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REVIEW ARTICLE

An overview of techniques for the characterization and quantification of microbial colonization on stone monuments

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Abstract Biodeterioration can be defined as any undesired change of the properties of a material caused by biological activity of living organisms. The biodeterioration of stone materials is related to the production of pigments (aesthetic action), to cell metabolism (biochemical action) and to the mechanical action of the biomass colonizing the material during its growth (physical action). Quantification of the sessile biomass and characterization of microbial communities colonizing stone are essential first steps to ensure the diagnosis of biodeterioration processes and to implement control strategies and appropriate treatment. Different destructive and non-destructive approaches can be used to sample stone specimens from monuments: scraping, swab using, and cutting. Different analytical methods can be used depending on the type of microorganism sought: determination of chlorophyll content and color analysis for pigmented microorganisms; measurement of in situ physiological activity of surface microcolonies by applying fluorogenic substrate analogues or confocal laser scanning microscopy observations after CTC staining for active biomass; scanning or transmission electron microscopy observation for biofilm visualization; enzyme-linked immunosorbent assay for the investigation of both microorganisms that can and cannot be cultured; classical microbiological methods, which consist in cultivation of microorganisms on synthetic media; and molecular methods for the study of microbial biodiversity based on the polymorphism of molecular markers using PCR, hybridization, classical or high throughput sequencing. The aim of this review is to present basics of the different biodeterioration mechanisms and to focus on the main techniques that can be used to characterize and quantify the biodeterioration biomass.

Keywords Biodeterioration · Stone · Monument · Microorganisms · Biofilm

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Introduction

The deterioration of stone material runs continuously all along the life of a monument and represents a significant loss of our cultural heritage. The extent of stone deterioration depends on physicochemical environmental factors, on the composition and the nature of the stone material itself, and on biological factors. These last factors are responsible for the so-called biodeterioration, which is defined as "any undesirable change in the properties of a material caused by the vital activities of organism" (Hueck 2001). The European standard EN15898 defines the main general terms used in the field of conservation of cultural property (2011). In this European standard, the terms deterioration and decay are defined as "gradual change in condition that reduces significance or stability", and weathering as "alteration due to exposure to outdoor environment". The economic costs of biodeterioration have been recently discussed by Allsopp (2011), but the cultural and historical value of many paintings, books, and monuments are inestimable and, thus, cannot be expressed merely in terms of money (Sterflinger and Piñar 2013). Microorganisms that play a potential role in biodeteriorative processes are autotrophic and heterotrophic bacteria, fungi, algae, lichens, and occasionally protozoa (Gómez-Alarcón et al. 1995a, b; Tomaselli et al. 2000; Urzi 2004; Gaylarde and Gaylarde 2005; Cutler and Viles 2010; Sterflinger 2010). This

consortium of heterogeneous microbial species forms a biofilm where microbial cells are embedded in extracellular polymeric substances (EPS) (Gorbushina 2007). The microbial colonizers are called epilithic when they are located on top of the rock. Microorganisms living inside the rock within cracks and fractures, or in the pore space of sandstone or granites are termed endolithic (Golubic et al. 1975, 1981). Microorganisms colonizing the depths of the stone can occupy different ecological niches. They are called chasmoendolithic and cryptoendolithic when they live in preexisting fissures and cavities, respectively, and euendolithic when they live in internal zones made by organisms, which are themselves capable of actively penetrating the rock substrate (Golubic et al. 1981). Several types of epilithic and endolithic microorganisms are usually observed on stone monuments (de los Ríos et al. 2004). Climatic conditions, nature of the substrate, and the duration of exposure of the surface to air greatly influence the microbial composition of these communities (Gorbushina and Broughton 2009). After surface stone conditioning by microorganisms colonization, macroorganisms such as insects and plants can also be recovered and may also have a biodeteriorative impact. Microbial biofilms can cause various damage on stone surfaces: (i) discoloration caused by pigments released from, or contained within, the microorganisms (aesthetic action), (ii) biocorrosion of the mineral support by acidolytic and oxidoreductive corrosion processes generated by products of the microbial metabolism (biochemical action), and (iii) physical damage caused by the mechanical action of the biomass colonizing the material during its growth (physical action).

Physicochemical environmental factors and biological factors participate together synergistically or antagonistically in the process of stone deterioration. The colonization of the outdoor surface of monuments is heterogeneous depending on the weathering level of the studied area (de los Ríos et al. 2009; Qi-Wang et al. 2011). Biodeterioration is sometimes regarded as a process that occurs after physicochemical alteration of stones. This theory suggests that microbial colonization is facilitated by inorganic agents and processes that condition the surface of the stone by altering its structure and enriching it in nutrients. However several studies have clearly shown that biodeterioration can be detected early after the exposure of the stone (de la Torre et al. 1991; Warscheid et al. 1996). Moreover, laboratory experiments simulating in situ conditions found on monuments have shown that the presence of microbial populations greatly accelerates the rates of deterioration of stone and concrete surfaces (Papida et al. 2000; Favero-Longo et al. 2009; George et al. 2013). Indeed, the combination of physical environmental factors and biological processes significantly enhances the extent of decay when compared with the physical environmental factors or biological agents acting alone.

Quantification of the sessile biomass and characterization of microbial communities colonizing stone are an essential first step to ensure the diagnosis of biodeterioration processes and to implement control strategies and appropriate treatments. Different methods can be used to quantify and describe the sessile biomass colonizing the surface of stone monuments, depending on the type of microorganisms that are looked for, such as autotrophic or heterotrophic, microorganisms that can or cannot be cultured (Bartosch et al. 2003; Prieto et al. 2004; de los Ríos and Ascaso 2005; Berdoulay and Salvado 2009; Qi-Wang et al. 2011; Cutler et al. 2012). This review is not intended to be exhaustive but to provide the essential knowledge concerning the different biodeterioration mechanisms with a particular focus on the techniques used to characterize and quantify the biodeteriorative biomass.

The impacts of environmental factors on microorganisms colonizing stone materials

Biodeterioration depends on many physicochemical environmental factors. Relative humidity, temperature, wind, rainfall, sunlight, and air pollution are weathering factors for stones that determine biodeterioration processes. The main cause of damages occurring to building stones is linked to the action of water that causes or aggravates the effects of all other factors. The penetration of water into the pores of the materials plays a central role in the deterioration of stones. During its crystallization at low temperature, it can increase in volume and exert physical pressure within the material. However, the damage induced is far more complex that the expansion of water upon crystallization which, as appears logical, is commonly accepted. Indeed, the deterioration is due to the phase change, similar to salt crystallization (Taber 1929; Taber 1930). In addition, water can carry organic and inorganic compounds that can dissolve the mineral matrix. Water is also a substrate essential to life, and, thus, it plays a key role in the development of the microbiota, which colonizes the stone. The relative importance of the different types of microorganisms varies from one continent to another, depending on the climate and microclimate surrounding the monument. A statistical analysis of the results from the study of the major biomass in 230 biofilms from buildings in seven Latin American (northern Argentina, Bolivia, Brazil, Colombia, Equador, Mexico, and Peru) and six European countries (Czech Republic, England, France, Italy, Poland, Portugal, and Spain) revealed that in Europe, algae are dominant followed by cyanobacteria while in Latin America, cyanobacteria are dominant followed by fungi (Gaylarde and Gaylarde 2005). Of course, differences in biodiversity within biofilms can be observed between different sites on the same continent as a function of altitude, distance or not from the tropics, etc., but the dominant type of microorganism (algae in Europe,

cyanobacteria in Latin America) is unchanged for each continent. Since cyanobacteria are resistant to desiccation and high solar irradiation, their growth on stone surfaces is favored in tropical countries (Gaylarde and Gaylarde 2005; Gaylarde et al. 2012). Proteobacteria (*Stenotrophomonas maltophilia* and *Methylobacterium radiotolerans*) and Firmicutes (*Bacillus* sp., *Bacillus niacini*, *Bacillus sporothermodurans*, *Lysinibacillus fusiformis*, *Paenibacillus* sp., *Paenibacillus panacisoli*, and *Paenibacillus zanthoxyli*) are also largely present in these biofilms (Gaylarde et al. 2012). Fungi have been shown to form extensive biofilms on areas previously colonized by autotrophic and heterotrophic microbial biofilms on sandstone at Bayon temple, Angkor Thom, Cambodia (Hu et al. 2013). In subtropical settings, many fungal species belonging to the *Ascomycota* can be isolated from limestone (Gómez-Cornelio et al. 2012). Several species of the common genera *Cladosporium*, *Alternaria*, and *Taeniolella* are abundant. Rare taxa are represented by the genus *Elasticomyces*, and several species of genera *Hyalodendron*, *Monodyctis*, *Papulospora*, *Curvularia*, and *Septoria*. Seasonal variations in rainfall affect the community composition of fungi colonizing the limestone in subtropical areas (Gómez-Cornelio et al. 2012). In temperate climates subject to regular rainfall, there is a high biodiversity in ecosystems colonizing the surface of monuments, with a higher bacterial biomass in winter and early spring than in summer and early autumn (Tayler and May 1991; Warscheid 2003). In Scotland, Actinobacteria belonging to the genus *Streptomyces* and *Arthrobacter* and fungi belonging to *Cladosporium*, *Penicillium*, and *Phialophora* genera are common on the outer surface of monuments (Suihko et al. 2007). Lichens and bryophytes are the dominant colonizers of rock-art on schist in the Côa Valley Archaeological Park, northeast of Portugal (Marques et al. 2014). The most common lichens in this environment belong to the species *Aspicilia contorta* subsp. *hoffmanniana*, *Caloplaca subsoluta*, *Collema ryssoleum*, *Lecanora pseudistera*, *Lepraria* sp., *Peltula euploca* and *Xanthoparmelia tinctina*. In the center of Italy, among the many lichens colonizing monuments, lichens belonging to *Aspicilia*, *Caloplaca*, *Lecanora* and *Lepraria* sp. have also been previously observed on tombs walls and/or on calcareous and siliceous walls of roman buildings (Seaward et al. 1989). *Lecanora muralis* has been shown to be a highly successful lichen in urban environments (Seaward, 1982). Strong evidence suggests that recent environmental changes have been conducive to increasing detrimental invasion by certain aggressive lichen species (Seaward et al. 1989). An index of lichen potential biodeteriogenic activity has been developped in order to provide a tool to evaluate the lichen impact on stonework (Gazzano et al. 2009). Climate changes in Europe are predicted to be conducive to an increase in temperature and precipitation in northern areas of the continent, which would lead to a higher accumulation of biomass at the surface of monuments (Gómez-Bolea et al. 2012). Otherwise, a significant reduction in precipitation is expected in southern areas of Europe, associated with a lower biomass accumulation in such areas. Tropical climates increase biodeterioration processes due to temperature and humidity.

Air pollution, mainly due to the combustion of fossil fuels, has been widely recognized as the main factor responsible for stone decay in urban area (Camuffo 1992). Indeed, many industrial pollutants affect the building stone in cities, and it is recognized that the damage of the building stone increases if the air contains sulphur and nitrogen oxides (Allen et al. 2000). Acid rain, caused by the solution of nitrogen and sulphur oxides in water and the formation of aggressive sulphuric and nitric acids, is responsible for deterioration of monuments (Bityukova 2006). In addition to chemical deterioration of stones, pollutant may affect microbial colonization and biodeterioration. The atmospheric pollution reduces the diversity of flora, by inducing the disappearance of the most sensitive species (in particular many lichens) and selecting resistant species among cyanobacteria, fungi, and also lichens (*Lecanora muralis* and *L. dispersa*), but it does not substantially reduce the total biological cover (Caneva et al. 1995). Microorganisms present on the stone surface can absorb gaseous and particulate air pollutants (such as SO_2) leading to the sulphation and decay of the stone due to crystallization of sulfate and other salts (Schiavon 2002). Moreover, the early presence of biofilms on exposed stone surfaces accelerates the accumulation of atmospheric pollutants (Steiger et al. 1993; Witteburg 1994). Thus, the exposition of monuments to air pollution, contributes to their deterioration by direct action but also indirectly via microorganisms (Warscheid and Braams 2000; Tecer and Cerit 2002; Herrera and Videla 2004; Nuhoglu et al. 2006).

Beside physicochemical environmental factors, the composition and the nature of the stone material itself control its bio-receptivity and, therefore, impact biodeterioration processes as well as the state of conservation (Miller et al. 2012). Microorganisms usually colonize different materials of a monument and/or different objects and surfaces of a site (limestone, cement, mortar, concrete, brick) (Seaward et al. 1989; Gaylarde and Gaylarde 2005). Thus, it has to be kept in mind that the impacts of environmental factors will vary according to the mineralogy, the porosity, the surface roughness, and the capacity to take up water and organic substances of a material (Krumbein and Gorbushina 1995). Thus, the colonization of the outdoor surface of monuments is heterogeneous depending on the weathering level of the area studied. On brick monuments encountered in China, bacterial communities are different in areas with different weathering states, and the abundance of bacterial communities positively and significantly correlates with the extent of weathering (Qi-Wang et al. 2011). Weathered areas are colonized by bacteria like *Bacillus*, *Massillia*, *Brevibacillus*, *Glacial ice bacterium*,

Acinetobacter, *Brachysporum*, and *Achromobacter* that contribute to biodeterioration of the surface. In central Spain in Segovia, church carbonate building rocks are mainly colonized by fungi (lichenized and non-lichenized) at sites showing signs of biodeterioration (de los Ríos et al. 2009). The study of different archaeological sites of southern Spain showed that mortar is a building material easily colonized by a diversity of calcicolous and rather nitrophilous lichens (Ariño et al. 1997).

Biodeterioration phenomena

Microorganisms organize themselves in the form of biofilms on the surface of the stone by producing exopolymeric substances (EPS) forming a matrix, also called slime, which encompasses the cells (Fig. 1). Since microbial biofilms make close contact with the surface, they are thought to cause significant deterioration of the stone (de los Ríos et al. 2009). The EPS matrix serves as an intermediate for the adhesion of bacteria to the mineral surface and plays a crucial role in the phenomenon of biodeterioration (Sand and Gehrke 2006). The quorum sensing system is involved in the formation of biofilm and bacterial attack of minerals (Jerez 2008). Biofilm formation gives many advantages to the colonizing microbiota as increased accessibility to nutrients, and protection against stress (biocides, UV radiation, drying). EPS of the matrix can produce mechanical stresses on the stone through the pores of the mineral structure (Dornieden and Gorbushina 2000; Guiamet et al. 2013). In inducing modifications of the distribution of pores of the stone, these alterations can modify water circulation within the material and its sensitivity to temperature variations (Garty 1990; Warscheid et al. 1996). Moreover, the presence of biofilm on stones accelerates the accumulation of pollutants in the material (Steiger et al. 1993; Witteburg 1994). Some adsorbed pollutants can serve as nutrients, which increase the aggressive biological activity against the stony support (Nuhoglu et al. 2006). Several biofilm and cellular components such as organic acids, anionic exopolymer, amino acids, and peptides and sugars acids are metal ions chelators (Jenneman et al. 1985; Kaplan et al. 1987; Tanner et al. 1991). The ability to chelate metal ions helps to dissolve ions out of minerals, even acid-insoluble ones, inducing their corrosion. Thus, EPS can concentrate metallic ions by complexing with uronic acid or other residues, thus, favoring oxidation (Kinzler et al. 2003; Sand and Gehrke 2006). The sulfur oxidizing bacteria produce extracellular EPS that are involved in adhesion to the mineral surface and interactions between microorganisms and minerals favoring oxidation of sulphide minerals (Sand and Gehrke 2006). The EPS matrix of the sulfur oxidizing bacteria *Thiobacillus ferroxidans* consists essentially of neutral sugars and lipids (Kinzler et al. 2003). An EPS cluster of five genes controls the biosynthesis of the matrix in *T. ferooxidans* (Barreto et al. 2005). In addition, the phospholipids of cellular origin, by their emulsifying action, are involved in the biodeterioration of insoluble compounds such as pyrite (Shively and Benson 1967).

Chemical, microbiological, and mineralogical techniques have revealed that colored coatings on monument surfaces can result from biomineralization (Garcia-Vallès et al. 1997). The coatings consist of mainly biogenic minerals (calcite, several oxalates, and phosphates) and their fabric and composition is independent of the underlying rock. The origin of the crusts or patinas has been attributed to bygone (sub-fossil) microflores, which colonized the surface of the limestones and marbles of the monument. Microflora living on and in the patina is not responsible for its formation; it can even modify and destroy partially the patina. Some current biological colonization can be seen with the naked eyes. Thus, the appearance of pigmented areas (greening, blackening, etc.), mainly due to photosynthetic microorganisms (algae, cyanobacteria, and lichens), is a type of biodeterioration (Gaylarde and Gaylarde 2005; Cutler et al. 2013a, b). Photoautotrophic organisms grow easily on inert supports poor in organic matter such as stone (Fig. 2). Algae are very often the first colonizers that form a visible biofilm on the surface of the material. Nevertheless, heterotrophic microorganisms can act as first colonizers in the areas rich in organic nutrients such as organic pollutants (Zanardini et al. 2000). Even if the stone coverage by photosynthetic microorganisms is thought to protect some

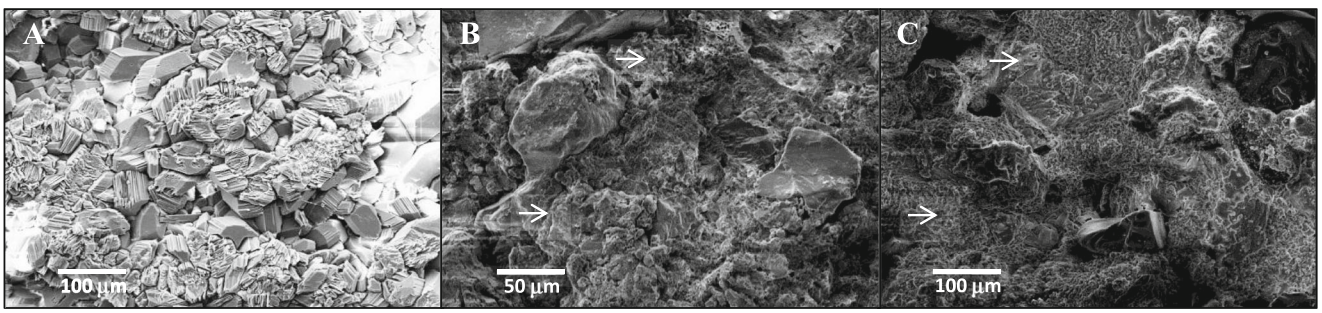

Fig. 1 Scanning electron microscopy view of biofilms on the surface of lutetian limestone. **a**, limestone lutetian uncolonized. **b**, biofilm on the surface of limestone Lutetian ruins of the abbey of Chaalis, France. **c**, limestone lutetian after 65 days of colonization by *Thiobacillus thiooxidans* in a sulfur-enriched environment. The arrows indicate areas of high colonization

Fig. 2 Stone surface of a sculpture from the park of Courances castle France (**a**), and of pillars from the "Les Salles-Lavauguyon" church France (**b**), colonized by algae. The arrows indicate areas of high colonization

monuments from weathering and air pollutants (Carter and Viles 2005; Concha-Lozano et al. 2012), it constitutes visual impairment that can also be associated with more severe alterations of the structure of the stone material (Danin and Caneva 1990). Other waves of colonization by heterotrophic bacteria having more complex nutritional needs may occur due to the presence of photoautotrophic microorganisms. This more complex microbial consortium can develop a more aggressive activity .

Surface temperature and moisture are the two major factors impacting the overall distribution of green algal biofilms on the surface of a monument (Cutler et al. 2013b). Indeed, greening occurs more rapidly and is more intense on north than on south side of historic monuments in Ireland (Adamson et al. 2013). Orientation of buildings plays a key role in the spatial distribution of colored patinas associated with the presence of the alga *Trentepohlia* and the coccoid cyanobacterium *Gloeocapsa* on Mayan monuments: red patinas, due to the presence of carotenoids (beta carotene and luteine), are predominantly associated with North-facing and East-facing walls, while extensive black patinas are mainly observed on South-facing and West-facing walls (Ortega-Morales et al. 2013). Algae and bacteria have also been associated with the appearance of an orange color on stone monuments (Urzı and Realini 1998). An orange color can be due to the development of the green algal genera *Trentepohlia* and *Haematococcus*, which contain high levels of carotenoid pigments (Adamson et al. 2013). Stone surface texture influences bioreceptivity and surface irregularities, such as roughness and porosity, that may provide microsites for algal colonization (Guillitte 1995; Barberousse et al. 2006; Giovannacci et al. 2013). Nevertheless, algal greening of sandstones is mainly related to climate and atmospheric chemistry and secondarily to stone texture. Indeed, coccoid cyanobacteria belonging to the genus *Chroococcidiopsis* are the major colonizers of discoloring biofilms from Cambodian temples (Gaylarde et al. 2012).

Blackening can also be due to fungi such as *Phialophora* sp., *Cladosporium tenuissimun*, and *Aspergillus* (Diakumaku et al. 1995; de los Ríos et al. 2009; Hu et al. 2013). This is mainly due to the production of melanoid pigments (Gorbushina et al. 1993). Blackening associated with fungi development is enhanced by climatic conditions (high irradiation, alternating cycles of extreme wetting and drying) and other environmental factors such as air pollution (Diakumaku et al. 1995). A black color can also occur by trapping particulate pollution in microbial exopolymers. The black crust coating monuments in urban environments supports microbial growth, as several nutrients required are available from external inputs (Gómez-Alarcón et al. 1995a). Beyond the unsightly appearance of this change in the color of the stone, the black areas will absorb more light energy which increases physical stress induced by cycles of expansion / contraction associated with temperature changes (Sand et al. 2002).

Among chemical or biochemical mechanisms induced by microbial metabolism, the production of inorganic and organic acids represents a significant part in biodeterioration processes (Herrera and Videla 2004). Indeed, sulfuric acid and nitric acid of biological origin are mainly produced by sulfur oxidizing bacteria of the genera *Acidithiobacillus* / *Thiobacillus* / *Sulfobacillus* / *Sulfolobus* and by nitrifying bacteria of the genera *Nitrosomonas* / *Nitrobacter*, respectively (Sand and Bock 1991; Sand 1997). The Thiobacilli are acidophilic and fix carbonic acid. For the majority of them, they draw their energy from inorganic sulfur compounds and many organic compounds inhibit their growth. Some species such as *T. thiooxidans* and *T. ferooxydans* can grow at extremely low pH and play an important role in corrosive aggressiveness of the stone. The sulfur-oxidizing bacteria usually live as biofilm communities on mineral surfaces (Beech and Sunner 2004; Jerez 2008). Fungi can also perform the oxidization of sulfur compounds. The colonization of stone by sulfur oxidizing fungi can contribute to the biodeterioration process by acid production (Li et al. 2010). Nitrifying bacteria are less resistant to highly acidic pH. Most of them fix CO_2 and draw their energy from nitrogen compounds such as urea, nitrites, and nitrogen oxides (Baumgärtner et al. 1990). The inorganic acids attacks cause severe corrosion of the stone and promote the formation of salts (calcium nitrate and gypsum). The crystal growth of salts in the porous structures of stone also conducts to damage of the material due to phase changes (Steiger 2005). Many microorganisms produce carbon dioxide as an end product of their metabolism. Although it is a weak acid, CO_2 contributes to acid attack monuments. Thus, the CO_2 reacting with $CaO/Ca(OH)_2/CaSiO_2$ induces the formation of $CaCO_3$. This compound lowers the pH and thereby promotes cell growth and activity. This highlights the lowering of pH and induces an acid attack. By fermentative processes, most bacteria and microscopic fungi produce organic acids when they metabolize organic or inorganic

compounds. The acid attack by the organic acids is similar to that of inorganic acids (Palmer et al. 1987; Dilling and Cypionka 1990). Acidogenic *Fusarium* strains producing oxalic acid have been isolated from the weathered sandstone of the church of Carrascosa del Campo, Spain (Gómez-Alarcón et al. 1995b). In addition to the biodeterioration mechanism shared by inorganic and organic acids, some organic acids also act as complexing agents of metal ions, so their action has two components.

Physical biodeterioration corresponds to action of the growth and the movement of microorganisms that lead to the disruption of the material or to its distortion. Binding and growing of cyanobacteria in small cracks of the stone can induce direct pressure on the material, increased pressure by water and subsequent development of other microorganisms (Danin and Caneva 1990). This pressure can lead to the detachment of the superficial layer of the stone at the colonized area level. Besides growing on stone surfaces, lichens and fungi can cause deterioration of the stone by physical penetration (Fig. 3). Fungal hyphae are able to penetrate deeply beneath the stone surface, contributing to mechanical deterioration (Fig. 4). This penetration simultaneously allows the transport of water and nutrients through the stone, which facilitates the colonization of the interior of the stone by bacteria and concomitantly triggers biochemical deterioration (Gómez-Alarcón and de La Torre 1994). Filamentous bacteria such as *Actinomycetes* penetrate their substrate by mechanisms similar to those employed by fungi (Scheerer et al. 2009). Penetration of masses of hyphae on the surface of granitic rocks also is conductive to biogeophysical weathering (Wierzchos and Ascaso 1994). Moreover, the hyphal penetration along intrinsic discontinuities of carbonate rocks is a relatively fast phenomenon (Favero-Longo et al. 2009).

Fig. 3 Wall from Les Salles-Lavauguyon church France, colonized by fungi. The arrows indicate areas of high colonization

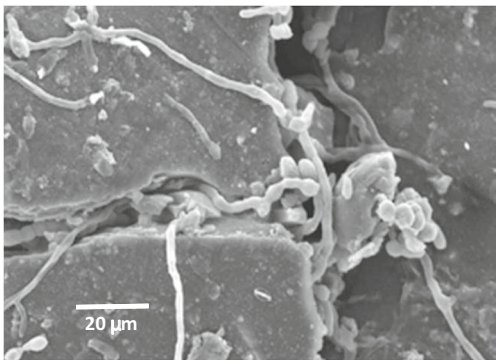

Fig. 4 Scanning electron microscopy view of the penetration of fungal hyphae under scales of mural painting

Techniques used to quantify and describe microbial communities colonizing stone materials

A wide variety of techniques can be used to quantify and describe microbial communities colonizing stone materials. Methods for the study of rock-inhabiting microorganisms have been previously reviewed (Hirsch et al. 1995). Such non-destructive methods correspond to the washing, scraping, or printing off of rock surface microorganisms for further cultivation and identification, and also the measurement of in situ physiological activity of surface microcolonies by applying fluorogenic substrate analogues. For destructive studies the rock samples are collected aseptically and fragmented to release the microorganisms in growth media or used to realize electron microscopy observations. The commonly used microbiological and molecular techniques as well as recently developed tools are the subject of the following paragraphs and are summarized in Fig. 5.

Contributions of in situ microscopy (electron and fluorescence microscopy, sometimes in conjunction with X-ray energy dispersive spectroscopy) to the current understanding of stone biodeterioration have been previously reviewed (de los Ríos and Ascaso 2005). Scanning electron microscopy (SEM) can be used to demonstrate the presence of microorganisms on rock samples (Figs. 1 and 4). SEM in black-scattered electron emission mode is particularly interesting to observe the microbial-rock interface (Wierzchos and Ascaso 1994; de los Ríos et al. 2004). Nevertheless, the microorganisms seen on such preparations cannot be precisely identified because only morphological information is obtained. Moreover, desiccation and high vacuum applied to biofilm samples during conventional electron microscopy protocols deform the biofilm morphology. To avoid such artefacts, wet-mode environmental scanning electron microscopy (ESEM) can be used under a moderate vacuum and without biofilm drying (Little et al. 1991; Priester et al. 2007). Transmission electron microscopy is also frequently used to observe the cytological features of lithobiontic microorganisms (Ascaso and Ollacarizqueta 1991). The use of combined SEM and TEM

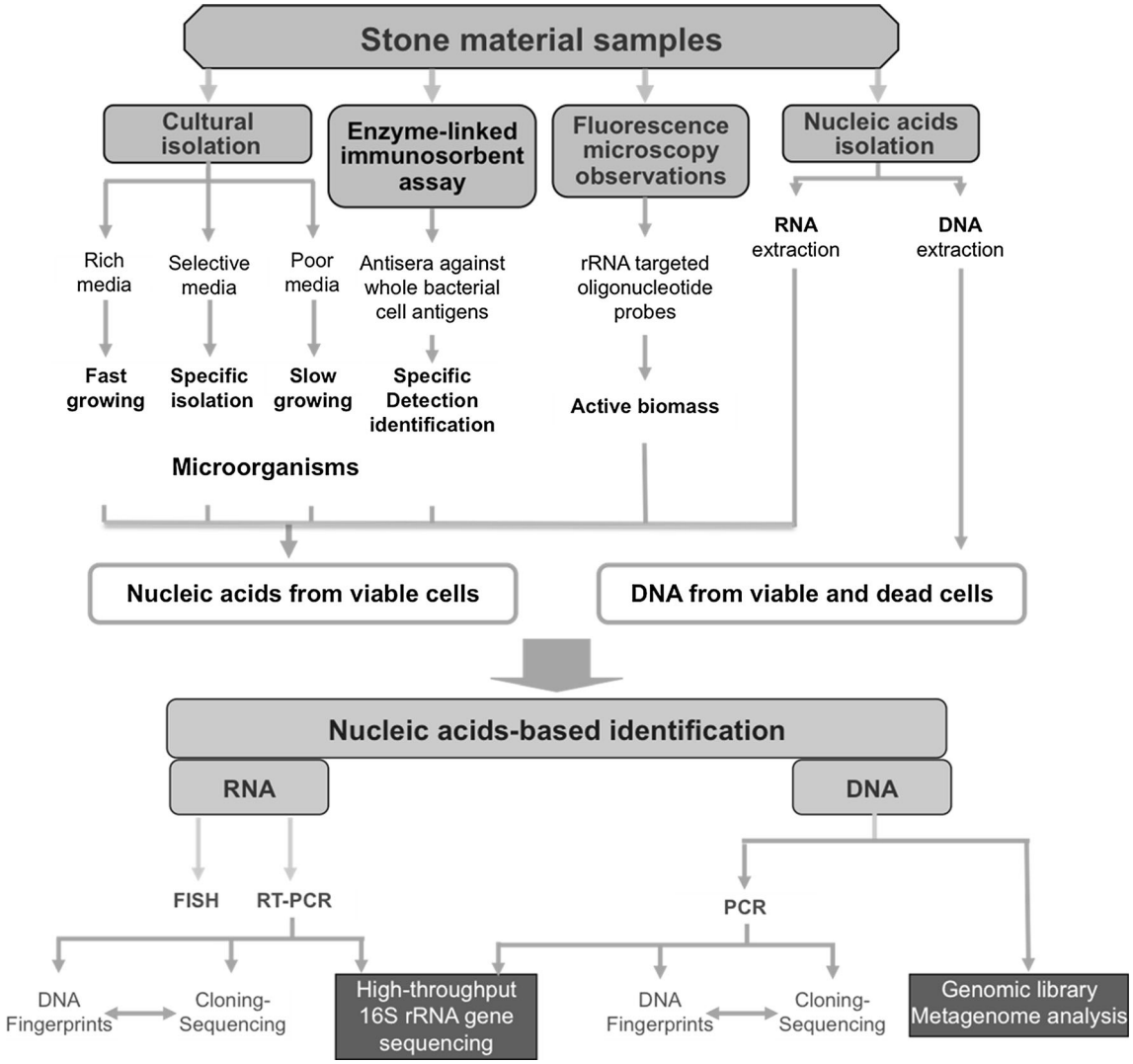

Fig. 5 Strategies for analysis of the microbial diversity on stone materials. Terms in black are commonly used strategies whereas terms in white represent recently developed tools that could be used to get more insight into microbial diversity and functions

brings out ultrastructural features inside the mycobiont, photobiont and/or other biological elements and also of their interaction with the stone surface (Ascaso et al. 2002). In addition to the analysis of the organisms involved in substrate biodeterioration, combined SEM and TEM gives the possibility of evaluating the efficiency of a biocide, without extracting the microorganisms from their rock microhabitat (Ascaso et al. 2002; Speranza et al. 2012). Checking the effects of biocide treatments on endolithic lichens can also be realised by epifluorescence microscope observations on cross-sections of treated and untreated samples (Tretiach et al. 2010). For chemoorganotrophic bacterial quantification, the combination of Confocal Laser Scanning Microscopy observations with CTC staining can be used as a fast method (Bartosch et al. 2003). One advantage of this microscopy technique is due to the small size of the samples required. Nevertheless, enumeration of total bacteria and active cells using fluorescence microscopy is not practical for large numbers of samples and only bacteria detached from stone particles can be easily counted. Thus, a fluorometric assay has recently been developped to monitor fungal biomass on a variety of cultural heritage materials non-destructively, and without the introduction of chemicals or solvents to the surfaces (Konkol et al. 2010). This assay measures the Beta-N-acetylhexosaminidase enzymatic activity specific for fungi using a fluorogenic labeled substrate and a microplate reader fluorometer. Assessing the microbial activity of stone samples from monuments can also be done by using triphenyltetrazolium chloride as a measure of dehydrogenase activity (Warscheid et al. 1990) or by measuring fluorescein diacetate hydrolysis releasing fluorescein dye, which causes active cells to fluoresce a brilliant yellow-green under ultraviolet light (Prieto et al. 2004).

For colonization associated with stone pigmentation changes, determination of chlorophyll content and the total colour difference in rock samples are two efficient methods (Dere et al. 1998; Prieto et al. 2004). A close correlation

between pigment content and color measurements enables formulation of predictive equations for estimating chlorophyll a and total carotenoid content of samples (Vázquez-Nion et al. 2013). Chlorophyll a fluorescence measurements can be used in combination with SEM and TEM images to evaluate the actions of biocides on endolithic microorganisms (Speranza et al. 2012). The limit of the chlorophyll content determination is that total extraction of chlorophyll is difficult to achieve. The colour measurement approach has several advantages: it is an in situ, non-destructive, and an on-site technique.

Microbiology studies carried out in the field of biodeterioration are mainly based on classical cultivation techniques. These techniques involve the isolation of microorganisms on solid culture media after a potential enrichment in liquid media and/or their detection and quantification using most probable number (MPN) techniques. Stone specimens used for microbiological incubating and counting can be taken by means of scraping off samples from the stone surface with different kind of tools like needles, microscalpels or scalpels (Nuhoglu et al. 2006), or using sterile swabs moistened in sterile saline (Ortega-Morales et al. 2013). A diamond-coated cutting disc can also be used to realize stone sampling (Bartosch et al. 2003). A non-destructive sampling technique for the analysis of microbial sessile populations is the use of sterile adhesive tapes (Gaylarde and Gaylarde 2005; Cutler et al. 2012). Tape sampling is straightforward, rapid, and cost effective. As a wide diversity of microorganisms can colonize stones, several types of culture media have been used. For example: Modified Bold's basal medium (MBBM) has been used for the isolation of green microalgae (Schumann et al. 2005), Chu and Detner media for algae and cyanobacteria (Polo et al. 2010), nutrient agar, Luria-Bertani and R2A for bacteria (Herrera et al. 2004; Suihko et al. 2007; Qi-Wang et al. 2011), *Thiobacillus* agar supplemented with bromocresol green to accelerate the detection and the enumeration of *T. thiooxidans* (Starosvetsky et al. 2013), and malt extract agar and potato extract agar for fungi (Piñar et al. 2009; Wiktor et al. 2009). Mycobionts of lichens can be inoculated on plates containing agarized, non-nutrient, mineral medium (Bold's Basal Medium, BBM) and incubated at 13–15 °C in the light for several months (Favero-Longo et al. 2009). Further transfer to agarized nutrient medium containing malt and yeast extracts, proteose peptone, casamino acids, and glucose can be done to perform identity checks by sequencing the ITS1-5.8SITS2 region of rDNA (Favero-Longo et al. 2009). The aerobic cultivable microbiota has been evaluated to be present on monuments in levels up to 10^5–10^7CFU g^{-1} (Suihko et al. 2007; Piñar et al. 2009; Qi-Wang et al. 2011). However, classical cultivating methods may overestimate the abundance of some microbial groups and underestimate the abundance of others (Laiz et al. 2003). For example, the presence of inhibitory and predatory microorganisms such as fungi, protozoa, and bacteria lead to an underestimation of cyanobacteria (Ward et al. 1990; Crispim and Gaylarde 2005). Moreover, it has been established that traditional cultural approaches reveal only 1 % of the microbial diversity present in samples from stone monuments; therefore, microbial investigations based on traditional approaches are not reliable in reflecting the microbial diversity present on stone surfaces (Ward et al. 1990; Herrera et al. 2009). Most of the "uncultivable" bacteria cannot develop on culture media because they are obligatory symbiotic or parasitic microorganisms, because the applied cultural technique used is not suitable for their growth or because they are at inactive stages. Even if cultural techniques are not suitable to evaluate the abundance and the diversity of microorganisms, efforts to develop new media and new culture approaches should be undertaken. Indeed, cultural techniques present great interest in generating isolates, which enable researchers to study their deteriorative potential in laboratory experiments (Qi-Wang et al. 2011). The investigation on stone of both microorganisms that can and cannot be cultured can also be done by enzyme-linked immunosorbent assay (ELISA) (Tayler and May 1994). ELISA allows rapid and specific detection of bacteria on stone in situ without the need for culturing, isolation, and subsequent identification. The main limitations of this technique are that it is necessary to have antibodies against the targeted microorganisms and that one does not reveal the unknown microorganisms.

As in many other environments, the use of molecular techniques in the field of biodeterioration has led to the identification of many more bacterial, archaeal, fungal, and algae species than cultural approaches do (Ortega-Morales et al. 2004; Ortega-Morales et al. 2005; McNamara et al. 2006; Suihko et al. 2007; Berdoulay and Salvado 2009; Laiz et al. 2011). Most of the studies dealing with stone biodeterioration are based on the analysis of ribosomal genes sequences. The small subunit ribosomal RNA gene (16S rDNA for bacteria and archaea and 18S rDNA for eukaryotes) is the most widely used molecular marker for studying microbial diversity. The identification of fungi to the species level has been mainly based on the analysis of the variable ribosomal DNA (rDNA) internally transcribed spacer (ITS) regions (Anderson et al. 2003). The analysis can be carried out by different kind of molecular techniques, such as fluorescent in situ hybridization (FISH) with ribosomal RNA targeted oligonucleotide probes, clone library analysis or community fingerprinting such as Denaturing Gradient Gel Electrophoresis (DGGE) and Single Strand Conformational Polymorphism (SSCP) (Dakal and Arora 2012). FISH applied on samples taken with adhesive tape strips is qualitative as well as quantitative and possesses a high sensitivity (La Cono and Urzì 2003; Cappitelli et al. 2007). Thus, FISH can be used to monitor microbial colonization of the same chosen surface after a specific period of time, and to look at interactions between different organisms, without being destructive for

the sampled surfaces. One limitation of FISH is the presence of phototrophic autoflorescent microorganisms and of inorganic material surrounding the bacteria that can be also fluorescent. Moreover, low ribosome content of some cells can underestimate the diversity of species observed. An additional problem can be due to the difficulty to make observations of thick microbial aggregates inside biofilms (Urzi and Albertano 2001). Higher fluorescence intensity of the targeted cells can be obtained by the use of several monolabeled oligonucleotides targeted to independent sites of the rRNA molecule (Müller et al. 2001). Using molecular techniques, Ortega-Morales et al. (2004), showed for the first time putative halophiles of the genera *Halothece* and *Salinibacter*, along with photosynthetic bacteria related to the *Ectothiorhodospiraceae*. De Felice et al. (2010) confirmed these findings by recovering for the first time on a historical monument, three clones showing 99 % similarity with *Salinibacter ruber* (De Felice et al. 2010). In another study, the comparison between microbial communities from old and new biofilms has established that the prokaryotic communities are more stable than eukaryotic ones (Lan et al. 2010). However, even if molecular techniques have broadened our knowledge of the microbial diversity colonizing the surface of monuments, some drawbacks have to be considered. Most of the studies using molecular approaches to study biodeterioration processes has been carried out from DNA extracts. However, DNA extracted from building materials does not necessarily reflect the living microorganisms present on the surface at the sampling time (Laiz et al. 2011). Molecular analyses provide information on the different and successive microbial colonization waves that the building materials suffered in the past. This is because the DNA from the microorganisms active many years before can be preserved and evidenced in the analysis thereafter. Assessment of the metabolically active fraction of the community should be done by analyzing RNA rather than DNA. However, RNA extraction requires special attention, as RNAs are highly vulnerable to RNAse degradation during the extraction process. Moreover, all the molecular methods used until now to study microbial diversity on the surface of monuments are based on PCR and, thus, are affected by PCR bias and cannot be quantitative (Anderson et al. 2003; Lueders and Friedrich 2003). Another limit of molecular methods is that identification of microbes is usually limited to the genus level, because identified microorganisms are often members of hitherto undescribed species (Suihko et al. 2007; Gaylarde et al. 2012). However, this information from molecular surveys can be very useful to design new suitable cultural media in order to cultivate newly identified microorganisms. A dual phenotypic and molecular approach is recommended to study the microbial colonization of monuments (Gaylarde and Gaylarde 2005; Qi-Wang et al. 2011). A further source of variability may be the nucleic acid extraction (Ettenauer et al. 2012). Not all microbial species have the same sensitivity to lytic agents, with the differences being due mainly to the organization of the cell wall. This affects the analyses based on in situ nucleic acid extraction, since a high yield in pure DNA/RNA is desired, as well as the detection of all the species occurring in that environment. The standardization of nucleic acid extraction protocols for molecular ecology analysis of building materials should be encouraged to allow comparisons of results between different laboratories (Ettenauer et al. 2012).

High throughput sequencing (HTS) and metagenomic approaches have so far been very little applied in the field of biodeterioration of stone (Cutler et al. 2013b). However, the current development of HTS techniques has revealed that the microbial diversity of communities that inhabit the biosphere is much higher than previously estimated by traditional cultural methods and SSU rRNA sequence analysis studies (Roh et al. 2010). HTS methods are cost effective and allow the study of thousands of sequences at the same time to ensure reliable identification of the majority of microorganisms occurring on stone surfaces. Depending on the desired level of sample coverage, many stone samples could be sequenced at the same time, saving much time compared to the approaches currently used. In microbial ecology, the most common application of HTS is the determination of phylogenetic biomarkers sequences. The 16S rRNA gene is the most widely used marker for bacteria (Ratogi and Sani 2011). To identify phylotypes, sequences are then compared to a reference database; a quantitative estimation is then given on the occurrence of each phylotype in the sample. Moreover, the development of HTS has allowed the development of metagenomic approaches. Metagenomics is the study of collective genomes in an environmental community. This approach, which allowed getting more insight into microbial activities has not so far been applied to the field of biodeterioration as well. However, these approaches have allowed getting new insights into the phylogenetic and functional diversity of microbial communities (Wilmes and Bond 2006).

Conclusion

There are many mechanisms involved in microbial deterioration of the stone surface of monuments. They result from a very high diversity of microorganisms. We have just begin to suspect the extent of this diversity. This microbial biomass exhibits varied properties of growth and capacity to respond to environmental changes. There is, therefore, no general rule and each site and each monument must be analyzed both in its stony materials and in its environment rather than in its microbial flora to identify the physicochemical and biological factors involved in deterioration. The next challenges will be the development of new cultivation techniques and new culture media but also the application of molecular biology

techniques such as high throughput sequencing and metagenomics to describe the biodiversity and behavior of microflora colonizing the stone monuments. Multidisciplinary approaches are needed more than ever to understand the phenomena of biodeterioration of stone monuments.

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