Biodeterioration

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Abstract

The biodeterioration of organic and inorganic materials, as well as polymers, is a complex of alteration processes induced by the growing and metabolic activity of organisms. It can be recognized on monuments, wall paintings, stone, wood, paper, vegetal/animal fibers, and parchment artworks. As defined by Hueck (1968), biodeterioration is "any undesirable change in the properties of a material caused by the vital activities of organisms"; this definition is

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accepted as the meaning of the phenomenon. Both macroorganisms (such as animals, plants and mosses) and microorganisms (such as autotrophic or heterotrophic bacteria, microfungi, cyanobacteria, algae and lichens) represent the triggers of biodeterioration for cultural heritage. Understanding the morphological and physiological features of the biodeteriogens is required to establish the kind of interaction that occurs with the material and to assess the cause-effect of the biodeterioration action of a specific identified biological agent. For a complete evaluation of biodeterioration, a proper sampling and identification of the majority of biodeteriogens are required. Therefore, in order to apply a prompt and effective conservation to limit further damage, evaluating and quantifying the presence of biological systems that induce damage in heritage materials is indispensable.

1.1   Biological Systems as Deteriogens

An object can represent a *microcosmos* in which several types of vital organisms – and their metabolic excretes or products – coexist, in response to climatic or environmental parameters and to chemical or physical materials properties. Both artifacts of artistic-historical value stored in museums or galleries, and monuments or historical buildings exposed outdoors, are largely subjected to deterioration processes by macro- and microorganisms (e.g., rodents, birds, plants, insects, lichens, algae, bryophytes, fungi, bacteria, cyanobacteria) commonly known as *biodeteriogens*. Biological systems are able to initiate, support, and accelerate some chemical and physical reactions, representing a serious threat for the integrity and conservation of cultural assets, in both indoor and outdoor sites (Dakal and Cameotra 2011).

The biodeterioration of a wide range of cultural heritage materials has been largely investigated, and the effects of biodeterioration caused by living organisms are well documented (Lombardozzi et al. 2012; Piervittori et al. 1996). In relation to nutritional requirements and metabolic properties, organisms show different responses to colonized materials and cause different types of damage; knowledge of the biodeteriogens, their nutritional requirements, their growing settings, and their manifestation once they have colonized cultural items is indispensable to recognize the severity of the biodeterioration process.

The biodeteriogens' varieties should be grouped according to their attendance in different materials and to their type of damage: Table 1.1 shows the main groups of biodeteriogens.

In biological colonization, there are always some pioneer cells that facilitate the colonization of other organisms, especially in those materials (e.g., stone) that do not support the growth of heterotrophic organisms but can support food and organic energy sources for them in the form of other microbes, insect fragments, and bird

Table 1.1 Major biodeteriogens affecting cultural heritage materials and related physical and chemical damages

	Physical damage	Chemical damage	Damaged materials
Higher plants	Cracks, detachment of stone	Roots excrete organic acids	Natural and artificial stones
Animals and insects	Holes, losses and surface erosion and disintegration, structural damages	Droppings and urine	Stone, wood, paper, parchment and leather, vegetal and animal fibers, and more
Mosses and liverworts	Physical intrusion by rhizoids	Extraction of mineral from substratum, production of carbonic acid	Natural and artificial stones
Lichens	Cracks and fissures	Releasing of highly corrosive organic acids	Limestone, sandstone
Algae and cyanobacteria	Powdering	Staining, disintegration of stone	Natural and artificial stones, wall paintings, wood
Fungi	Contraction and expansion of thallus, fissures, loss of materials	Production of organic and inorganic acids and pigments, chelating properties	Stone, wall paintings, wood, paper, parchment and leather, vegetal and animal fibers, and more
Heterotrophic bacteria	–	Staining, production of acids and pigments	Stone, wall paintings, wood, paper, parchment and leather, vegetal and animal fibers, and more
Autotrophic bacteria	–	Production of organic and inorganic acids, biofilm formation, staining	Stone, wall paintings, wood, paper, parchment and leather, vegetal and animal fibers, and more

droppings (Sharma et al. 1985; Hochella 2002; Vaughan et al. 2002; Warscheid and Braams 2000).

The bioaerosol is also a potential source of biodeteriogens, due to the large number of biological particles suspended in the air (fungal and bacterial spores, lichen and algal cells, pollen grains); the spores transported by air or dust can represent a threat of paper and graphic collections (books, photographs, etc.) preserved in libraries, archives, and repositories. Throughout, these locations are generally characterized by poor ventilation, and the absence of periodic cleaning can compromise the integrity of stored objects and in addition can facilitate the proliferation of dangerous fungi. An object evidently has different levels of susceptibility to biodeterioration or colonization, also depending on human contribution in terms of care of cultural heritage.

The following biological systems have been largely known to induce biodeterioration phenomena on cultural heritage, in both organic and inorganic materials.

Fig. 1.1 Higher plant
massive growth on
Cambodian temple
(Angkor Park)

1.1.1   Macro-systems

Higher plants pose a severe threat especially for the conservation of archaeological remains and buildings, as they can involve physical and chemical diseases (Celesti-Grapow and Blasi 2004; Mishra et al. 1995). The damage caused by plants on monuments or archaeological sites is well known and is linked either to particular biological forms or to the characteristics of root systems (Caneva et al. 1994). Archaeological sites are characterized by rich flora and vegetation (Caneva et al. 2003; Capotorti et al. 2013) where the plants can develop in various structures (e.g., vertical/inclined wall surfaces, horizontal surfaces) and in different ecological conditions (Fig. 1.1). Lisci et al. (2003) described two types of colonization; the first regards an attack on the wall structure, usually the result of abiotic factors that create suitable conditions for microbial growth (bacteria, fungi, and lichens), which accelerate the deterioration of the structure and lead to the formation of a substrate for the germination of seeds. It is well known that some wall plants, especially perennial ones, with their radical apparatus and biomass can compromise the integrity and structural stability of ancient monuments (Mishra et al. 1995; Caneva et al. 2006). The second mode of colonization ensues mostly on horizontal surfaces with

a water supply; the pioneer plants are mosses that trap atmospheric dusts, allowing the formation of appropriate substrate for the germination of other plants. Moreover, higher plants with a root system may penetrate deep into the structure and grow, causing physical and chemical damage, through the exudation of organic acids. The deep penetration of the roots of tree species (such as *Pinus pinea* and *Quercus ilex*) can be dangerous for hypogenous structures, producing the detachment of plaster and the collapse of walls, their mechanical force can open cracks, cause crumbling, loosen stones and large fragments of the wall (Tiano 1986; Bettini and Villa 1976; Caneva and Galotta 1994; Lisci et al. 2003; Almeida et al. 1994).

Mosses and liverworts (bryophytes) are able to penetrate some types of stone with their rhizoids. The damage they cause is mainly associated with aesthetic appearance (usually green-gray patches). The capacity of the mosses to accumulate calcium ions from the substratum is associated with their biodeterioration capacity; the carbonic acid produced as a result of their cellular respiration can cause damage to stone. Moreover, the death of mosses causes indirect damage to monuments and stones by enriching and increasing the humus content which supports the growth of successive higher plant species (Dakal and Cameotra 2012).

As regards the role of animals in the biodeterioration of cultural heritage, direct and indirect actions can be distinguished; for example, the direct action of birds is both physical, caused by crushing and scraping, and chemical, caused by the release of acid excrements containing high amounts of nitrate and phosphate compounds. Indirect damage occurs as a result of organic substances accumulated on stone surfaces, which can serve as nutritive substrata for heterotrophic microflora.

Molds affecting archival and library materials can display different types of interaction with insects that feed on the materials of stored objects, and the biological particles can be vehicle by visitors or, sometimes, by synanthropic rodents, eating wood, leather, and other soft organic materials. Many insects are able to damage wood using it as a nutrient source and for shelter and egg laying. The way adult insects or larvae digest cellulosic compounds varies (Allsopp et al. 2004). Among insects, silverfishes are a threat to any heritage collection; in particular they eat glue and starch in books and in paper objects; termites eat cellulose-based materials (e.g., wooden furniture and artifacts, textiles, ethnographic artifacts); beetles can cause extensive damage to a variety of proteinaceous materials (e.g., wool, feathers, silk) and cellulosic textiles.

Furthermore, submerged or waterlogged materials, such as ceramics and wood, can suffer damage by several marine organisms that use them as a support for their growth. Depending on their chemical composition, the submerged stone artifacts are susceptible to the action of corrosion by perforating animal and plant organisms, such as sponges and bivalves that can induce macroboring (Ricci and Davidde 2012; Casoli et al. 2015).

1.1.2 Micro-systems

The term "microorganism" covers a wide variety of life forms, including bacteria, cyanobacteria, algae, lichens, and fungi. All cited microorganisms have different ecological properties and cause different damage in organic and inorganic materials of cultural heritage.

Favorable environmental conditions and the presence of energy sources with organic or inorganic nutrients allow the biological settlement of an exposed surface (Urzi and Krumbein 1994). The consequence of the biological activity is the formation of biofilms, colored patinas, encrustations and the presence of vegetative and reproductive bodies. In addition, fungi and bacteria are commonly isolated from libraries, archives, and museums' collections. Moreover, in indoor environments, the high presence of certain microbial species may involve irritation of the eyes and respiratory tract and can induce headaches, drowsiness, skin rash, and itching of the skin (Green and Scarpino 2002).

1.2   Microbial Colonizers

Microorganism biodiversity includes bacteria, archaea, and eukaryotes, which are extraordinarily diverse in their requirements for growth, and their proliferation is greatly affected by the nutrients available in their environment. Different kinds of microorganisms colonize artworks: chemoheterotrophic bacteria, chemolithotrophic bacteria, phototrophic bacteria, algae, and fungi. In the case of microbial-induced biodeterioration, alteration processes can arise when environmental conditions are favorable to growth (Nielsen et al. 2004; Nittérus 2000; Caneva et al. 1994) or when the existence of nutrients favors the colonization (Saiz-Jiménez 1993; Sterflinger and Piñar 2013). Microbial development depends on a combination of factors including the relative humidity (RH, %), temperature fluctuation, natural or artificial lighting, moisture content, dust content, osmotic pressure, and carbon dioxide concentration in the atmosphere (Valentin 2003; Woese 2000; Caneva et al. 2008).

1.2.1   Algae and Cyanobacteria

Among *micro*-biodeteriogens, photosynthetic ones are potentially the most aggressive, due to their ability to develop on stone surfaces, causing colored patinas and incrustations (Tomaselli et al. 2000). Algae and cyanobacteria are usually the first colonizers of historical monuments due to their photosynthetic nature, causing aesthetic damage and indirectly supporting the growth of other microorganisms (Urzi and Krumbein 1994). These organisms can develop on exposed stone when a suitable combination of dampness, warmth, and light occurs and inorganic nutrients (e.g., calcium and magnesium minerals) are present. Algae may also cause biochemical deterioration, producing small quantities of organic acids (they dissolve and powder the stone), proteins, and sugars, which can promote the growth of bacteria.

Cyanobacteria are organisms traditionally included among algae, but they have a cell structure typical of bacteria. Cyanobacteria are a morphologically diverse group of phototrophic prokaryotes with the ability to synthesize *chlorophyll a* and *phycobilin* pigments. Depending on the kind of organism and on the cycle phase,

dark-green-, brown-, gray-, and pink-colored patinas may occur (Huer 2008). All cyanobacteria are unicellular, though many grow in colonies or filaments, often surrounded by a gelatinous or mucilaginous sheath.

The photosynthetic activity of these microorganisms enriches the substrate with organic carbon in various forms, and subsequently the growing biomass entraps dust and soil particles providing further nutrient enrichment (Hirsch et al. 1995; Souza-Egipsy et al. 2004). The tolerance to desiccation, ability to utilize efficiently low light intensity and tolerate high levels, and resistance to high temperatures are important features of cyanobacteria which explain their widespread occurrence (Lamenti et al. 2003). Cyanobacteria have the ability to survive under drying and rehydration conditions on exposed monument surfaces and can protect themselves from harmful UV radiation by pigment production; they are also recognized in hypogeal environments (Albertano et al. 2003; Hoffmann 2002; Roldan et al. 2004).

1.2.2   Bacteria

Bacteria are a group of prokaryotic unicellular or colonial organisms of various shapes, classified into five groups according to their shapes: cocci, bacilli, spirilla, vibrios, or spirochaetes. The bacterium has a fairly thick cell wall made of peptidoglycans (carbohydrate polymers cross-linked by proteins); such bacteria retain a purple color when stained with a dye known as crystal violet and are known as gram positive (staining procedure). Other bacteria have double cell walls, with a thin inner wall of peptidoglycan and an outer wall of carbohydrates, proteins, and lipids. Such bacteria do not stain purple with crystal violet and are known as Gram negative (La Placa 2005).

Bacteria involved in deterioration of monuments and artworks mainly belong to three nutritional groups: photoautotrophs, chemolithoautotrophs, and chemoorganotrophs. Various species in photoautotrophs and chemolithoautotrophs groups (cyanobacteria, sulfur-oxidizing, and nitrifying bacteria) produce strong inorganic acids (sulfuric and nitric, respectively). Chemolithoautotrophic bacteria can derive their energy from the oxidation of reduced inorganic substances, using carbon dioxide as main carbon source. Endolithic nitrifying bacteria are the main representatives of the chemolithoautotrophic microflora in building stones; Kauffmann (1952) and Wagner and Schwartz (1965) first indicated the significance of nitrifying bacteria for biodeterioration.

Chemoorganotrophic bacteria produce several organic acids that can solubilize the mineral components of stones, such as the genera *Flavobacterium*, *Pseudomonas*, and *Microbacter* (Tiano and Tomaselli 1989; Tayler and May 1995; Swings and Descheemaeker 1995; Ortega-Calvo et al. 1993). Among the microorganisms dwelling on stone monuments, the autotrophic ones are considered the pioneering inhabitants.

Biodeterioration of organic substrates is a process involving several types of bacteria and represents a severe problem for archives, museums, and libraries. Bacteria also display wide diversity in enzyme production, including lipases, proteases, and oxidoreductases (Neelakanta and Sultana 2013). Intense research on advanced

microbiological systems based on the use of microorganisms for the removal of alterations on works of art has shown them to be a viable alternative for cultural heritage restoration (Ranalli and Sorlini 2008; Palla et al. 2013, Martino et al. 2015; Barresi et al. 2015).

1.2.3 Fungi

Fungi are usually classified in four divisions: the *Chytridiomycota*, the *Zygomycota*, the *Ascomycota*, and the *Basidiomycota*. There are also two conventional groups which are not recognized as formal taxonomic groups; these are the *Deuteromycota* and lichens. *Deuteromycota* include all fungi which do not reproduce themselves sexually, and lichens are a group of composite organisms formed by the association of algae and fungi. Frequently associated with biodeterioration of rocks and outdoor stone, lichens are highly resistant to desiccation and extreme temperatures. They are able to produce pigments, such as chlorophyll, carotenoid, and melanin, that may generate chromatic variations toward yellow, orange, red, or even brown (Krumbein 2003; Tiano and Tomaselli 1989; May et al. 1993; Palla et al. 2010).

Fungi are metabolically more versatile than other biodeteriogens because they are able to colonize on a wide variety of substrates (Sterflinger 2010; Onofri et al. 2014). They can exert both a biomechanical action through the disaggregation and reaggregation of the mineral fraction of stones and a biochemical action by producing metabolic organic and inorganic acid and by the absorption of metals by mycelia felts (Burford et al. 2003; Sterflinger 2000). Due to their enzymatic activity, fungi are also able to inhabit and to cause decay in paintings, textiles, paper, parchment, leather, oil, casein, glue, and other materials used for historical- artistic objects. Several fungal metabolic products are strongly colored, and the phenomenon of staining produced on the damaged areas on monuments can be the consequence of fungal development. In indoor environments (e.g., archives), when relative humidity increases and no adequate ventilation occurs, the conidia may be deposited more quickly on documents and deteriorate the document supports (Borrego et al. 2012).

Fungal flora inside environments are representative of the outdoor atmosphere, since airborne spores penetrate through doors and windows and the spores in the airborne particulate can cause an increase in allergies and respiratory and skin diseases, headaches, asthma, and weariness (Borrego et al. 2012).

1.3   Microbial Metabolic Activities and Deterioration of Cultural Assets

Both in organic and inorganic matters, evidence of biodeterioration is related to physical and chemical processes induced by microorganisms; physical damage is generally induced by growth or penetration within the material with formation of micro-fractures, loss of cohesiveness, and disaggregation of the substrate

(López-Miras et al. 2013; Cataldo et al. 2005). Physical damage caused by microbial colonization is less extensive than chemical damage, which can arise when products of metabolic activity interact with the material, causing alteration of the substrate, which at times is irreversible. Biochemical deterioration induced by microbial colonization consists both in digestion processes, when microorganisms use the substrate as nourishment, especially when organic compounds are utilized, and in metabolic excretions, waste products, or other substances (e.g., organic and inorganic acids, pigments) (Strzelczyk 2004; Saiz-Jimenez 1999; Perry et al. 2003). Many factors can contribute to biodeterioration, including the properties of the original materials, sources of nutrients, and the environmental conditions in which the objects are stored or exposed (Warscheid and Braams 2000; Prieto et al. 2006; Pavlogeorgatos 2003). However, the complete understanding of biodeterioration processes is difficult, being the result of complex microbial interactions (Mandrioli et al. 2003; Warscheid and Braams 2000).

1.3.1   Inorganic Substrates

Artworks made of inorganic materials are exposed to natural processes of biodeterioration, especially when they are located outside (Cutler et al. 2013; Kumar and Kumar 1999; Dakal and Cameotra 2012). The biodeterioration process affects monumental stones, statues, historical buildings, wall paintings, archaeological remains, and, to a lesser extent, glasses and metals (Giustetto et al. 2015; Biswas et al. 2013; Gorbushina and Palinska 1999; Gorbushina et al. 2004; Piñar and Sterflinger 2009).

Numerous parameters influence the succession of microorganisms on stone; firstly the properties of the stone itself determine the colonization pattern. The mineral composition, structure-texture, porosity, and permeability of stone may influence the distribution of such organisms in the monuments (Miller and Macedo 2006). Favorable environmental conditions and the presence of nourishment sources allow the biological colonization of an exposed stone surface (Miller et al. 2000; Palla et al. 2003; Anagnostidis et al. 1991). It is known that biological growth on stone is highly dependent on climatic and microclimatic conditions, such as humidity, temperature, light, and atmospheric pollutants (Moroni and Pitzurra 2008; Mansch and Bock 1998; Zanardini et al. 2000; Nuhoglu et al. 2006). To avoid microbial proliferation, it is necessary to control environmental factors, which becomes difficult in archaeological sites or in urban spaces, but is more easily achieved in closed environments where the control of climatic and microclimatic conditions is easier (Salvadori and Charola 2011). Occasionally, the metabolic activities of microorganisms (autotrophic and heterotrophic) induce different types of damage: physical, when pressure is exerted by the growth of vegetative structures (e.g., lichenic and fungal thalli); chemical, when the excretion of enzymes, the production of inorganic and organic acids, and the liberation of chelating compounds occur; and aesthetical, as in the effect of releasing pigments (colored patches or patinas) (Ascaso and Wierzchos 1995; Ortega-Calvo et al. 1995; Albertano and Urzì 1993). Microbial colonization of cultural stone heritage may cause aesthetic changes due to the growth of pigmented

Fig. 1.2 Differently pigmented biological systems (algae and cyanobacteria) colonizing wall surface in hypogeal environment

microorganisms; cyanobacteria, algae, fungi, lichens, and some pigmented bacteria
are responsible for these effects (McNamara and Mitchell 2005; Crispim and
Gaylarde 2005). The algae and cyanobacteria can be considered the pioneering colo-
nizers of a stone surface (Tomaselli et al. 2000), and as a result of their development,
green-, brown-, and gray-colored patinas may also occur (Fig. 1.2) (Hauer et al.
2015; Golubíc et al. 2015). Lichens can be identified by directly observing the stone
surface and are regarded as major biodeterioration agents in outdoor stone monu-
ments (de los Ríos et al. 2009; Chen et al. 2000). Lichens and fungi can cause serious
degradation by physical penetration: fungal hyphae are able to penetrate deeply
beneath the stone surface, contributing to mechanical deterioration. This penetration
simultaneously allows the transport of water and nutrients through the stone, facili-
tating internal colonization (Fernandes 2006).

   Many microorganisms play an important role in the deterioration of stone often
through the action of organic and inorganic acids produced as metabolic products
(Adamo and Violante 2000; Sazanova et al. 2014). Generally, most microorganisms
excrete organic acids while metabolizing organic and inorganic compounds. Organic
acids react with the substrate through the action of protons and chelation of metal
ions. The excretion by the lichen of low-molecular-weight organic carboxylic acids,
such as oxalic, citric, gluconic and lactic acids, with combined chelating and acidic
properties, is a phenomenon of high intensity (Adamo and Violante 2000).

   The production of inorganic acids is limited to some systematic groups, in par-
ticular to sulfobacteria and nitrobacteria; due to the action of nitrous and nitric acid
excreted by nitrifying bacteria (*Nitrosomonas* spp. and *Nitrobacter* spp.), as well as
sulfuric acid by sulfur-oxidizing bacteria (*Thiobacillus* spp.), stone dissolution and
the formation of nitrate salts of stone (biocorrosion) can ensue (Fernandes 2006;
Mansch and Bock 1998; Bock et al. 1990). Endolithic nitrifying bacteria are the

main chemolithoautotrophic microflora in building stones. On the other hand, heterotrophic microorganisms develop on inorganic materials if nutrients, e.g., settled particulate matter, organic residual substances, and organic resins, glues, or binders by restoration practices, have been deposited on them (Caneva et al. 1998).

The fungal genera which have been demonstrated to be more abundant on inorganic materials, especially on stone, are *Cladosporium*, *Penicillium*, *Trichoderma*, *Fusarium*, and *Aspergillus* (de la Torre et al. 1993; Sazanova et al. 2014; Torre et al. 1993; Gu et al. 1998; Hu et al. 2013; Prasad Gupta and Sharma 2012; Sharma et al. 2011). The reduction and oxidation of mineral cations are characteristic activities of fungi (de la Torre and Gomez-Alarcon 1994). Several fungal metabolic products are strongly colored; dark spots are attributed to the presence of fungi of the family of *Dematiaceae*, which contains melanin pigments inside the mycelium. Black microcolonial fungi (MCF) play an important role in structural and aesthetic alteration and are considered among the most harmful microorganisms associated with monumental stone biodeterioration (Marvasi et al. 2012; Wollenzien et al. 1995; Isola et al. 2016; Salvadori and Municchia 2016). The dark gray and black crusts and patinas generally found on stone monuments, in particular, calcareous stones, can be derived by secreted dark compounds such as melanins and/or humic acids (Delgado Rodrigues and Valero 2003).

The organisms are often organized as microbial biofilms that cover the surface of the material and/or penetrate the substratum (Hoppert and König 2006). Biofilms often excrete polysaccharides, lipids, pigments, and proteins (Hauer et al. 2015). Through in situ microscopy, the zones where biodeterioration processes take place can be detected as biofilms composed of different microorganisms; endolithic microbial colonization seems to be induced by enhanced moisture availability in cracks and fissures (de los Rìos and Ascaso 2005). The enrichment of sulfur- and hydrocarbon-utilizing bacteria in the biofilms may contribute to dissolution of the stone (Mitchell and Gu 2000).

Detection and identification of damage induced by microorganisms' activity within inorganic substrates is not easy. Anyway, some researchers are using several methodologies and integrated techniques in order to recognize the different microorganisms and their associated biodeterioration (de los Ríos et al. 2009; Suihko et al. 2007; Gutarowska 2010; Fernandes 2006; Herrera et al. 2009).

1.3.2   Organic Matter

Organic-based materials of cultural heritage (e.g., paper, fibers, wood, papyri, leather and parchment) are generally subject to the aggression of several heterotrophic microorganisms that use them as a source of nourishment (Strzelczyk et al. 1997; Manente et al. 2012; Kavkler et al. 2015; Sterflinger 2010; Vukojević and Grbić 2010). Additionally, the presence of organic residues (e.g., glue, dirt, dust) may accelerate the processes of degradation, such as the loss of strength and elongation, oxidation, discoloration and breakdown of molecular structures (Guiamet et al. 2014).

Most microorganisms are specialized in producing hydrolytic or proteolytic enzymes, necessary for the degradation of cellulose or collagen, respectively (Strzelczyk 2004; Sterflinger and Pinzari 2012). The production of extracellular enzymes and the extraction of aggressive metabolic products increase the loss of material (López-Miras et al. 2013). Enzymatic activity (due to cellulases, glucanases, laccases, phenolases, keratinases) plays an important role in the decay of organic materials, especially for libraries and archives heritage; moreover, if collections are exposed to high humidity, high temperature, and insufficient air circulation, fungal colonization is more dangerous (Sterflinger 2010; Rakotonirainy et al. 2015; Guiamet et al. 2014). Fungi often cause serious aesthetical spoiling and chromatic alteration due to the formation of colonies and fungal pigments. When they grow on paper, they degrade all its carbon-containing components such as cellulose by excreting enzymes or organic acids (oxalic, fumaric, succinic, and acetic acids), which settle over the substrate and acidify (López-Miras et al. 2013; Canhoto et al. 2004; Kavkler et al. 2015). Microscopy is also useful in revealing the biological structures related to the development of hyphae in cellulolytic fungi (Guiamet et al. 2014). In paper, leather, or fibers, microbial growth mainly appears as patches of different colors (purple, yellow, brown, black, red, and green), shapes and sizes, related to the presence of pigmented fungal mycelium and spores or to exo-pigments produced by bacteria and fungi (Fig. 1.3a, c).

Paper documents have received particular attention in the past, mainly for a staining phenomenon called *foxing*. Foxing, a typical phenomenon in paper or in textile, is the name of random circular and irregular yellowish to brownish-red stains, fluorescent under ultraviolet rays, on the surface of old books, documents, maps, etc. (Montemartini Corte et al. 2003; Kraková et al. 2012; Rakotonirainy et al. 2015). The role of fungi, and whether they accompany the formation of stains, is still not clearly understood. Many authors dealing with the phenomenon of foxing on paper point out its microbiologic origin, but, in fact, foxing-causing fungi can rarely be cultured. Karbowska-Berent et al. (2014) showed foxing as well as numerous hyphae and fluffy coatings and were able to isolate five strains, belonging to the *Eurotium*, *Aspergillus*, and *Penicillium* genera. Due to their enzymatic activity (exoenzymes such as cellulases, glucanases, laccases, phenolases, keratinases, monooxygenases), fungi are able to inhabit and decay not only paper heritage but also paintings, textiles, parchment, oil, casein, glue, and other materials used for historical art objects.

In paper decay, the most numerous bacteria are heterotrophs (*Cytophaga*, *Cellvibrio*, and *Actinomyces*). Deterioration by bacteria and fungi leads to loss of strength of the natural fibers, causing odor emissions, aesthetic damage, staining, and loss of fiber structure. Fungal damage of textiles can result in discoloration, staining, and smell due to the production of volatile compounds and the enzymatic and mechanical degradation of the material through the activity growth of fungi (Guiamet et al. 2014; Kavkler et al. 2015). Other important properties of fungi are related to their pathogenicity for workers involved in collection maintenance (Borrego et al. 2010; Sahab Ahmed et al. 2014).

Fig. 1.3 Microbial colonization on organic materials: (**a**) paper document affected of pigmented spots originated by microorganisms, (**b**) an oil painting on canvas with fungal colonization on *verso* and (**c**) a lather sword holder completely damaged by white fungal colonization

More complex biodeterioration process occurs in composite materials, such as paintings on canvas, where it starts on the reverse side, due to the presence of support polymers and glue; these components can be substrates for microbial growth. On the other hand, the organic materials present on the *recto* of the paintings are susceptible to attack by specialized microorganisms and by occasional contaminants (López-Miras et al. 2013). Moreover, fungi penetrate cracks and migrate underneath paint layers (Fig. 1.3b), rapidly developing when the environmental relative humidity and temperature increase (RH > 70 % and T > 25 °C).

The enzymatic degradation by microorganisms regards also hemicellulose and lignin. The decomposition activity in wood can induce physical-mechanical damage (brown rot, soft rot, and white rot) and chromatic alterations (chromogenic fungi). Chromogenic fungi involve chromatic alterations in wooden objects, producing pigments or metabolic substances. These fungi cause the pinkish, bluish, or grayish discoloration of wood, which may occur either in depth or superficially. Instead, bacteria belonging to *Bacillus*, *Pseudomonas*, *Cellulomonas*, *Clostridium*, and *Cytophaga* genera may induce several alterations in wooden items, with characteristic conformation, i.e., erosion and tunneling (Blanchette 2000).

Textiles, particularly those composed of natural organic fibers, such as cotton, linen, wool, etc., are readily attacked by microorganisms. Microbial growth on textile items causes loss of strength and elongation, discoloration, and changes in appearance (Szostak-Kotowa 2004).

The biodegradation of proteinaceous materials – such as vellum, parchment, and leather – can be induced by bacterial (*Bacillus*, *Pseudomonas*, *Clostridium*, *Streptomycetes*) and fungal (*Mucor*, *Chaetonium*, *Aureobasidium*, *Thrichoderma*, *Epicoccum*) strains. Both groups of microorganisms produce many strong enzymes and acids which can efficiently hydrolyze organic materials, such as extracellular proteolytic enzymes (collagenase, keratinase, etc.). Among actinomycetes (that find proper conditions to proliferate in humid and poorly ventilated environments), there are several strains with strong proteo- and collagenolytic properties, especially within the genus of *Streptomycetes*. Chromatic alterations in leather artifacts, induced by microbial activity, are generally originated by pigmented microbial species (Piñar et al. 2015; Koochakzaei and Achachluei 2015), such as *Streptomyces fimicarius*, generally isolated from miniature and responsible for red- and purple-colored stains (Karbowska-Berent and Strzelczyk 2000).

1.4   Integrated Approach to Reveal and Identify Bacteria and Fungi Colonization on Work of Art Surface

The evaluation of those factors promoting microbial activity and proliferation on artworks and the understanding of related deterioration mechanisms is essential in designing appropriate conservation and restoration strategies. Knowledge of microbial contamination of cultural heritage is of great interest, not only in terms of recognizing microbial communities with potential dangerous effects for stored materials or monuments but also in terms of recognizing microorganisms which represent a risk for human health. This justifies the need for performing systematic microbiological sampling to estimate the prevalence of microbial contamination (Borrego et al. 2010); for an evaluation of the biological risk, it is important to measure both the total microbial load in the indoor environment and on artwork surfaces.

Most of the literature reports on studies identifying biodeterioration on cultural heritage using invasive sampling and culture methods. However, traditional culture methods do not always succeed in isolating microbial agents (Michaelsen et al. 2010). Studies using noninvasive sampling and molecular approaches to investigate the role

of microorganisms in the deterioration process allow a wider understanding of the biodeterioration phenomena. Generally, a total understanding of the conservation situation can be obtained by an integrated approach using traditional culture techniques, microscopy investigations, and molecular analyses. Instead, the diversity of the cultivable microorganisms adhering to a surface can be analyzed directly by molecular techniques (e.g., RAPD analysis, DGGE fingerprinting). In addition, molecular techniques are increasingly applied in the field of conservation and represent very useful tools for detecting and identifying different microbial strains, but they can never be helpful in the evaluation of damage-causing biodeterioration on the substrate. If combined with microscopic observation, molecular analysis enables interrelations between microorganisms and substrates to be detected (Salvadori 2000).

The study of the mechanisms underlying the microbiological attack of historical materials has been widely practiced and continues to represent one of the main focuses of those institutions and laboratories involved in cultural heritage conservation.

1.4.1   Sampling by Noninvasive Methods

In order to reveal microbial communities and understand their relationship with colonized substrates, the use of noninvasive sampling techniques is often required to minimize invasive actions. Depending on the importance, features, materials, and state of conservation of cultural items, it is not always possible to practice invasive sampling (e.g., use of scalpel to sample small pieces from substrate). This step is also required to monitor microbial colonization before and after conservation and restoration treatments of artistic objects. Several nondestructive methods have been proposed for direct or indirect evidence of microbial colonization or biofilms on different substrates. Current sampling procedures involve sterile needles, adhesive tape, and sterile swabs (Fig. 1.4a, b); the use of these tools is closely related to the nature of sampling objects (Sterflinger 2010; Pasquarella et al. 2015).

In the case of paper items, needles and adhesive tape may cause severe surface damage, so it is preferable to use dry swabs, but unfortunately these are very often ineffective (Michaelsen et al. 2009). In order to overcome this problem, other noninvasive sampling methods involving the use of nitrocellulose or nylon membrane filters may be adopted and then transferred onto solid culture media to isolate bacteria and fungi (Fig. 1.4c, d), or used to direct DNA extraction (Montanari et al. 2012; Palla 2011).

Nitrocellulose and nylon membranes can be applied to investigate the presence of viable microbial communities that may be the result of aesthetic or structural damage on several materials. In particular, the use of these filters is indicated for ancient paper or leather items (Palla 2006; Pasquarella et al. 2015; Pasquariello et al. 2016).

Microbiologists have generally used sterile adhesive tape to collect samples from skin, food, or medical devices, to detect pathogenic microorganisms in a noninvasive way (Miller 2006); adhesive tape may be associated with microscopic analysis, for example, to detect the presence of fungal conidia on stone surfaces (observation

Fig. 1.4 Noninvasive sampling tools: (**a**) cotton swab on stone, (**b**) adhesive tape on lather, (**c**) nylon membrane fragment on book and (**d**) nitrocellulose membrane on paper (drawing)

in light microscopy), or with other techniques, as well as fluorescent in situ hybridization (FISH), molecular and culture techniques (La Cono and Urzì 2003; Bisha and Brehm-Stecher 2009). Adhesive tape sampling can be widely and safely applied in the field for the conservation of cultural heritage, due to its several advantages (i.e., it is cheap and does not require the expertise of specialized staff). Recently, Urzì and De Leo (2001) proposed the use of adhesive tape strips as a nondestructive sampling technique for the study of microbial colonization on monument surfaces. Cutler et al. (2012) used adhesive tape to collect fungi samples suitable for molecular analysis from stone buildings.

1.4.2   Microscopy

The most common tools used in the field of cultural heritage (assessing the biological contribute to depleting of cultural items) are microscopy techniques applied to the study of biodeterioration processes in cultural assets. Different kinds of microscopes are used to observe biofragments from needle, scalpel, or adhesive tape samplings; to recognize the biodeteriogen morphology, species, secondary metabolites, and products; or to provide direct evidence of biofilm formation.

Microscopy techniques are also widely used to observe the morphology of microbial taxa; several microscopy techniques have been used to study the
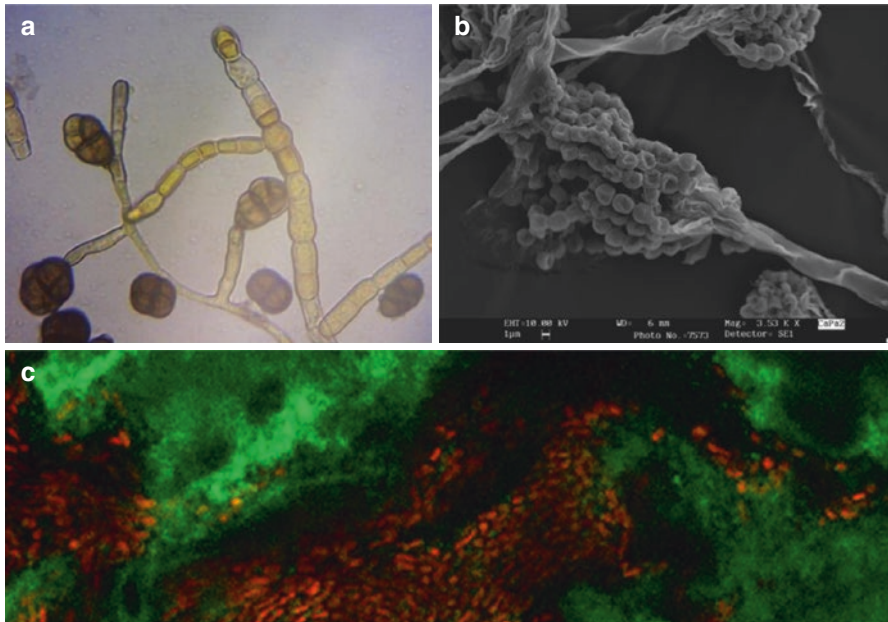
Fig. 1.5 Microscopy techniques used to reveal microbial systems: (**a**) fungal structures (*Alternaria* spp.) stained by Lugol's solution observed by OM – 40× magnification, (**b**) micrograph by SEM of fungal reproductive structure and spores (*Penicillium* spp.), and (**c**) *green and red autofluorescence patterns* related to cyanobacteria and algae cells by CLSM

relationship between microorganisms and rock applying nondestructive methods; both light microscopy and light microscopy combined with scanning electron microscopy were used (de los Rìos and Ascaso 2005).

The most common microscopy techniques used in studying microbial colonies are optical microscopy (OM), or light microscopy (LM), scanning electron microscopy with back-scattered electron imaging (SEM-BSE), transmission electron microscopy (TEM), confocal laser scanning microscopy (CLSM), and environmental SEM (ESEM) (Macedo et al. 2009; Herrera and Videla 2009; Rakotonirainy et al. 2007).

Optical or light microscopy can provide information simply by observing the samples and is useful in the detection of several morphological structures (Fig. 1.5a) (e.g., fungi, cyanobacteria, algae, lichens) (Golubíc et al. 2015; Macedo et al. 2009). This type of microscope is useful for first stage identification of bacteria as cellular morphology and reaction with the Gram stain can be verified (Bergmans et al. 2005).

Electronic microscopes are widely used in scientific applications, the most frequently utilized being SEM and TEM, which allow observation and surface characterization at a higher resolution and depth of field than the optical microscope by giving a 3D effect to the images (Fig. 1.5b) (Nuhoglu et al. 2006; Troiano et al. 2014; Golubíc et al. 2015; Florian and Manning 2000; Cappitelli et al. 2009). Unlike SEM, in ESEM analysis the sample is hydrated and nonconducting, without prior dehydration or conductive coating (Bergmans et al. 2005).

CLSM is extremely useful for revealing the chemical and biological relationship between a microorganism (biodeteriogen) and its microenvironment (colonized substrate); CLSM has many advantages, as its nondestructive and "real-time" technique can be used in situ to detect the growth, metabolism, and gene expression of organisms including the microenvironment surrounding each cell (Fig. 1.5c) (Caldwell et al. 1992; Villa et al. 2015). A full range of multi-microscopic techniques (CLSM/SEM) is often used to detect biota communities (fungi, bacteria, cyanobacteria) on samples from particular cultural items (e.g., mummies, fossilized bones), to confirm the presence of certain organisms, or to establish which dominant biota is causing deterioration in biofilm (Palla et al. 2011; Janssens and Van Grieken 2004; Marano et al. 2016; Polo et al. 2012; de los Rìos and Ascaso 2005).

1.4.3   *In Vitro* Culture

Traditional culture methods for isolation and identification of microorganisms are very useful in assessing biodeterioration in cultural heritage. The cultivation methods (nutritive media) are used for the specific isolation of fungi and bacteria or specific growing of other biological systems, such as cyanobacteria or lichens. Some nutritive media may be designed for specific strains proposing the growth condition that originally existed on the cultural heritage (e.g., high pH level for strains isolated from wall paintings) (Gorbushina et al. 2004).

In order to reveal microbial contamination of cultural items (organic or inorganic), it is possible to place a piece of item (sample) directly onto the nutritive medium (Abrusci et al. 2005) or sampling tools, such as swabs, adhesive tape, or charged membranes (Pasquarella et al. 2015; Palla et al. 2015). No medium has been recommended for the isolation of certain microorganisms; as regards the isolation of fungal communities from paper heritage, for example, Sabouraud dextrose agar and malt extract agar, sometimes supplemented with an antibiotic such as chloramphenicol or streptomycin to inhibit bacterial growth, and Czapek agar have been used. For determining the total number of cultivable bacteria, tryptic soy agar is used, while MacConkey agar is used to detect gram-negative bacteria (López-Miras et al. 2013).

The microbial samples may be put into a nutritive broth (generally composed of a peptone solution with meat extract and mineral salts, agar-free) and incubated for many hours (18–72 h) to allow microbial growth.

Thus, it is possible to perform morphological analysis detecting and evaluating macroscopic parameters, e.g., color, shape, and appearance (Fig. 1.6). Colony-forming units (CFU) on solid growth media are counted using a simple visual inspection. Besides pH, the availability of nutrients, the temperature, and the duration of incubation are also important for successful isolation. However, methods based on the cultivation of microorganisms may detect only a minor fraction (2–5 %) of the total number of microbial communities, this cultivable fraction representing only the living part of the microbes in the sample (Otlewska et al. 2014). In any
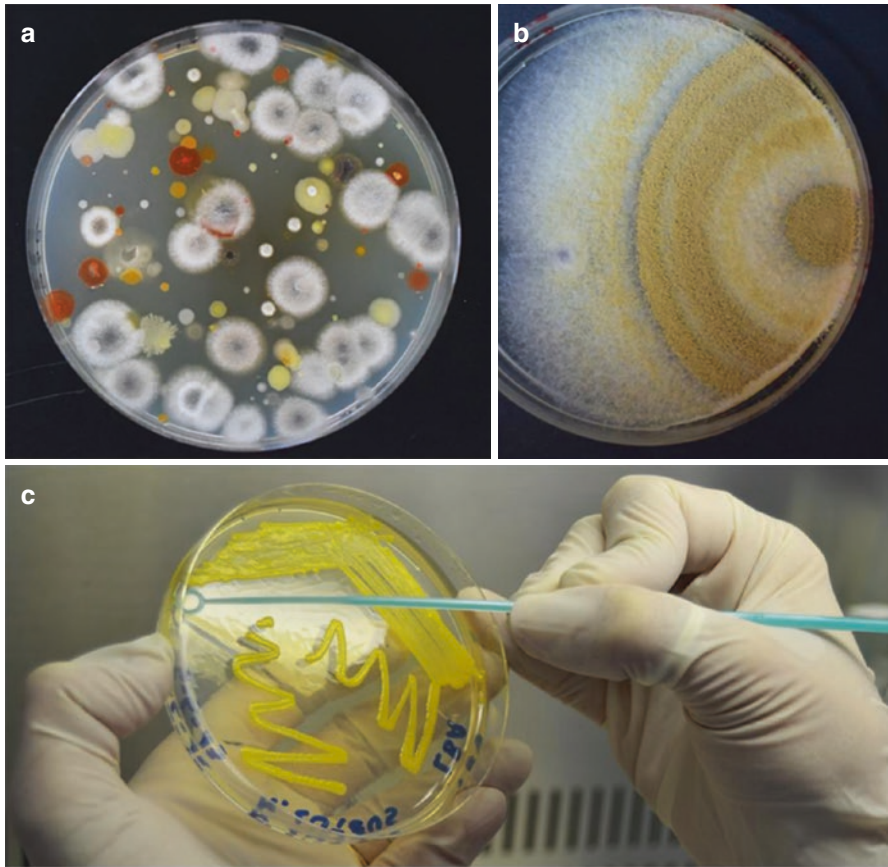
Fig. 1.6 Bacteria and fungi isolated by culture-dependent methods: (**a**) huge microbial growing on nutritive medium, (**b**) fungal colony on Sabouraud media (*Scopulariopsis* spp.), and (**c**) subculture onto N-Agar medium (*Micrococcus luteus*)

case, culture-based approaches alone cannot provide exhaustive information on the real microbial consortia, also because only a small fraction of the microorganisms can be cultivated (Dakal and Arora 2012). For this reason, for correct evaluation of biological contaminants, culture techniques should be supported by molecular investigations.

1.4.4   Molecular Investigations

While traditional microbiological methods based on culture procedures provide important but limited information on microbial communities, molecular techniques offer a great overview on the diversity of microbiota involved in the biodeterioration of monuments and artworks (Otlewska et al. 2014; Gonzáles and Saiz-Jiménez 2005).

The development of recent molecular techniques has improved the sensitivity, specificity and speed of detecting microorganisms. Primarily, the molecular detection of microorganisms begins with the extraction of nucleic acids from collected samples, using specific *ad hoc* protocols or commercial kits. The detection of microorganisms is mainly based on the sequence of small subunits (16S for bacteria and 18S for fungi) ribosomal RNA (rRNA) genes (Gonzáles and Saiz-Jiménez 2005). Molecular investigation continues with *in vitro* amplification of specific genomic regions by polymerase chain reaction (PCR), analyses of amplification products using gel electrophoresis technique, determination of nucleotide composition of DNA fragments (sequencing) and analyses of homology of sequence using international data banks (e.g., NIH, USA; EMBL, Germany). The existence of DNA database guarantees optimal identification of the microorganisms detected and the possibility of carrying out phylogenetic analysis (Palla 2004; Gonzáles and Saiz-Jiménez 2005).

At present, molecular methods, including polymerase chain reaction (PCR), denaturing gradient gel electrophoresis (DGGE) and the creation of clone libraries, are used as a sensitive alternative to conventional cultivation techniques. DGGE is the most frequently reported technique for separating DNA fragments during microbial diversity studies of art objects (Dakal and Arora 2012; Otlewska et al. 2014; González and Saiz-Jiménez 2004; Michaelsen et al. 2010). Genetic fingerprinting is a rapid and useful method for studying diversity in microbial communities including nonculturable and inactive microorganisms (Otlewska et al. 2014).

Polo et al. (2010) studied microbial contamination using denaturing gradient gel electrophoresis and established that cyanobacteria and green algae genera were responsible for green staining. Fluorescence in situ hybridization (FISH) has been applied in the field of conservation and restoration to study microbiota involved in biodeterioration. Furthermore, the application of FISH directly on adhesive tape strips added another advantage to this non-destructive sampling method and led to the identification in situ of the microorganisms present on a given area, without destruction of the valuable surfaces and with little biofilm disturbance (Andersen et al. 2001; Sterflinger 2010).

The application of molecular biology on cultural heritage has been applied to identify the development of microorganisms in inorganic materials or in organic materials, in museums, and in archives (Piñar et al. 2013; Valentin 2003). Molecular biology provides a sensitive study on the microbial contamination of works of art, based on the analysis of specific DNA genomic sequences.

In order to increase the screening of a large number of microorganisms, oligonucleotide microarray protocols have been applied for the detection and genotyping microbial variety (Maynard et al. 2005) and can be used efficiently in the identification of microbial communities affecting cultural heritage.

Microarray analysis is based on hybridization between fluorescent-labeled DNA sequences and thousands of probes immobilized on a glass slide (array), providing a cultivation-independent characterization of microbes on works of art (Gargano et al. 2012; Neugebauer et al. 2010). The core principle behind microarrays is hybridization between two DNA strands, the property of complementary nucleic acid sequences to specifically pair with each other by forming hydrogen bonds

between complementary nucleotide base pairs. Each DNA spot contains a specific DNA probe sequence, used to hybridize a sample called target under high-stringency conditions. Fluorescently labeled target sequences that bind to a probe sequence generates a signal that depends on hybridization conditions (such as temperature) and washing after hybridization. These sample microarrays contain a large number of samples that could be analyzed, for instance, through FISH techniques for the detection of specific microorganisms or the expression of specific genes (Gonzalez 2003; Andersen et al. 2001; Kononen et al. 1998).

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