM measurements confirmed the linkage between biofilm and lithic substrate and showed that cyanobacteria were closely adhered to the tuff whereas algae appeared superficially. After treatment to remove the microorganisms, SEM images showed that a significant reduction and morphological alterations of them had occurred. However, the authors also pointed out the limitation of light microscopy relative to other techniques such as molecular techniques, for identifying species without characteristic morphological features. Rakotonirainy and Dubar [52] observed structures that resembled filamentous fungi in 'foxing' spots (red or brown blemishes that emerge in the presence of fungi) on old paper, by using light microscopy and SEM. The species cannot be identified by this technique, and their viability cannot be determined by DNA analysis alone.

Along the same lines, Krakova and colleagues [39] reported that use of SEM observation and data from previous culture-dependent studies [53] together enabled the detection and characterization of the white biofilms in poorly lit areas of the Roman catacombs (Italy). In the same study site, Hsieh and colleagues [54] showed the effectiveness of endogenous protochlorophyllide precursor d-aminolevulinic acid (D-ALA), on red light exposure during a photodynamic antimicrobial chemotherapy (PDT) process (biocontrol method), to suppress phototrophic biofilms predominated by cyanobacteria and associated microorganisms. The viability of cells during the assay was monitored by CLSM imaging. SytoX Green® dye was used to mark the damaged cells in the biofilm. Similarly, in a study published by the authors 1 year later, the formation of reactive oxygen species (ROS) was measured by electron spin resonance (ESR) spectroscopy, and the physiological state of biofilm after light treatment was assessed by SytoX Green staining [55].

Finally, Troiano and colleagues [56] showed, using fluorescence microscopy, the dominance of fungi over bacteria in seven parchment manuscripts dating from the 1600s. In the same studied surfaces, they also demonstrated that the brown discoloration was not related to the microbial communities currently present in the manuscripts.

Light Methods, Fluorometric and Spectroscopic Techniques

Konkol and colleagues [57] fine-tuned a simple fluorometric method for detecting fungal biomass on a variety of CH materials: paper, canvas, and marble substrata. Two years later, the same authors applied the method to old paper [7]. They developed a rapid (less than 45 min) fluorometric assay to facilitate early detection of fungi (at an early stage of growth) on paper. The assay is based on the breakdown of fluorogenic 4-methylumbelliferyl (MUF)-labelled substrate (N-acetyl-beta-D-glucosamine (NAG) (MUF-NAG) by fungal beta-N-acetylhexosaminidase (NAGase), which permits detection of minute amounts of fungi on paper. *A. niger* ATCC® 10535 was selected for the study. A standard curve between the *A. niger* biomass and its fluorescence output was constructed ($R^2 = 0.9856$). The lower detection limit of the measurement method was established as 0.45 μg of *A. niger* biomass, and thus, the fungal presence could be detected before it became visible to the human eye. In a more recent study, Konkol and colleagues [58] developed this assay even further and embedded the MUF-NAG in Whatman 5 filter paper. This filter paper strip was then used to swab samples that were potentially colonized with fungi. After moistening the strip and

incubating it in the dark for 15–30 min, a fluorescent signal was obtained on the filter paper if fungi were present. The short time and the need for only a UV lamp to perform this assay make it an attractive option for conservators to use in settings that might not have access to sophisticated equipment. This assay can be semi-quantitative and fluorescence measured via image capturing software (e.g., the software commonly used for reading agarose gels).

Use of digital cameras and image processing is increasingly being incorporated into contact-type color measuring devices (for details, see [59, 60]). In a study carried out in 2007, but published more recently, Cámara and coworkers [10] used a field-oriented spectrophotometer to assess the efficacy of biocide treatments applied to several plots of a bioweathered dolostone quarry. In terms of CIELAB color coordinates [61] measured by the device, the ratio between the lightness and chroma color coordinates (L^*/C^*_{ab} diagram) was found to be the most appropriate to determine differences in color between the untreated and treated plots. In a later study on the biodeterioration of limestone, Miller and colleagues [62] used in vivo chlorophyll-a fluorescence and digital image analysis (both of which are easy-to-handle, portable, non-destructive, and non-contact techniques) to estimate microalgal biomass and quantify both epilithic and endolithic cover in artificially colonized limestone samples. In a previously mentioned study, Coutinho and coworkers [4] analyzed the biofilm-covered area of artistic tiles from Pena National Palace (Sintra, Portugal) by digital image analysis. Digital images of different colonized glazed tiles were subjected to PCA, to facilitate identification of the biofouled area, following the method proposed by Miller and colleagues [62]. The technique proved successful and provided information about the whole samples. Similarly, in 2009, Gazzano and colleagues [63] developed two new procedures, based on image analysis through color-based pixel classification, to quantify epilithic and endolithic lichen from stone. Both methods were successfully tested on different materials from CH (viz. marble, travertine, and mortar) colonized by different lichen ecology, which give rise to different coverage levels.

The microbial communities present on the wall surfaces of Roman catacombs (Italy) have been extensively investigated since 2001, when the deteriorative role of cyanobacteria communities (which occurred in the wake of the opening of the catacombs to visitors) began to be studied within the framework of the European project Cyanobacteria ATtack RockS (CATS) [64]. During this time, strategies aimed at controlling and even reducing and/or eliminating growth of microbiota (primarily composed of cyanobacteria, a few eukaryotic microalgae, and in some cases also actinobacteria and/or fungi) have been designed, and the most appropriate type of artificial lighting has been installed inside the catacombs. These strategies include restricting the duration of lighting, changing the amount of light, and applying light strong enough to cause "photoinhibition" (which is observed as a reduction in photosynthesis in response to increasing irradiance, although the term may refer to any growth inhibition that is due to a change in light applied) or the light quality, by using different wavelengths of monochromatic LED light. Hsieh and colleagues [54] tested several monochromatic lights and found that red light (620–650 nm) was effective for inhibiting the growth of cyanobacterial isolates and biofilms, whereas blue light (460–480 nm) had the adverse effect of promoting rather than inhibiting biofilm growth. In a later study [55], the same authors found that red light (630 nm) was the most effective for inhibiting cyanobacteria rich in the phycobiliproteins phycocyanin and allophycocyanin and green light (520 nm) was effective for inhibiting species rich in phycobiliprotein phycoerythrin. White light was effective for controlling grayish and black cyanobacteria, while blue light (480 nm) again proved to be the least effective as it favored the formation of cyanobacteria-derived biofilms due to formation of ROS. A chlorophyll fluorometer was also used in the study to determine the effective quantum yield of photochemical energy conversion in the PSII reaction centers in response to light.

ATP Measurements

The adenosine triphosphate (ATP) bioluminescence assay is a widely used sensitive method for establishing microbial viability. In a previously mentioned study, Rakotonirainy and Dubar [52] successfully applied an ATP-based protocol in the specific case of the "foxing" spots (in which microorganisms are not visible) in books dating from the 1900s and 2000s. The ATP content of the colored spots was compared with that of clear zones in the book pages, and no ATP was detected in the clear zones. However, as the authors themselves pointed out, the method is invasive and the fragments of paper under analysis must be removed from the whole sample (book); the method is therefore not appropriate for routine detection of microorganisms. On the contrary, in another already-mentioned study, Troiano and colleagues [56] did not always find such differences between discolored and non-discolored surfaces in samples of stored parchment manuscripts dating from 1600s. The authors explained this by the fact that ATP assay kits are primarily designed for bacteria, and the predominant microorganisms in their samples were fungi; they also noted that cases in which ATP kits have worked reliably have been those involving artificially contaminated paper or cut fragments, such as in [52]. In both studies, ATP bioluminescence assay results confirmed that the discoloration was not related to current active microbial colonization as the primary source of damage. In the previously mentioned study carried out in the Luca Signorelli frescoes in St. Brizio Chapel, Cappitelli and colleagues [27] used ATP measurements to test the efficiency of two biocides based on benzalkonium chloride (*Neo Desogen* and *Metatin*) to correct

rosy discoloration. The results of the treatment, which proved successful, suggested that *Metatin* was the most effective biocide.

Biocontrol Methods

In the context of this review, a biocontrol method denotes the cleaning or removal of fouling substances using living organisms, as well as the microbial abatement using biocides and related products. In both cases, the process must respect the chemical-physical nature of the artwork's material and its aesthetic appearance. Integrating both approaches, biocleaning and biocide methods, Polo and colleagues [65] carried out a study in two oolitic limestone statues (Demetra and Cronos) from the second half of 1800s, found in the courtyard of the Buonconsiglio Castle in Trento (Italy), and that presented black crust and other discolorations of biotic and abiotic origin. First, the chemical alterations (due mainly to gypsum) were successfully removed using the sulfate-reducing bacterium (SRB), *Desulfovibrio vulgaris* subsp. *vulgaris* ATCC 29579. Subsequently, the biocide *Biotin N* was efficaciously applied for the reduction of dematiaceous fungi, cyanobacteria, and green algae, which are present in the black crust.

Biocleaning

Although microorganisms are commonly associated with negative effects to the integrity of buildings, materials, and structures (whether cultural heritage related or not), there is growing evidence that they can be used in a positive manner as cleaning agents in the biocleaning process (for details, see [66–69]). The first studies that showed the positive role of microorganisms in remediation/cleaning processes on CH date from the late 1980s/early 1990s. The application of this new technology is not limited to but consists mostly of using non-pathogenic, enzyme-producing bacteria. In many cases, anaerobic microorganisms, mainly SRB, were employed. However, despite some good results, there was a decline in interest in this topic shortly afterwards. At present, though, the biocleaning and biorestoration of CH have received renewed attention with the development of new microbial biotechnological approaches, and some examples are shown below.

Several studies have reported the successful role of hydrolase enzymes (lipase, protease, amylase) in the removal of biological patina from canvas, paper, and polychrome surfaces. The enzymatic approach has clear advantages compared with conventional chemical methods, according to users of the technique. In the study of Valentini and colleagues [70], the glucose oxidase enzyme was used to remove the biofilm present on the travertine and peperino samples, which have been collected in "Main Entrance" from beginning of 1800s and "False Ruins monument" dated around to 1830, both located in Villa Torlonia (Rome, Italy). The glucose oxidase enzyme is able to produce H_2O_2 from the catalytic reaction of the glucose. It works like a cleaning agent (bleach and biocide) due to its oxidizing properties. The cleaning efficiency in situ was highest toward the peperino surface; the authors suggest that it is due to the low porosity of the lithic material. Thus, despite being affected by the same biodeterioration phenomenon, travertine and peperino responded differently to the biocleaning treatment. Finally, the authors noted that the bleaching effect and the antimicrobial properties typical of the H_2O_2 were confirmed for a period of 3 years in the place under study, Villa Torlonia. Alfano and colleagues [71] presented another case study of biocleaning, addressing the removal of nitrate and sulfate salts from the sandstone (tuff stone) surfaces of the 1100s cathedral of Matera (Italy). Matera Cathedral is placed in its historical center called "Sassi," which has been considered a World Heritage Site by UNESCO since 1993. *Pseudomonas pseudoalcaligenes* KF707 (cultivated in aerobic conditions) and *D. vulgaris* subsp. *vulgaris* ATCC 29579 [65] cells (cultivated in anaerobic conditions), both nitrate- and sulfate-reducing bacteria respectively, were applied. Both bacterial strains were used separately and their consortium (1:1 *v*/*v*). Although it has been possible to eliminate the two different salts (nitrate and sulfate) simultaneously, the best results were obtained by separate applications of sulfate and nitrate-reducing bacterial microorganisms. Indeed, after 24 h, 55 % of the nitrate and 85 % of the sulfate deposits had been removed. Long-term on-site monitoring of the cleaned area (viz. after 8 and 72 months) showed that the nitrate presence had not increased, microbial growth had not been favored, and no significant change in the color had occurred during that time. Bosch-Roig and colleagues [72] also obtained interesting results when using a white cotton wool delivery system impregnated with *Pseudomonas stutzeri* bacterial cell suspension to clean nitrate salt efflorescence and animal glue from frescoes located on the Santos Juanes church in Valencia, Spain.

In 2011, Gioventu and colleagues compared the biocleaning technologies to chemical and laser treatments and concluded that the biocleaning method was the most satisfactory for the removal of sulfate from stone materials [73]. Lustrato and colleagues [74] removed organic matter from past restorations (e.g., thin layers of animal glue and casein) on the surface of the fresco "Stories of the Holy Fathers." Fresco by Buffalmacco Buonamico, dated from 1300s and located at the Monumental Cemetery in Pisa (Italy). Viable bacterial cells of *P. stutzeri*, A29 strain, were applied for periods of 2 h for this purpose. Bacterial treatment was performed after a preliminary cleaning with (i) water and brush, (ii) two treatments of fixing, and (iii) two chemical cleanings failed to eliminate the large quantity of restoration material present in the fresco. Bioremediation using the A29 strain of

P. stutzeri was highly successful and rapid. Moreover, viable cells were not present in the fresco after biological treatment.

Troiano and colleagues [75] demonstrated the synergic effect of combining chemical and biological treatments for the cleaning of stone artworks. *D. vulgaris* subsp. *vulgaris* ATCC 29579 [65, 71], coupled with a soft non-ionic detergent *Tween 20* (applied before the biological treatment), was tested on a stone column affected by black crusts and subsequently on a one-century-old artistic marble statue weathered by sulfate-based crusts and gray deposits (Fig. 3). The results indicate that a single application of Tween 20 softens the black crust, helping in the biocleaning process by reducing the cleaning time and the number of treatment applications. It was later established that contact times of 22 h in successive cycles were required for the complete removal of black crust.

Mazzoni and colleagues [76] carried out a case study to clean deposits—classified "hard-to-remove" by curators—from mural paintings of the end of the 1500s in the loggias (exterior galleries) of the Casina Farnese on the Palatine Hill (Rome, Italy). The deposits were composed of gypsum, calcium oxalate dihydrate (weddellite), calcium carbonate, apatite, and a protein compound (most likely aged casein). *Cellulosimicrobium cellulans* TBF11E, *Stenotrophomonas maltophilia* UI3E, and *Pseudomonas koreensis* UT30E bacteria were applied in of three ways: singly (which produced the best results), in a consortium, and in succession. The strain TBF11E removed the inorganic (gypsum and carbonates) darker layer, UI3E dissolved the protein brownish layer, and UT30E removed the mixed (phosphates and proteins) deposits. Contact times of between 24 and 48 h were established.

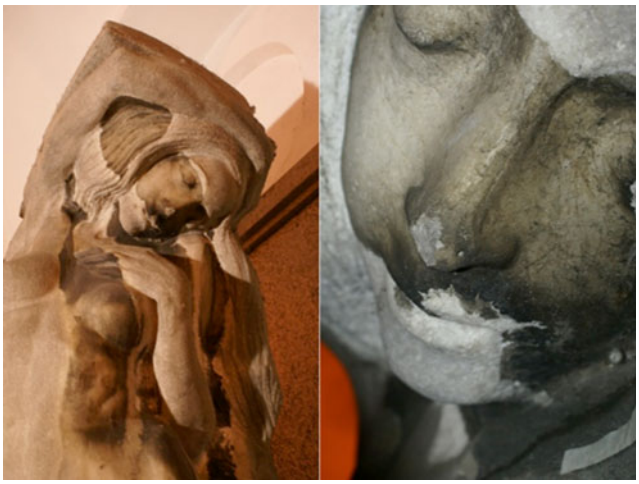

Fig. 3 Marble statue artwork, showing sulfate-based crusts and gray deposits. The statue, which is a funeral monument, made in 1921 by Lina Arpesani in Milan (Italy), was successfully cleaned by a combination of chemical and biological treatments [75]. Photos: Prof. Francesca Cappitelli (Università degli Studi di Milano, Italy) and SME Micro4You (Italy)

Biocides

Before the use of one biocide or related product, it is necessary that the microbial ecology of the target community should be fully characterized. In more recent years, the trend has been the search for natural biocides (for details, see [77, 78]). The use of antagonistic organisms or their metabolic products against the biodeteriorating agents offers a new approach. It is not harmful to human health, has a low environmental impact, is highly selective, and is available at low cost. Some examples are reported below.

Essential oils of many plants are known to have antimicrobial activity. Borrego and colleagues [79] used essential oils of plants as biocides against fungi and bacteria isolated from the National Archive of the Republic of Cuba (Cuba) and the Historical Archive of the Museum of La Plata (Argentina). Seven essential oils of the plants *Pimpinella anisum* L. (anise seed), *Syzygium aromaticum* L. (clove), *Cuminum cyminum* L. (cumin), *Allium sativum* L. (garlic), *Laurus nobilis* L. (laurel), *Citrus sinensis* (L.) *Osbeck* (orange sweet), and *Origanum vulgare* L. (oregano) were analyzed. The bacterial strains used were *Bacillus polymyxa*, *Bacillus cereus*, *Bacillus thuringiensis*, *Bacillus* sp., *Enterobacter agglomerans*, and *Streptomyces* sp. The fungal strains were *A. niger*, *Aspergillus clavatus*, *Penicillium* sp., and *Fusarium* sp. The oils exhibit different antibacterial effects. Thus, the essential oil of clove showed positive activity against *B. cereus*, *B. thuringiensis*, and *Streptomyces* sp. The garlic oil, at 25 %, was effective against *B. polymyxa*, *E. agglomerans*, and *Streptomyces* sp. and to a lesser extent against *Bacillus* sp. and *B. polymyxa*. Anise oil, however, had no antimicrobial activity on bacteria. Against fungi, anise, clove, garlic, and oregano oils showed the best antimicrobial activity, whereas orange sweet and laurel oils were ineffective. Laurel oil showed low antibacterial and antifungal activity.

Sasso and colleagues [80] tested secondary metabolites from *Solanaceae* extracts (glycoalkaloids), *Burkholderia gladioli* pv. *agaricicola* (Bga) ICMP 11096 strain and Bga cell-free filtrate as natural biocides against microorganisms isolated from two bridges located in Potenza and in Campomaggiore (Italy). Glycoalkaloid extracts inhibited all bacterial strains tested, while the Bga broth and the cell-free filtrate were more selective, especially against bacteria belonging to *Firmicutes phylum*. Antifungal activity was less evident, probably due to the structural complexity of fungi, according to the authors. Stupar and colleagues [81] tested the antifungal activity of *O. vulgare* L., *Rosmarinus officinalis* L., and *Lavandula angustifolia* essential oils and biocide benzalkonium chloride against fungi isolated from stone (*Bipolaris spicifera* and *Epicoccum nigrum*) and wooden substrata (*A. niger*, *Aspergillus ochraceus*, *Penicillium* sp., and *Trichoderma viride*) of cultural heritage objects in Serbia. The oil of *O. vulgare* and the biocide benzalkonium chloride

displayed the strongest antifungal activities followed by *R. officinalis* and *L. angustifolia* essential oils. The fungus that was most susceptible to essential oil treatments was *E. nigrum*.

The effects of usnic acid, norstictic acid, and parietin, secondary metabolites produced by saxicolous lichens, were investigated by Gazzano and colleagues [82]. In vitro tests against commonly occurring microcolonial fungi (*Coniosporium apollinis*, *Coniosporium perforans*, *Coniosporium uncinatum*, *Phaeococcomyces*-like sp.), coccoid cyanobacteria (*Chroococcus minutus*), and green algae (*Scenedesmus ecornis*) showed that all the three metabolites (approx. 10^{-2} mM) inhibited the growth of the tested species, displaying an effect similar to that of the control benzalkonium chloride. Their findings propose that lichen secondary metabolites can be used to abate biofilm-forming microorganisms. Furthermore, it is important to note that their results showed that chromatic alteration caused by lichen metabolites to a white marble was negligible, thus making these secondary metabolites potential natural sources for the control of microorganisms on stone materials in the preservation of CH.

Outlook and Future Trends

The goal of studying microorganisms on objects of historic and cultural significance is to identify and characterize their deterioration potential in order to prevent them from causing damage. Therefore, it is of paramount importance that the methods used provide a holistic picture of the ecology of the microorganisms in their environment.

The usefulness of methods recently used on CH materials is demonstrated in this review paper; however, there are also some drawbacks to these methods. For instance, as previously stated, no information about metabolic potential can be obtained on the basis of ribosomal DNA-based analysis. Therefore, it cannot be used as the sole approach for microbial community characterization. Troiano and colleagues [83] claimed that the molecular approach can be time-consuming, requires skilled personnel, and is often expensive [84]. A major intrinsic limitation of non-invasive techniques is that it is not possible to collect microorganisms growing in the substratum that do not produce emerging structures [28]. The culture-based approach also greatly limits the microbiota that can be studied. Thus, microbial studies based on cultivation strategies are not reliable on their own because they yield only a limited fraction of the present microbial diversity. However, we note that this varies greatly between microorganisms as well as the types of microbiological growth media used. Thus, while cultivation methods generally recover less than 1 % of the total amount of bacteria present in environmental samples, the recovery rate for fungi is estimated to be more than 70 %. Culture-based approaches are therefore still extremely useful in mycology. It is impossible to study the physiological functions of microorganisms by the sole use of molecular techniques. It is therefore recommended that a combination of microscopy, molecular techniques, and cultivation techniques be used to assess the diversity and ecology of microorganisms on CH.

The use of integrated microbiological risk management, supported by well-managed information, is crucial in the field of cultural heritage, in which human and financial resources are often limited. A combination of rapid, invasive, and non-invasive methods has generally proved effective for preserving CH from biodeterioration, thus ensuring long-lasting conservation. As this review attempts to illustrate, prevention is the most practical answer to the problem of biodeterioration caused by microbial activity on CH materials. Thus, studies involving preventive conservation should aim to maximize information and minimize the volume of sample consumed and should also aim to develop improved instruments to favor the use of non-invasive techniques [85].

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