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**THE TWO SIDES OF MICROORGANISMS IN
CULTURAL HERITAGE CONSERVATION**

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Biodeterioration is any irreversible change in the properties of a material caused by the activity of organisms belonging to different systematic groups. Microorganisms (bacteria, fungi, yeasts, algae and lichens) are considered one of the most important biodeteriogens, thanks to their ability to colonise the surface of both organic and inorganic artworks. Cultural heritage biodeterioration commonly results from the complex interaction established by different kind of microorganisms co-existing simultaneously and the substrate. But then, microorganisms can also be considered biological agents rich in virtues; in fact, they can be used for the conservation of stone works of art. The potential of microorganisms for effective bioremediation of deteriorated cultural heritage materials is gradually being unveiled, and promising results of this methodology have been obtained in field sites. As a consequence, it should not come as a surprise that conservators are forever asking microbiologists how advances in biotechnologies could contribute to the conservation of heritage.

The aim of this work was to investigate the two sides of microorganisms in the cultural heritage conservation, starting from some case-studies. In particular the objectives were to:

- ✓ characterise the microbial communities on parchment manuscripts together with the study of the microbial airborne communities and the environmental physical conditions in close proximity to where the manuscripts are stored, suggesting some general guidelines for the correct management of historical documents;
- ✓ reduce the biological treatment time and assess the effects of a chemical and sulfate-reducing bacteria (SRB) co-treatment for the bioremoval of black crusts and grey deposits on a marble statue;
- ✓ broaden the fields of application of biocleaning technologies on stone conservation, proposing a valid alternative to the traditional cleaning methodologies for the bioremoval of synthetic polymers from stone monuments.

The first step to assess the microbiological risk of a cultural heritage object is to identify the entire microbial community colonising art objects, using non-invasive sampling, or sampling that needs only small amounts of material. Most of the literature dealing with microbial spoilage on documents use invasive sampling methods and culture approaches for the identification of the species causing spoilage. However, traditional culture methods can be time-consuming, do not always succeed in isolating microbial agents, and do not necessarily provide exhaustive information on the real microbial community load because only a small fraction of the microorganisms can be cultivated. In addition, aerobiological investigations of conservation environments are also helpful in choosing interventions aimed at establishing the sources of contamination and preventing the microbiological deterioration of artworks. During the last decade, in fact, the environmental conditions of the museum exhibition facilities and storage areas have been shown to be the most crucial factor, concerning the preservation of collections and artifacts.

Until now few microbiological studies have been conducted on parchment. **Chapter 3** reports the evaluation of the microbial risk for the conservation of seven 16th century manuscripts written on finely illuminated parchment. The aims of the work were: (a) to clarify any relationship between the presence of an active microbial community and discolouration, (b) to study microbial air quality and environmental conditions in the repository, and (c) to investigate the relationship between airborne and surface-associated microbial communities. For the first time,

the microbial community on historical parchment has been investigated by both non-invasive sampling and fully culture-independent approaches, coupling an aerobiological monitoring of the repository by an exclusively biomolecular approach.

On discolouration putatively caused by microbial colonisation, two non-invasive sampling techniques were chosen: nitrocellulose membrane and fungi-tape. The nitrocellulose membranes were gently pressed onto the surface of the manuscripts to collect the cells; the DNA was directly extracted from the membrane and culture-independent molecular approach based on PCR-DGGE was adopted to fully characterise the surface-associated communities. Fungi-tape was chosen for investigating the spatial distribution of microorganisms, staining the tape with two fluorescent dyes (SYTO9 and Fluorescent Brightener 28, to label bacteria and chitin in the walls of fungi, respectively). Epifluorescence microscopy observations of the tape samples, coupled with ATP assay on the surfaces of the manuscript, showed a low contamination level and that the discolouration was not related to currently active microbial colonisation. The airborne microflora was sampled with a MAS-100 portable bioaerosol sampler, for monitoring the microbial loads, and the AGI-30 impinger, for fully characterising airborne communities with culture-independent molecular methods. Potential biodeteriogens, as the fungus *Aspergillus fumigatus*, and microorganisms ecologically related to humans, as *Candida sp.*, were found, suggesting the need to control the conservation environment and improve handling procedures. The proteolytic activity of *A. fumigatus*, often isolated from libraries and museums, represents a potential risk for library materials; besides all these potential deterioration effects, *A. fumigatus* is an opportunistic human pathogen. Air microbial loads and thermo-hygrometric measurements showed that the repository was not suitable to prevent parchment microbial deterioration, suggesting the environmental remediation of the repository and the routine monitoring of air.

In addition to microorganisms, other important causes of stone deterioration are physical and chemical agents; a chief factor in the weathering of stone monuments is the atmospheric pollution. Sulfur dioxide is one of the major gaseous components of polluted atmospheres in urban areas; when dissolved in water, sulfur dioxide forms sulfurous acid, which is oxidised to sulfuric acid, that attacks stone resulting in the formation of gypsum. During the crystallization of gypsum, airborne organic pollutants, carbonaceous particles and dust are accumulated at the surface of buildings and trapped in the mineral matrix, resulting in the formation of the black crusts. The black crust formation causes a heavy decay phenomenon, due to solubilization and recrystallization of calcium sulfate. Mechanical and chemical treatments are traditionally adopted for the black crust removal, but they are not always selective; moreover, when there are different weathering forms on a stone surface, it is difficult to use a single cleaning procedure. In this respect, an enormous contribution in the preservation of stonework can come from the microbial world. In **Chapter 4** the removal of sulfate-based crusts from stone artworks using an alternative cleaning technology employing the sulfate-reducing bacteria (SRB) is discussed. SRB reduce sulfate to gaseous hydrogen sulfide and, in the last decade, they have been effectively used for the removal of gypsum and black crust. This biological methodology has been proven to be an effective cleaning procedure if compared with the traditional cleaning techniques. However, some concerns still remain: it is a time-consuming process when the crust is thick, and it is not clear how the biotechnological approach can fit within a complex conservation treatment. To address these challenges, the effects of an SRB strain (*Desulfovibrio vulgaris* subsp. *vulgaris* ATCC 29579) coupled with the non-ionic detergent Tween 20 pretreatment was studied on a stone column

affected by black crusts. Stereomicroscope observations, X-ray diffraction, Fourier Transformed Infrared Spectroscopy and Environmental Scanning Electron Microscopy analyses were performed. The coupling of the two cleaning treatments removed the black crust without affecting the original sound marble, with 38% reduction in cleaning time. The combined method was later applied to the one-century-old artistic marble statue, a funeral monument realized by Lina Arpesani in 1921 in memory of 'Neera', the poetess Anna Zuccari. The chemical, mineralogical and morphological analyses confirmed that the statue was weathered by sulfate-based crusts and grey deposits. A barium sulfate layer was found in the black crust stratigraphy, most probably deriving from an undocumented conservation treatment employing the barium hydroxide technique. The detergent Tween 20 used alone effectively removed the grey deposit, but not the black crust. However the co-treatment synergy resulted in the complete removal of the black crust layers, with the added advantage, compared to the biocleaning alone, of fewer biological applications and a 70% reduction in total cleaning time, but still retaining all the advantages of the biocleaning approach. Bacteria were also able to reduce the barium sulfate being completely removed by the microbiological method.

Over the past decades, synthetic resins have been applied on stone monuments, both as consolidants, protectives and adhesives, to enhance their long-term preservation. Although it is generally thought that synthetic resins are less prone to chemical, physical and biological deterioration than other organic products, there are some reports in the conservation of cultural heritage literature claiming the contrary. The chemical degradation of synthetic polymers include changes in chemical structure, reduction or increase in molecular weight due to chain scission and crosslinking respectively. Microorganisms are also able of degrading synthetic resins, producing enzymes that can attack the resins, causing changes in the viscosity, pH and colour of polymer emulsions. At present, the traditional way for removing a degraded synthetic polymer is the use of mixtures of solvents, that pose some health risks. In **Chapter 5** a method to select bacteria able to remove synthetic polymers from cultural heritage surfaces is proposed; synthetic polymers, in fact, can act as a growth substrate for microorganisms.

The ability of five bacteria to attack Paraloid B72, the most commonly used polymer in conservation treatments, was evaluated by optical and scanning electron microscopy observations, weight loss measurements, Fourier Transform Infrared Spectroscopy and Differential Scanning Calorimetric analysis. As all these techniques have proved to be very useful techniques in both polymer science and microbiological investigations, they were applied to evaluate naturally aged Paraloid B72 susceptibility to bacteria. *Pseudomonas rne* (PA01), *P. stutzeri* (ATCC 23856), *P. putida* (DeFENS collection, isolated from wastewater treatment plant), *Escherichia coli* (ATCC 25404) and *Bacillus licheniformis* (DeFENS collection, isolate from a biodeteriorated acrylic painting on canvas by a contemporary artist) were the test bacteria used as inocula for biodegradation tests. Although none of the bacteria were able to attack Paraloid B72, the methodology developed can be applied to select other bacteria with this ability. Therefore the results offer insightful guidance to a better design of bioremoval experiments of synthetic resins used in conservation. Biotechnology has been proved promising for a selective and environmental/health safe approach in the cultural heritage field, for the removal of altered layers on stone monuments. Consequently, each study that relates with the use of viable microorganisms for bioremoval is an advance in the cultural heritage conservation field.

In conclusion, this project shows that:

- non-invasive sampling coupled with selective molecular techniques have proved effective for an exhaustive investigation of both stained surfaces of historical documents and air quality of the repository where the documents are stored, and very useful for their correct management;
- bioremediation techniques, using SRB, can fit within a complex conservation treatment using a soft detergent Tween 20, resulting in homogeneous, satisfactory and rapid removal of black crusts;
- bioremediation techniques, promising in the cultural heritage preservation, can be implemented, opening new horizons of applications, e.g. the removal of synthetic coatings from stone monuments.

Biotechnology for the preservation and restoration of culturally relevant artworks not only deals with the identification of the living organisms by molecular-based techniques but also with the development of bio-based methodologies that contribute to the biocleaning of weathered artworks. Presenting three exemplary case-studies, this thesis makes a contribution to cultural heritage preservation and restoration.

Introduction

Biodeterioration is any irreversible change in the properties of a material caused by the activity of organisms belonging to different systematic groups (Hueck 1968). Bacteria, fungi (including yeasts), algae and lichens, because of their biodeteriorative potential, are considered one of the most important biodeteriogens, causing problems in the conservation of cultural heritage; this holds true for both historic artefacts and art made of modern materials. Art is made of both organic and inorganic materials, equally subjected to biodeterioration phenomena. Despite this, the mechanisms and phenomenology of biodeterioration can be different, involving physical and chemical processes, or aesthetic damage. Physical deterioration occurs as biodeteriogens penetrate into the materials (e.g. fungal hyphae), causing changes in the physical structure of the material or, in the worst case, loss in matter. Biodeteriogens can also produce acids or enzymes able to degrade the substrate, using it for their growth; this results in chemical damages. Microorganisms can also cause serious staining of buildings, paintings, costumes, ceramics, mummies, books and manuscripts, releasing coloured metabolites (Sterflinger and Piñar 2013). Cultural heritage biodeterioration commonly results from the complex interaction established by different kind of microorganisms co-existing simultaneously and the substrate. The biodeterioration phenomena observed on materials of cultural heritage are determined in particular by two factors: (1) the climate and exposure of the object and (2) the chemical composition and nature of the material itself. The degree of biological colonization of a surface depends not only on environmental factors but also on the intrinsic properties of the material (Guillitte, 1995). The bioreceptivity of a material is defined as ‘the totality of material properties that contribute to the establishment, anchorage and development of fauna and/or flora’ (Guillitte, 1995). Thus two different types of material may undergo different degrees of colonization under the same environmental conditions.

Stone is one of the most important inorganic material traditionally used for both construction and ornamental purposes. Most of the worldwide cultural heritage monuments are built using this porous material (Miller et al. 2012). On stone and other building materials, such as concrete, mortar, slurries and paint used in architecture, fungi may be the most important biodeteriorative organisms, contributing to the deterioration phenomena (Piñar and Sterflinger 2009). In particular, black fungi have been considered by several authors the most harmful microorganisms of outdoor stone materials and they have been isolated on marble and limestone in several environments, including outdoor monuments, archeological sites and indoor environment (Gorbushina et al. 1993; Saarela et al. 2004). Cyanobacteria, algae and lichens also contribute to the weathering of stone in humid as well as in semi-arid and arid environments (Lamprinou et al. 2013).

Biodeterioration of heritage collections caused by microorganisms is a worldwide problem, involving not only inorganic but also organic materials. Organic material biodeterioration is an essential process in the environment that recycles complex organic matter and is an integral component of life (Coleman and Williams 2002). Synthetic polymers have been applied in the past especially on stone monuments to enhance their long-term preservation, e.g. water repellents to preserve the artefacts from further deterioration (Amoroso 2002; Cappitelli et al. 2007a). With advancements in materials science over the past few decades, there has been a dramatic increase in the use of synthetic polymers by both artists, as original constituents of works of art, and conservators (Cappitelli and Sorlini 2008). Some reports in the scientific literature claim that microorganisms are capable of degrading polymeric materials protecting lithoid materials. Biodeterioration of these new materials is due to enzymatic action of hydrolases, that can attack the resins, causing changes in the viscosity, pH and colour of polymer emulsions. Generally, filamentous and black meristematic fungi were the agents causing deterioration of these materials, although some bacteria, yeasts, algae, and lichens that are capable of growing on synthetic polymers have been isolated (Cappitelli and Sorlini 2008). Among black fungi, *Cladosporium* sp. has been found to attack a freshly dried synthetic polymer used to consolidate marble of the

Milan Cathedral, causing chromatic changes due to pigment excretion, and aesthetic decay processes due to the presence of hyphae or pigment production (Cappitelli et al. 2007a). A yeast able to degrade the coating Incralac, an ethyl methacrylate and butyl acrylate copolymer widely used for protecting outdoor bronze monuments, was isolated from the bronze monument of George Washington at New York City (McNamara et al. 2004). A colonization by the alga *Stichococcus bacillaris* was noticed in the Roman archaeological site at Luni in northern Italy after treatment with an epoxy resin and an acrylic-siliconic resin (Favali et al. 1978). Lichens were reported to deteriorate a synthetic polyester resin that was used as a consolidant of stucco walls and column capitals in the Roman city Baelo Claudia in Spain (Ariño and Saiz-Jimenez 1996). Other cases of modern materials, increasingly important art objects in modern collections, undergoing rapid deterioration, are reported in the literature (Cappitelli et al. 2006; Gu 2007).

Biodeterioration of organic materials also results in a loss of valuable cultural properties stored in libraries, archives, and repositories. Since the beginning of mankind, human beings have strived to pass on their thoughts and knowledge to other people and to future generations (Cappitelli et al. 2010); although many materials have been employed during the history of humankind, the most commonly used graphic supports include papyrus, parchment, and paper (Cappitelli and Sorlini 2005). Archives, museums and libraries conserve many historical document collections of important cultural value for all humankind. In this respect, the cultural role played by papyrus, parchment and paper in knowledge dissemination has been immense. Unfortunately, biodeterioration phenomena pose a serious risk for the cultural heritage conservation of these materials. The mechanism of deterioration usually depends on the structure of the materials on which the microbes have been growing, while the chemical composition of the substrate determines the genera of the microorganisms (Szczepanowska and Cavaliere 2003).

The susceptibility of parchment to microbial deterioration is dependent upon the raw material, methods of production, and conditions of preservation (Cappitelli and Sorlini 2005). Until now, few microbiological studies aimed at investigating the role of microorganisms in parchment biodeterioration have been conducted; the majority of published papers have focused on the biodeterioration of books, maps and other historical paper documents. Parchment, known in Europe as early as the second century A.D., is originated in Asia Minor (Ustick 1936). Parchment replaced papyrus rolls as medium for the dissemination of the knowledge, because parchment was more durable under poor traveling conditions, it could be written on both sides and it was more suitable for easy reference than the cumbersome scroll. Moreover, parchment was easily available, as the raw materials were available everywhere. Sheep, cows, goats, rabbits, and squirrels all provide the skins for various qualities of parchment. Parchment was used as the common writing material from the second century BC to the end of the Middle Ages, when it was joined by paper (Reed 1972). However, it has continued to be used for special purposes, such as bookbinding or official documents. The chemical composition of parchment is based on collagen, a natural biopolymer, constructed from a triple helix with random coil telo-peptides (Reed 1972). Works of art supported on parchment represent highly composite objects, as parchment is subjected to mechanical and chemical treatments to receive writing, making the material more susceptible to biodeterioration. As a consequence, the degradation of ancient parchment is a complex phenomenon which involves several processes other than the hydrolytic cleavage of the peptide backbone (Florian 2007). The literature dealing with the biodeterioration of this material mainly examined fungal contamination; however bacteria with proteolytic activity and, in particular, collagenolytic activity, able to grow on parchment as the sole source of carbon and nitrogen, were isolated (Kraková et al. 2012). Microorganisms can hydrolyse collagen fibres and other proteinaceous structures of parchment, but can also act modifying its inorganic components, or can produce pigments and organic acids which discolour parchment and cause indirect damage (Pinzari et al. 2012). Environmental conditions, such as air flow, temperature and relative humidity, influence the microbial prevalence inside archives and cultural heritage repositories. Aerobiological investigations of conservation environments are indeed helpful in

choosing interventions aimed at establishing the sources of contamination and preventing the microbiological deterioration of artworks (Favali et al. 2003). During the last decade, in fact, the environmental conditions of the museum exhibition facilities and storage areas - such as relative humidity, environmental temperature and the temperature of exhibits, as well as improper lighting and atmospheric pollution - have been shown to be the most crucial factors concerning the preservation of collections and artifacts (Pavlogeorgatos 2003). Today's challenge in microbial investigations on historic documents is to characterise both airborne and superficial communities using culture-independent molecular approaches that do not affect the integrity of the documents (Cappitelli et al. 2010). However, until now, investigations of surface-associated microflora have been focused mainly on paper manuscripts (Michaelsen et al. 2012; Principi et al. 2011). As the historical value of parchment manuscripts is inestimable, it is necessary to make meaningful the microbiological studies on this material.

Generally, microorganisms are considered biodeterioration agents. They can deteriorate both organic (e.g. wood, paper, textiles, leather, parchment and paintings) and inorganic materials (e.g. glass, metals, stone and related materials). From a different point of view, microorganisms can be considered biological agents rich in virtues. In fact, they play a very important role in soil and water bioremediation, they are indispensable in the production of some food and beverages. Recently they have been used as means of conservation of stone, playing a very important role in bioconsolidation and biocleaning of works of art made of stone (Sorlini and Cappitelli 2008). Microorganisms are considered consolidant agents as microbial activity may lead to the accumulation of hydrogen carbonate and carbonate ions, and indirectly to pH increase that favors CaCO_3 precipitation and stone consolidation (Castanier et al. 1999; Jroundi et al. 2010). The first article describing the effect of calcifying bacteria on deteriorated stones was by Atlas (1988) who, using *Desulfotribrio desulfuricans* to remove black crusts from stone, also evidenced the effects of biocalcification. New findings related to biocalcification showed that bacterial carbonatogenesis is not species or strain specific, but depends on the type and mineralogy of the substrate; moreover, the precipitation of calcite can be achieved by the indigenous bacterial community of stone monuments directly applying a sterile medium on the surface itself (Rodriguez-Navarro et al. 2012). Biocleaning strategies employ both viable cells and enzymes to prevent the deterioration of works of art and to restore them when they are altered (Sorlini et al. 2010). The use of microorganisms to deal with contaminated soils, oil spills or chemical waste are in fact well-developed biotechnologies (Atlas 1995; Jördening and Winter 2005), but the application of bioremediation to ameliorate the effects of stone deterioration is still in its infancy (Webster and May 2006). During the last ten years this new technology, based on the use of microorganisms to clean the surfaces of altered works of art, have been extended to different weathering forms (Sorlini and Cappitelli 2008). Until now, viable cells of bacteria have been applied with successful results to remove the following alterations from the surfaces: (i) 'sulfatation' of stones, resulting in black and white crusts; (ii) 'nitratation' resulting in crumbling and pulverization. Bioremediation of artworks is mainly based on the use of sulfate- and nitrate-reducing bacteria (SRB and NRB, respectively) and hydrocarbon-degrading bacteria (HDB) (Fernandes 2006). SRBs (e.g. *D. desulfuricans* and *D. vulgaris*) have been proven to be the most satisfactory and efficient treatment for the removal of gypsum on stone exposed to polluted environment, reducing sulfate to gaseous hydrogen sulfide (Gioventù et al. 2011). Biocleaning of calcium sulfate has been tested, successfully, on many materials including white and coloured marbles (Cappitelli et al. 2007b) and porous limestone (Polo et al. 2010). NRBs, which reduce nitrates to gaseous nitrogen or nitrous oxide, have been used for the removal of nitrates. The nitrate-reducing bacterium *Pseudomonas pseudoalcaligenes* has been used to remove nitrates from the external walls of the Cathedral of Matera, affected by nitrification (Alfano et al. 2011). HDBs are capable of removing organic matter applied in the past to stone surfaces during restoration interventions. One example is the application of aerobic heterotrophic viable bacterial cells to a fresco of 14th century by Spinello Aretino in the Monumental Cemetery of Pisa (Ranalli et al.

2005). The potential of microorganisms for effective bioremediation of deteriorated cultural heritage materials is gradually being unveiled, and promising results of this methodology have been obtained in field sites. Despite the variety of methods used for cleaning stone surfaces, one reason to explore new treatments is to find a method able to remove, with great precision and efficiency, even very thin layers (down to a few micrometers) of materials, thus allowing the operator to stop the cleaning process at any selected level (Sabatini et al. 2000). However, until now, the conservation treatments employing viable cells do not represent a routine treatment in the cultural heritage preservation. Moreover, although microbiological cleaning appears to be a valid tool, the feasibility of using these treatments needs to be assessed on a case-by-case basis. Along with this, the application of biocleaning in the preservation of stoneworks needs to be developed, and efforts need being made to implement bio-based methodologies that may actively contribute to the bioremediation of weathered historic artworks.

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Aims of the work

Microorganisms are considered biodeteriogen agents, due to their ability to colonise both organic and inorganic historic artefacts. On the other hand, they are rich in virtues for their ability to degrade the organic and inorganic matter disfiguring stone materials.

The aim of this work was to investigate these two sides of microorganisms in the cultural heritage conservation, starting from exemplary case-studies.

1. The first case-study is represented by seven 16th century manuscripts written on finely illuminated parchment, reporting liturgical music used in religious ceremonies, presenting brown stains all over the sheets. The first objective was to characterise the surface-associated microbial communities on parchment manuscripts using non-invasive sampling followed by molecular analysis. The surface-associated community study was coupled with aerobiological monitoring in close proximity to where the manuscripts are stored, by an exclusively molecular approach. The intent was to supply some general guidelines for the correct management of historical documents, increasing the few reports about the biodeterioration of parchment, a precious material support.
2. The second case-study was a marble funeral monument, realized by Lina Arpesani in 1921 in memory of 'Neera', the poetess Anna Zuccari, located at the Cimitero Monumentale of Milan. The statue was characterized by two alteration forms: a grey deposit covering all the surface and black crust alterations. The aim here was to explore new application fields of the biocleaning of black crusts and assess the effects of interaction between biocleaning and other chemical cleaning procedures like the use of detergents, coupling the two treatments for the removal of black crusts and grey deposits on the marble statue.
3. Finally, a methodology to select bacteria able to remove synthetic polymers was designed in a laboratory scale, with the aim of implementing the bio-based methodologies that until now have actively contribute to the bioremediation of weathered stone artworks, proposing a valid alternative to the traditional cleaning methodologies for the bioremoval of synthetic polymers from surfaces of cultural heritage.

Assessing the microbiological risk to stored 16th Century parchment manuscripts: a holistic approach based on molecular and environmental studies¹

Abstract

The microbial risk for the conservation of seven 16th century parchment manuscripts, which showed brown discolouration putatively caused by microorganisms, was evaluated using non-invasive sampling techniques, microscopy, studies of surface-associated and airborne microflora with culture-independent molecular methods, and by measuring repository thermo-hygrometric values. Microscopic observations and ATP assays demonstrated a low level of contamination, indicating that the discolouration was not related to currently active microbial colonisation. Nevertheless, a culture-independent molecular approach was adopted to fully characterise surface-associated communities searching for biodeteriogens that could grow under appropriate thermo-hygrometric conditions. Indeed, potential biodeteriogens and microorganisms that are ecologically related to humans were found, suggesting the need to control the conservation environment and improve handling procedures. Microbial loads of air and thermo-hygrometric measurements showed that the repository was not suitable to prevent the microbial deterioration of parchment.

A holistic approach to the assessment of risk of microbial deterioration of documents and heritage preservation is proposed for the first time.

1. Introduction

Since early times, humans have strived to transmit thoughts, information and knowledge to other people and to future generations. Evidence of the intellectual and cultural efforts of the human race can be found in historical documents, including those made of parchment. The major component of parchment is collagen, an organic polymer that is susceptible to deterioration by various microorganisms, especially bacteria and fungi, and which serves as an energy and carbon source (Cappitelli et al. 2005; Michaelsen et al. 2009; Jurado et al. 2010; Sterflinger & Pinzari 2012). The microbial degradation of parchment causes various kinds of damage: fungi and bacteria with collagenolytic and proteolytic activities can hydrolyze collagen fibres and other proteinaceous molecules of parchment, and they can also act by modifying inorganic components or produce pigments causing discolouration (Pinzari et al. 2012).

The biodeterioration of historical parchment is a cause of great concern for libraries and archives all over the world (Cappitelli et al. 2010). Although the microbial attack of parchment can occur as part of a natural process, today's scientific research aims at preventive and active conservation that is aimed at slowing down the rate of deterioration significantly. Considering the health hazards of microbial contamination, the low cost of prevention compared to recovery costs, and human health and environmental concerns raised by the use of chemicals for disinfection treatments, the modern trend is to focus on preventive measures (Florian 2002; Cappitelli et al. 2005). Researchers are united in considering the following key steps as crucial to the assessment of the level of actual or potential biological risk, and to properly plan long-term conservation for historical documents: i) non-invasive sampling techniques, ii) quantification of microbial colonisation and of airborne populations in the conservation environment, iii) identification of potential biodeteriogens on surface and air by highly sensitive molecular methods, and iv) control of environmental conditions, particularly temperature and relative humidity (Michaelsen et al. 2006; Cappitelli et al. 2010; Sterflinger & Pinzari 2012). As the culture-dependent methods traditionally used in conservation detect only small amounts of effective surface-colonising and airborne organisms (Michaelsen et al. 2006), today's challenge in microbial investigations on historic documents is to characterise both airborne and superficial communities using culture-

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independent molecular approaches that do not affect the documents' integrity (Cappitelli et al. 2010). To date, no work has been done to face the conservation problem with such a holistic approach. In recent years, investigations of surface-associated microflora have been focused mainly on paper manuscripts (Michaelsen et al. 2006, 2009, 2010, 2012; Principi et al. 2011), while the few studies on parchment were conducted using only culture-based approaches (Pinzari et al. 2012; Kraková et al. 2012) or invasive sampling (Jurado et al. 2010). In any case, all these studies were performed without considering either climate control or aerobiological investigations. The present study evaluated microbial risk in the conservation of seven 16th century manuscripts written on finely illuminated parchment, reporting liturgical music used in religious ceremonies. An initial inspection of the parchment revealed two types of discolouration putatively caused by microbial colonisation: (1) brown stains all over the pages, and (2) brown rings on the bottom edges of the sheets (Figure 1).

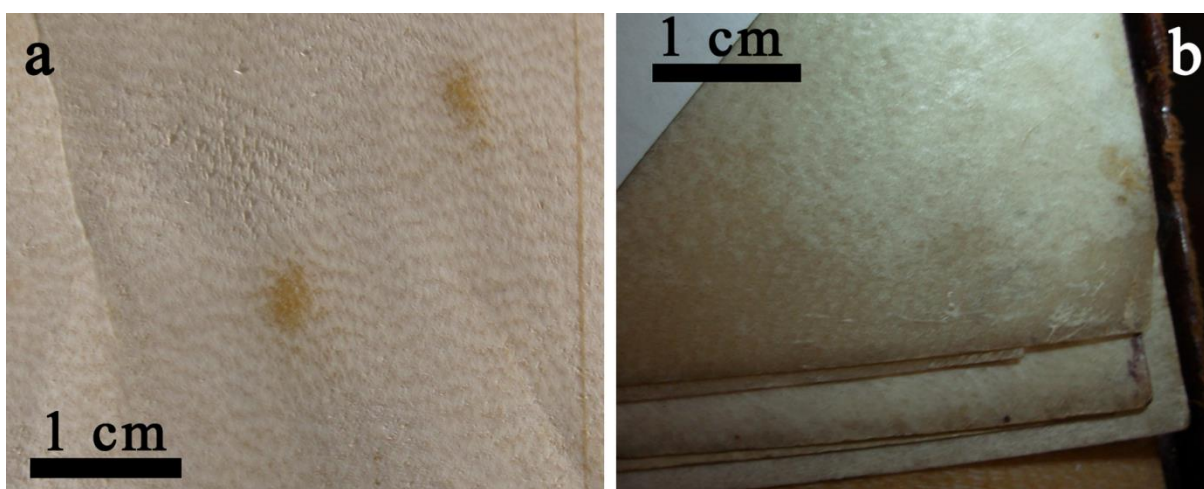


Figure 1. Brown stain on pages (a) and brown ring on lower-external edges of sheets (b).

The aims of this work were: (a) to clarify any relationship between the presence of an active microbial community and discolouration, (b) to study microbial air quality and environmental conditions in the repository, and (c) to investigate the relationship between airborne and surface-associated microbial communities. In this way it was possible to: i) supply exhaustive guidelines for the correct conservation of manuscripts and ii) set up, for the first time, a holistic and routine method to assess the risk of microbial deterioration in documentary heritage preservation, taking into consideration studies of microorganisms on the parchment surface (using non-invasive sampling) and in the air, and the environmental parameters of the repository.

This is the first time that the microbial community on historical parchment has been investigated by both non-invasive sampling and fully culture-independent approaches. Furthermore, it is the first time that a surface-associated community study was coupled with aerobiological monitoring, again by an exclusively biomolecular approach.

2. Materials and Methods

2.1 Sampling

Thirty-three samples from areas of discolouration, putatively caused by microorganisms, and apparently non-discoloured areas (7 samples) were collected by sterile nitrocellulose membranes following the non-invasive method reported by Principi et al. (2011), and subjected to molecular analysis. In brief: nitrocellulose membranes (Sartorius AG, Göttingen, Germany), 47 mm in diameter (corresponding to an area of 17.34 cm²) and handled with sterile forceps, were gently pressed for 30 s onto the surface of the manuscript using sterile swabs, then immediately transferred into tubes containing phosphate buffered saline (PBS, Sigma Aldrich, Milan, Italy) and transported to the laboratory for processing.

Adhesive tape strip (Fungi Tape™, DID Milan, Italy) was used to collect samples of biological structures from stained and apparently non-discoloured parchment as described by Michaelsen et al. (2012). The adhesive tapes were stored on sterile plates and transferred to the laboratory for microscopic analysis.

Airborne microorganisms were collected at five sites in the repository (Figure 2) in both summer and winter. Three replicates of 120 l of air for each sample were collected with a MAS-100 portable bioaerosol sampler (Merck), flow rate 100 l min⁻¹, onto Petri dishes containing two different media viz. plate count agar medium (PCA, Merck) and potato dextrose agar medium (PDA, Merck), to determine the microbial charge of aerobic heterotrophic bacteria and fungi respectively. The agar plates were kept at 28°C for 48 h. After growth, the colonies were counted and the results expressed as colony forming units per cubic meter of air (CFU m⁻³). Two replicates of 275 l of air for each site were collected also with an AGI-30 impinger in 25 ml of sterile PBS, rate flow 4.55 l min⁻¹, as reported by Polo et al. (2012), and transported to the laboratory for molecular processing. During the air sampling days, the temperature and relative humidity were monitored with the sensor Hygrolog-D (Rotronic AY, Swiss).

Table 1 shows the details of the samples, code, location, type of discolouration and performed analyses. The sample codes are presented in the form XY.Z.(I), where X indicates the sample source (P for parchment and A for air), Y represents the manuscript (numbered 1 to 7) for samples from parchment, or the sampling season for air samples (S for summer and W for winter), Z indicates the sampling page (for samples from parchment) or the sampling sites in the repository (for air samples) (see Figure 2). Only for the samples taken from the manuscripts, 'I' represents the page number.

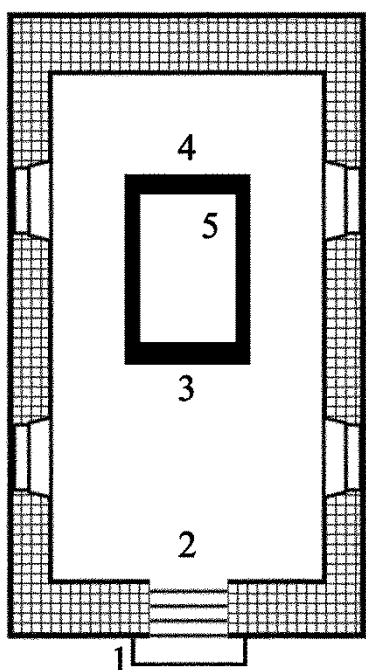


Figure 2. Repository plan. The rectangle inside the repository indicates the bookcase. Numbers indicate the air sampling sites both outside (1) and inside (2-5) the repository door.

2.2 Microscopic analysis of the chromatic changes

Adhesive tape strips were mounted on a microscope glass slide and fixed in 4% paraformaldehyde solution (Sigma-Aldrich) in 0.1 M PBS pH 7.2 for 2 h on ice. After three PBS washing steps, a portion of the tape confined by *in situ* frames (1 cm² area; Eppendorf) was stained with 100 µl of 10 mM SYTO 9, a green-fluorescent nucleic acid stain (Invitrogen) and 100 µl of 0.4 mg ml⁻¹ Fluorescent Brightener 28 (Sigma-Aldrich) for 20 min in the dark at room temperature, to label bacteria and chitin in the walls of fungi, respectively. After three washes with demineralized water, all samples were observed by epifluorescence microscopy with a Leica DM 4000 B (Leica Microsystems, Milan, Italy) and images were acquired by the CoolSNAP CF camera (Photometrics Roper Scientific, Rochester, USA).

TYPE OF SAMPLE (CODE XY.Z.(T)), POSITION AND ALTERATION				TYPE OF ANALYSIS			
SURFACE		AIR		MICROSCOPY	CELL VIABILITY	MOLECULAR METHODS	MICROBIAL LOAD
ON THE PAGE	LOWER-EXTERNAL EDGES OF SHEET						
NON-DISCOLOURED SURFACE	BROWN STAIN	BROWN RING					
		P1.1.1 P1.10.1 P1.21.1 P1.32.1 P1.42.1 P1.52.1 P1.108.1 P2.1.2 P2.139.3 P3.69.3 P4.1.1 P5.30.2 P5.49.2 P6.1.1 P7.41.2 P7.77.22				X	X
	P1.10.2 P1.10.3 P1.21.2 P1.32.2 P1.52.2 P1.108.2 P2.1.1 P3.69.1 P3.69.2 P5.30.1 P5.49.1 P6.1.2 P7.41.1 P7.77.1					X	X
	P2.139.2 P4.1.2			X	X	X	
P2.139.1				X	X	X	
P1.108.3 P3.69.4 P4.1.3 P5.30.3 P6.1.3 P7.77.3					X	X	
			AS.1 AS.2 AS.3 AS.4 AS.5 AW.1 AW.2 AW.3 AW.4 AW.5			X	X

Table 1. Samples collected for microbiological analyses: sample code, type of discolouration, location and analyses. The sample codes are presented in the form XY.Z.(T), where X indicates the sample source (P for parchment and A for air), Y represents the manuscript (numbered 1 to 7) for samples from parchment, or the sampling season for air samples (S for summer and W for winter), Z indicates the sampling page (for samples from parchment) or the sampling sites in the repository (for air samples). Only for the samples taken from the manuscripts, T represents the page number.

2.3 Viability assessment of colonising community

The viability of the surface-associated microbial community was assessed *in situ* by measuring the relative light units per second (RLU s⁻¹), using a surface hygiene test kit (Promicol) and a FB 14 Vega bioluminometer (Berthold Detection Systems). The surfaces studied were 17.34 cm² for each site investigated. The sampling was conducted by means of a circular frame corresponding to the area of the cellulose-membrane used for the non-invasive sampling of the discolourations. For each manuscript, one site apparently without discolouration was used as a control. The ATP content was measured according to the manufacturer's protocol. The RLU s⁻¹ values were converted to ATP concentrations (nmol) using an ATP Standard Kit (Promicol) as standard, and thus in nmol cm⁻², by dividing by sampled surface values. Viability measures of each site (both with and without discolouration) were performed in duplicate. For each manuscript, the mean values, the standard error of the mean, and analysis of variance (ANOVA) were calculated using GraphPad Prism 4 to assess the significance of differences in nmol ATP cm⁻² among several of the surfaces investigated. Differences were considered significant with P-values < 0.05. Individual comparisons were made *post hoc* with the Tukey-Kramer test.

2.4 DNA extraction and amplification

On the same day as sampling, the nitrocellulose membrane filters with the sampled cells were vortexed for 15 min to detach cells from the membrane, and centrifuged at 6000 rpm for 30 min to concentrate cells. The pellet was resuspended in 1ml of lysis buffer (EDTA 40 mM, Tris HCl 50 mM pH 8, sucrose 0.75 M) and vortexed for 10 min. Both replicates of each air sampled by impinger were filtered through a sterile polycarbonate membrane (pore size 0.2 mm), and then put into tubes with 1.8 ml of lysis buffer and vortexed for 10 min in order to detach the cells. All samples were stored at -20°C. Total DNA was extracted directly from the surface and air samples as described by Ausubel et al. (1994), with the addition of three thermal cycles -80°C/ +70°C before the addition of lysozyme to break the cellular walls.

Bacterial communities were analysed by amplifying 16S rRNA gene fragments with primers 357 F (3'-ACGGGGGGCCTACGGGAGGCAGCAG-3') and 907 R (5'-CCGTCAATTCCTTTGATGTTT-3') with the following chemical conditions: 1X of PCR run buffer, 1.8 mM of MgCl₂, 0.2 mM of dNTP mix, 0.3 μM of each primer, 2 μg μl⁻¹ of bovine serum albumin (BSA) and 1.25 U of Taq DNA polymerase (GoTaq, Promega), and a thermal cycling program as reported by Polo et al. (2010). Fungal communities were analysed by amplifying the 18S rRNA gene fragments by semi-nested PCR performed as follows: a first amplification step using the combination of primers NS1 (CCAGTAGTCATATGCTTGTC) and EF3 (5'-TCCTCTAAATGACCAAGTTTG-3') with 1X of PCR buffer, 1.8 mM of MgCl₂, 0.2 mM of dNTP mix, 0.5 μM of each primer and 0.3125 U of Taq DNA polymerase (GoTaq, Promega); the cycling program consisted in an initial denaturation at 94°C for 5 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 45 s and extension at 72°C for 3 min, and a final extension at 72°C for 10 min. The second amplification step was performed using the first PCR product as template, with the primers NS1-GC (5'-CCAGTAGTCATATGCTTGTC -3' with GC clamp CGCCCGCCGCGCGGGCGGGCGGGGCGGGGCACGGG) and NS2 (5'-GAATTACCGCGGCTGCTGGC-3'). The reaction mixture was identical to first-step PCR except for 0.625 U of Taq DNA polymerase. The cycling program consisted in an initial denaturation at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 45 s and extension at 72°C for 2 min, and a final extension at 72°C for 10 min. All the PCR were performed in a final volume of 25 μl.

2.5 Denaturing gradient gel electrophoresis (DGGE) and profile analysis

The obtained amplicons were analysed by DGGE, as previously described by Polo et al. (2010). Amplicons from both superficial and air samples were loaded in the same gel to make the

DGGE profiles comparable. DGGE gels were performed with 40–60% and 30–55% denaturant gradients for the bacterial and fungal communities, respectively. After excising, the DGGE bands were eluted in 50 μ l milli-Q water by incubation at 37°C overnight and re-amplified with the same conditions as above, except for the absence of the GC clamp for primers. Reamplified PCR products of excised DGGE bands were purified with a QIAquick PCR purification kit (Qiagen) according to manufacturer's instructions and identified by sequencing (Primm, Milan). The sequences were analysed in September 2012 using BLASTN software (www.ncbi.nlm.nih.gov/BLAST).

The DGGE gels were run simultaneously and 16S rDNA and 18S rDNA band profiles were converted into computer digital images using the gel imaging system GelDoc (Biorad). Lanes were normalized to contain the same amount of total signal after background subtraction, and individual lanes of the gel images were straightened and aligned using Adobe Photoshop (Adobe Systems, Inc. Mountain View, CA, USA). The DGGE images were then transformed into line plot profiles using the ImageJ software (Rasband 2008), and then imported into an Excel file as x/y values. The X-axis represented distance along the line and the Y-axis was the pixel intensity. The matrix of x/y values of DGGE line profiles was analyzed using the principal component analysis (PCA). Multivariate investigations were conducted by XLSTAT (version 7.5.2 Addinsoft, France). The PCA type used during the computations was the Pearson's correlation matrix. The significance of the PCA-analysis model was tested by a cross-validation procedure.

3. Results

3.1 Epifluorescence microscopy

Tape samples from both the discoloured and non-discoloured surfaces showed a few microbial cells of filamentous shape. In general, most of the cells were stained with Fluorescent Brightener 28 (blue fluorescence) ascribable to eukaryotic microorganisms. Figure 3 shows the detail obtained from sample P2.139.2 (brown stain) and an apparently non-discoloured area (sample P2.139.1).

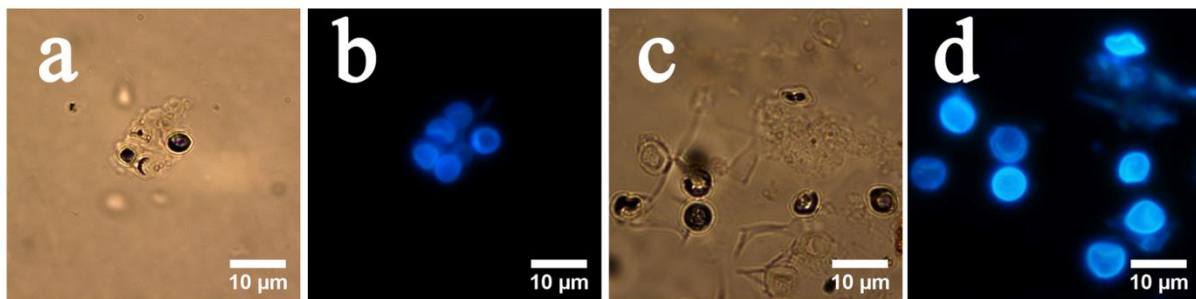


Figure 3. Cells stained with Fluorescent Brightener 28 (blue fluorescence) and SYTO 9 (green fluorescence) on tape samples: bright field (a) and epifluorescence (b) microscope images of apparently non-discoloured area (sample P2.139.1); bright field (c) and epifluorescence (d) microscope images of P2.139.2 showing brown stain.

3.2 Viability assays

On most of the surfaces of the manuscripts studied, cellular activity on the discoloured surfaces showed no significant changes compared to non-discoloured surfaces (P -values > 0.1), the exception being P1.52.1 (brown ring), P5.49.1 and P7.41.1 (brown stains) where the P -values were < 0.031 . The ATP values were between $6.9 \cdot 10^2$ and $3.0 \cdot 10^3$ nmol cm^{-2} .

3.3 DGGE, sequencing and community profile analysis

Figure 4 shows the DGGE profiles and Table 2 reports the strains identified from sampling the manuscripts and the air. To assess the role of the microflora evidenced by 16S and 18S DGGE, the microbial communities on discoloured and non-discoloured areas were compared by PCA-analysis. A plot of the two-dimensional scores for PCA-analysis from superficial samples

accounted for 83.3% of the variability in the input data for bacteria and 60.2% for fungi. The bacterial PCR product from discoloured surfaces was obtained only from samples collected on manuscript 1, the exception being sample P2.139.2 from manuscript 2. PCA-analysis showed that the bacterial communities on discoloured and non-discoloured (control samples P2.139.1 and P1.108.3) surfaces were statistically different, the exception being samples P1.10.1 and P2.139.2 (Figure 5a). The fungal PCR product was obtained only from 14 samples collected on all the manuscripts. Whilst samples from manuscripts 2-7 and control samples from non-discoloured surface presented statistically significant similar fungal communities, samples P1.1.1, P1.10.2, P1.21.2, P1.32.1, P1.52.2, P1.108.2, P2.139.2 and P3.69.1 presented separate clusters (Figure 5b). PCA-analysis of 16S and 18S DGGE profiles from both superficial and air samples was adopted to study the relationship between airborne and surface-associated microbial communities. The plot of the two-dimensional scores for PCA-analysis from both superficial and air samples accounted for 71.8 % of the variability in the input data for bacteria and 73.4% for fungi. Whilst control samples and samples P1.32.1, P1.108.1, P2.139.2, AS.2, AW.2, AW.4 and AS.1 presented statistically significant similar bacterial communities, the other samples from air and manuscript 1 showed statistically different bacterial communities (Figure 6a). No similarity was found between fungal airborne and surface associated communities (Figure 6b).

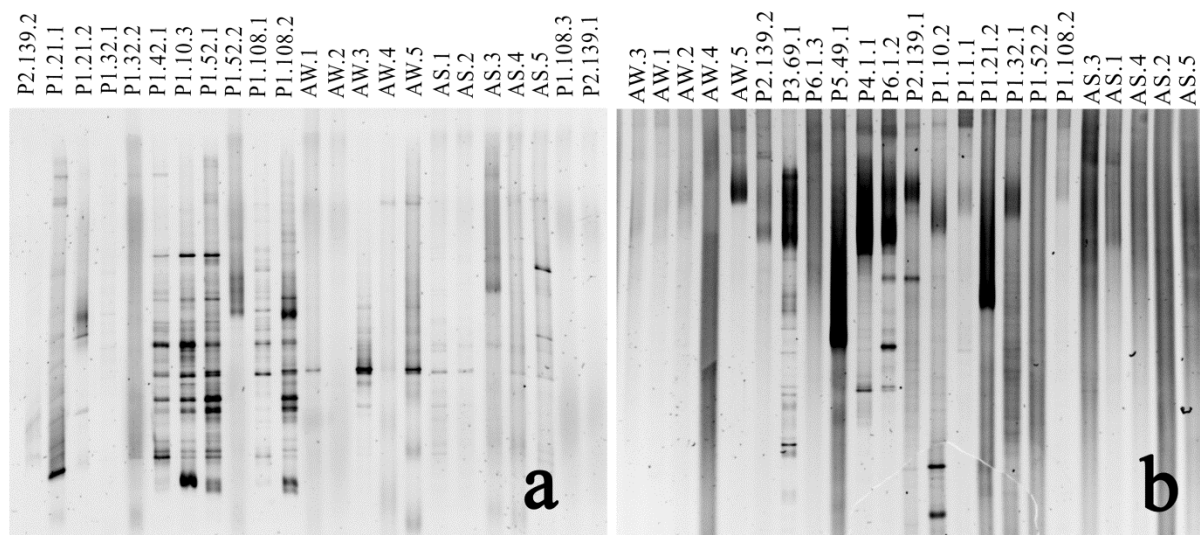


Figure 4. 16S (a) and 18S rRNA gene (b) DGGE profiles of the samples (P2.139.2, P1.21.1, P1.21.2, P1.32.2, P1.42.1, P1.103.3, P1.52.1, P1.52.2, P1.108.1, P1.108.2, AW.1, AW.2, AW.3, AW.4, AW.5, AS.1, AS.2, AS.3, AS.4, AS.5, P1.108.3 and P2.139.1 for 16S DGGE profiles; P2.139.2, P3.69.1, P6.1.3, P5.49.1, P4.1.1, P6.1.2, P2.139.1, P1.10.1, P1.1.1, P1.21.2, P1.32.1, P1.52.2, P1.108.2, AW.1, AW.2, AW.3, AW.4, AW.5, AS.1, AS.2, AS.3, AS.4 and AS.5 for 18S DGGE profiles).

3.4 Airborne community charges and microclimatic parameters

Cultural analyses of airborne communities showed that heterotrophic bacteria and fungi were present in the repository air. The microbial airborne loads (CFU m^{-3}), temperature (T °C) and relative humidity (RH %) monitored during the air sampling campaigns are reported in Table 3. In the semi-confined environment outside the repository (sampling site 1) the microbial airborne loads for heterotrophic bacteria and fungi were, respectively, 213 ± 155 and 533 ± 103 CFU m^{-3} in winter, and 486 ± 332 and 869 ± 164 CFU m^{-3} in summer. Inside the repository (sampling sites 2-5) the microbial airborne loads for heterotrophic bacteria were between 73 ± 12 and 267 ± 99 CFU m^{-3} in winter, and between 817 ± 92 and 1461 ± 141 CFU m^{-3} in summer; for fungi they were between 420 ± 191 and 480 ± 72 CFU m^{-3} in winter, and between 211 ± 54 and 481 ± 51 CFU m^{-3} in summer.

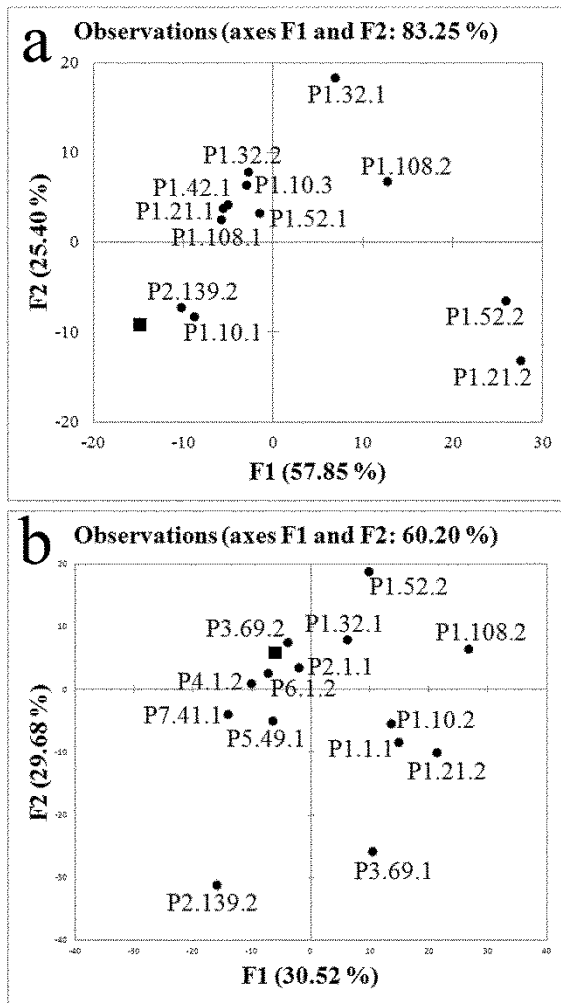


Figure 5. bacterial (a) and fungal (b) DGGE band patterns from samples collected on parchment surface. In panel (a) ■ represents sample P1.108.3 from the non-discoloured surface of manuscript 1 and P2.139.1 from manuscript 2; in panel (b) ■ represents the samples from non-discoloured surface of the manuscripts from 1 to 7.

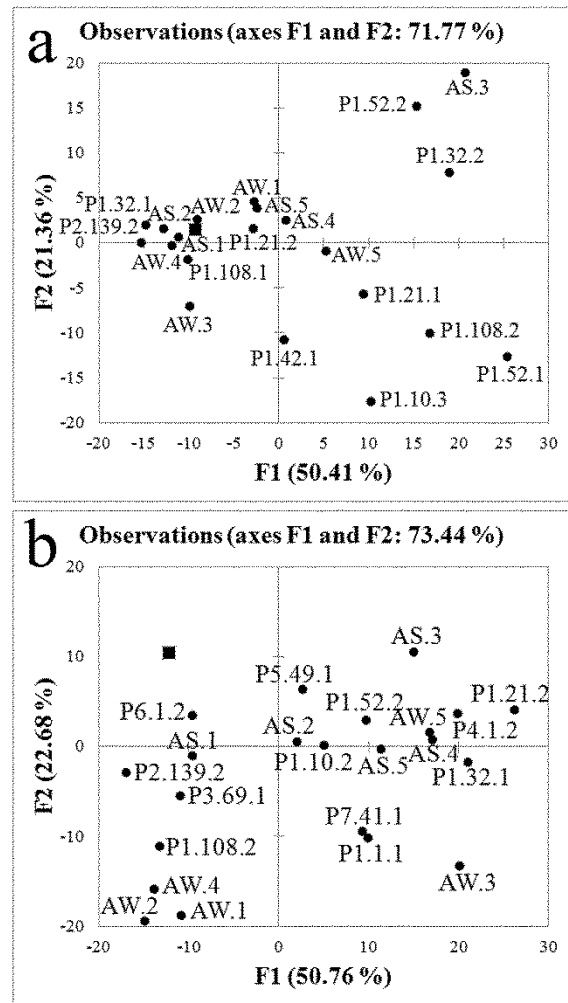


Figure 6. PCA-analysis of bacterial (a) and fungal (b) DGGE band patterns from parchment surface and air samples. In panel (a) ■ represents the sample P1.108.3 from the non-discoloured surface of manuscript 1 and P2.139.1 from manuscript 2; in panel (b) ■ represents the samples from non-discoloured surfaces of the manuscripts from 1 to 7.

Samples														BlastN reference strains			RDP taxonomic Classifier					
P1	P2		P3		P4		P5		P6		P7		Air summer	Air winter	B	Closest relative strain	Accession number	Similarity (%)	Most probable taxon	Similarity (%)		
BS	BR	BS	BR	BS	BR	BS	BR	BS	BR	BS	BR	BS	BR	in	out	in	Out					
X	X															X		<i>Burkholderia thailandensis</i>	DQ388537	99	<i>Burkholderia</i>	100
	X															X	X	Uncultured Betaproteobacterium	JQ937380	98	Betaproteobacteria	100
																X	X	Uncultured <i>Methylobacterium</i> sp.	HM565053	96	Rhizobiales	100
																X	X	Uncultured <i>Mesorhizobium</i> sp.	GU271769	96	Rhizobiales	100
X	X															X	X	<i>Methylobacterium</i> sp.	EU303272	99	<i>Methylobacterium</i>	100
X	X																X	<i>Microbacterium</i> sp.	JF279926	99	Microbacteriaceae	100
X	X																	<i>Lactobacillus sanfranciscensis</i>	JN863669	98	<i>Lactobacillus</i>	87
X	X																X	<i>Microbacterium</i> sp.	JX434128	98	Micrococcineae	100
	X													X		X		<i>Sphingomonas</i> sp.	JQ917912	99	Sphingomonadaceae	100
X	X														X	X		Uncultured <i>Methylobacterium</i> sp.	JF274019	98	<i>Methylobacterium</i>	100
X	X														X	X		<i>Aeribacillus</i> sp.	KC551235	100	<i>Aeribacillus</i>	98
X	X	X		X		X		X		X		X	X	X	X	X	X	Uncultured <i>Aspergillus</i> sp.	AJ635506	99		
X	X																	<i>Candida</i> sp.	HM161746	99		
	X																	<i>Aspergillus</i> sp.	HE814598	99		
X	X												X	X	X			<i>Aspergillus fumigatus</i>	JQ665711	100		
X	X																	<i>Candida albicans</i>	JN940588	99		

Table 2. Identification of 16S and 18S gene sequences of DGGE profiles from brown stains (BS) and brown ring (BR) on several manuscripts (P1-P7), and air during summer and winter, both inside (in) and outdoors (out) of the repository. (B) indicates the control sample from a non- discoloured area. X and - indicate, respectively, presence or absence of the strain.

Sampling site	Winter				Summer			
	Bacteria (CFU m ⁻³)	Fungi (CFU m ⁻³)	T (°C)	UR (%)	Bacteria (CFU m ⁻³)	Fungi (CFU m ⁻³)	T (°C)	UR (%)
1	213±155	533±103	6.7	27.4	486±332	869±164	24.5	67.7
2	73±12	420±191	5.2	45.5	1461±141	481±51	24.3	64.6
3	147±81	480±72	4.0	49.9	1292±258	394±5	24.3	62.4
4	207±136	440±80	2.1	61.2	1397±59	364±34	27.7	60.8
5	267±99	420±92	4.1	54.6	817±92	211±54	24.4	62.9

Table 3. Microbial airborne loads (CFU m⁻³), Temperature (T°C) and Relative Humidity (RH%) values registered at the air sampling sites (see Figure 2) during sampling days in winter and summer.

4. Discussion

In order to assess the role of the microflora dwelling on the discoloured areas, the microbial component of discoloured and non-discoloured surfaces was evaluated by microscopic observations and an ATP bioluminescence assay. The adhesive tape strip technique was chosen for microscopic analysis, as it has been shown to be a useful sampling method for monitoring microbial colonisation as well as the spatial distribution of microorganisms (Urzi & De Leo 2001; Villa et al. 2009). However adhesive tape could cause the removal of fragments from physically damaged parchment (Cappitelli et al. 2010), therefore, for the present study, only three surfaces were chosen as representative of the seven manuscripts: P4.1.2 and P2.139.2 as brown stain on page (P2.139.2 was the surface with the largest stain) and P2.139.1 as non-discoloured surface. No sample was collected by fungal tape from a brown ring as the parchment on the lower-external edges of the sheet was physically damaged (Figure 1b). Epifluorescence microscopy showed that, although in low number, both filamentous and circular structures, respectively ascribable to fungal hyphae and spores, dominated compared with bacteria. The small number of cells detected by epifluorescence microscope on both the discoloured and non-discoloured surfaces was the same, demonstrating that the discolouration on the surfaces studied was not caused by current microbial colonisation.

The determination of ATP via the firefly bioluminescence assay has been applied previously in the cultural heritage field for the detection of viable fungal spores contaminating paper documents (Rakotonirainy et al. 2003) and graphic documents (Rakotonirainy & Arnold 2008). In the present work, no statistically significant differences of ATP content on discoloured and non-discoloured surfaces were observed for most of the surface studied. It is important to note that ATP assay kits available on the market have been developed essentially for bacteria, from which ATP is more easily extracted compared to from fungi (Rakotonirainy et al. 2003). Thus the ATP results are more representative of the bacterial component of the community. At present, ATP extraction methods for fungal cells have been devised, but have been only applied to artificially contaminated paper or fragments cut from old documents (Rakotonirainy et al. 2003; Rakotonirainy & Dubar, 2013). The ATP data are in agreement with the few bacterial cells observed by microscopic investigation. Although samples P1.52.1, P5.49.1 and P7.41.1 showed cellular activities that were significantly higher than the control surfaces, the changes were less than one order of magnitude and the mean values of ATP per cm² did not differ from those detected in the other manuscripts on both discoloured and non-discoloured surfaces.

Microscopy and ATP results confirmed that the discolouration on the parchment was not related to current active microbial colonisation as the primary source of damage. However, the biological origin of discolouration due to past microbial activity cannot be excluded and it is known that microbial attack can result in loss of structure and irreversible distortion and staining (Szczepanowska 2013). In addition, the cause of the discolouration may not related to biological

agents. Prior to the development of the printing press, manuscripts were often written using iron gallotannate inks. Iron ions can leach from the ink to the substrate, causing brown discolouration (Brown & Clark 2002). Furthermore, degradation of the iron gallotannate ink complex can introduce a yellow to brown colour associated with oxidation to quinoid structures (Ciglanská et al. 2013).

Despite the low contamination level, it is important to study microbial communities on parchment surfaces as identification can highlight potential biodeteriogens, which could grow under favourable thermo-hygrometric conditions (Cappitelli et al. 2010; Principi et al. 2011). A culture-independent molecular approach based on PCR-DGGE from DNA directly extracted from environmental samples was adopted to fully characterise the surface-associated communities, as reported in several works in the cultural heritage field (Michaelsen et al. 2006; Polo et al. 2012). Indeed, Jurado et al. (2010) reported the use of a culture-independent molecular method for a microbiological study on parchment, but an invasive sampling procedure was applied. In this study, nitrocellulose membranes were used to collect cells because they provided a non-invasive sampling procedure, which has already been successfully applied on frescoes and paper (Pitzurra et al. 1999; Principi et al. 2011). To date, no study coupling a non-invasive membrane-sampling procedure with a culture-independent molecular method has been reported for parchment. The statistical approach based on PCA-analysis was used to study the structural changes in the bacterial and fungal communities between discoloured and non-discoloured surfaces evidenced by the 16S and 18S DGGE profiles, as previously reported by Principi et al. (2011). On manuscript 1, the bacterial community of the greater part of the samples from both brown stains and brown rings was dissimilar to that of the non-discoloured surface. Fungal communities were detected on all the manuscripts, in agreement with the microscopy analysis. Only the fungal samples from manuscript 1 presented statistically significant differences, compared to the non-discoloured surface. Of the manuscripts studied, manuscript 1 was the only one that had recently undergone conservation treatment. As conservation treatments involving organic materials (eg as adhesives and consolidants) could potentially support the growth of microorganisms, thus accelerating the biodeterioration process (Cappitelli et al. 2010), the results suggested that the conservation treatment might have contributed to conditions favourable for microbial growth. Although in the present investigation work no data were available for the microbial communities prior to the conservation work, such information should be taken into account in further studies. The sequences obtained in this study were phylogenetically most closely related to bacteria belonging to *Burkholderia thailandensis*, Betaproteobacteria, *Methylobacterium* sp., *Microbacterium* sp., *Lactobacillus* sp., *Sphingomonas* sp. and *Aeribacillus* sp., and fungi belonging to *Aspergillus* sp. and *Candida* sp.. Although species belonging to Betaproteobacteria have been mainly isolated from soil and water (Wongprompitak et al. 2008), *Burkholderia thailandensis* has been isolated from breathing apparatus (Glass et al. 2006), therefore its presence on the surface of manuscript 1 could be of human origin. Members of the genus *Methylobacterium* are aerobic phototrophic bacteria distributed in a wide variety of natural habitats, including soil, dust, air and fresh water as well as in man-made environments. Because of carotenoid and photopigments production (Hiraishi et al. 1995), *Methylobacterium* strains could potentially be responsible for discolouration. Strains belonging to the genus *Microbacterium* have been isolated from coloured stains on historical documents made of parchment (Kraková et al. 2012). Furthermore, they have proteolytic properties (Kraková et al. 2012) and can colonise subsurface layers along collagen fibres (Petushkova & Koesler 1999). Fermenting bacteria belonging to the genus *Lactobacillus* are common inhabitants of the human gastrointestinal tract as well as of the oral cavity (Müller et al. 2001; Walter & Ley 2011), so their presence on both the sheets and the external edges of the manuscript could be of human origin. *Sphingomonas* have been isolated from biofilm on deteriorated bas-relief walls (Lan et al. 2010) and mural paintings (Heyrman & Swings 2001), and they were reported as being responsible for the degradation of ceramic tiles covered by a green and/or black patina (Coutinho et al. 2013) and waterlogged

archaeological wood (Landy et al. 2008; Palla et al. 2013). Bacteria belonging to *Aeribacillus* sp. have never been isolated on cultural heritage and historical documents. However, the role of *Aeribacillus* spp. in the fermentation of starch, a substance often used in parchment conservation, makes these bacteria potentially dangerous (Fratkin & Adams 1946; Woods 1995). Fungi belonging to the genus *Candida* are commonly isolated from human skin and mucosal surfaces as harmless commensal organisms (Samaranayake & MacFarlane 1990; Heo et al. 2011) and their presence on manuscript 1 can be ascribed to the hand of readers as the manuscript was used in religious ceremonies and has, in the past, been subjected to conservation treatment. *Aspergillus* species are common biodeteriogens of organic and synthetic materials (Cappitelli & Sorlini 2005) and are frequently associated with paper spoilage as they are able to degrade cellulosic materials and cause discolouration (Pinzari et al. 2006; Zotti et al. 2008; Principi et al. 2011). Members of the genus *Aspergillus* are also known as the most active biodeteriogen agents on both ancient and modern parchments (Polacheck et al. 1989; Matè 2002). *Aspergillus* spp. secrete a range of pigments and proteolytic enzymes that respectively cause aesthetic and chemical damage, whilst hyphal growth exerts mechanical pressure on the substrate, causing weakness (Cappitelli & Sorlini 2005; Michaelsen et al. 2010; Kraková et al. 2012). *Aspergillus* spp. were isolated from both brown stains on all the manuscripts and from the brown rings of manuscript 1.

Aspergillus fumigatus is one of the most ubiquitous airborne saprophytic fungus. Being a xerophilic and xerotolerant fungus, it has often been isolated from the indoor aeromycoflora of libraries (Zielinska-Jankiewicz et al. 2008), museums (Gäuzère et al. 2013) and hospitals (O’Gorman, 2011). The proteolytic activity of *A. fumigatus* represents a potential risk for library materials as it might hydrolyse different complex proteins available as substrates, including collagen (Lee & Kolattukudy, 1995; Farnell et al. 2012). Besides all these potential effects on manuscript, handling mould-contaminated objects constitutes a health risk as *A. fumigatus* is an opportunistic human pathogen (O’Gorman, 2011; Pinheiro et al. 2011). Interestingly, there are other case studies in the literature reporting the identification of only one fungal genus, i.e. *Aspergillus* or *Penicillium*, in the air of archives (Borrego et al. 2010; 2012).

Although in this research the suitability and usefulness of using non-invasive sampling methods and molecular techniques to determine the presence and diversity of bacteria and fungi on heritage material has been demonstrated, these methods also have some drawbacks. The molecular approach can be time-consuming, requires skilled personnel and is often expensive (Cleland et al. 2013) and a major intrinsic limitation of non-invasive techniques is that microorganisms growing in the substrate without producing emerging structures cannot be collected (Cappitelli et al. 2010). The study of the microbial communities on the manuscript surfaces was undertaken together with the study of the microbial airborne communities and the environmental conditions in close proximity to where the manuscripts are stored as well as outside the repository. It is widely reported that microbial cells reach surfaces mainly through transport in air (Borrego et al. 2010) and that a close relationship exists between culturable airborne microorganisms and the microflora colonising paper documents (Cappitelli et al. 2010). Aerobiological and thermo-hygrometric investigations of conservation environments are therefore helpful in choosing interventions aimed at preventing the microbiological deterioration of historical documents. In the present work, the air-sampling procedure and the study of airborne microbial community were carried out without any cultivation step, in order to fully investigate the source of surface-associated biodeteriogens. This is the first time that such an approach has been proposed for historical document repositories. The microbial community fingerprints from DGGE gels were compared by PCA-analysis to investigate the relationship between surface-associated and airborne communities. The analysis showed the lack of a clear relationship between bacterial communities in air and on manuscript 1, and although some samples from both the brown stains and the rings were similar to some airborne communities detected inside and outside the repository during both winter and summer, most samples showed no such similarity. In addition, PCA-analysis excluded the presence of a relationship between

fungus communities in air and on manuscript surfaces. This lack of similarity was in contrast to data based on conventional culture-dependent methods (Cappitelli et al. 2010). Indeed, Polo et al. (2012) in a culture-independent investigation excluded a close similarity between airborne and surface-associated microflora on stone surfaces in an outdoor environment. There could be several reasons for this: the culture-based approach greatly limits the microflora that can be studied; the diluted concentrations of airborne microorganisms make detection difficult; environmental factors cause important quantitative and qualitative changes of airborne communities in space and time and the chemical and physical features of surfaces select colonising microorganisms.

The sequencing of bands of airborne microflora demonstrated that in addition to microorganisms belonging to *Burkholderia*, Betaproteobacteria, *Methylobacterium*, *Microbacterium*, *Sphingomonas* and *Aeribacillus* identified on the discoloured surfaces, airborne communities also included bacteria belonging to *Mesorhizobium*. *Mesorhizobium* sp. is a soil bacterium (González-López et al. 2005) and is most likely present because the repository is located in a rural area. *Aspergillus* was the only fungal genus detected inside and outside the repository in both winter and summer. *Aspergillus* spp. have been isolated from the air of indoor and outdoor environments (Arya et al. 2001; Borrego et al. 2010; Vanhee et al. 2010; Docampo et al. 2011; Borrego et al. 2012), and are considered primary colonizers: when the relative humidity increases, and indoor environments are without ventilation for long periods of time, conidia can be deposited quickly over documents and deteriorate them (Borrego et al. 2012).

Airborne *Methylobacterium*, *Microbacterium*, *Sphingomonas* and *Aspergillus* spp. are potential biodeteriogens. However, air microbiota are known to coexist with document collections without causing significant damage under suitable conditions of temperature and relative humidity.

However, if there is an increase in thermohygrometric values, microorganisms can accelerate deterioration (Cappitelli et al. 2009; Borrego et al. 2012). Furthermore, parchment absorbs water, expanding when the relative humidity rises and shrinking when it falls (Pavlogeorgatos 2003; Giacometti et al. 2012). Therefore suitable thermo-hygrometric conditions inside the repository are crucial. The UNESCO program 'Memory of the World' (UNESCO 1995) suggests 18 °C (maximum daily variability of 2°C) and 50-60% relative humidity (maximum daily variability of 5%) as optimal values to prevent the deterioration of parchment (UNESCO 2000). At present, the repository environment is not suitable to protect the parchment as in both winter and summer the temperatures are respectively below and above the threshold values. In addition, the microclimatic parameters are subject to marked seasonal change, possibly because the windows of the repository are not fully insulated, allowing the outdoor environment to contribute to temperature and humidity fluctuations.

Microbial loads inside and outside the repository (confined and semi-confined environment, respectively) were determined, because current museum regulations consider only microbial load thresholds in order to establish air contamination. Indeed, the museum standards set down by the Italian Ministry of Cultural Heritage (MIBAC 1998) recommend less than 750 CFU m⁻³ of heterotrophic bacteria and less than 150 CFU m⁻³ of fungi for museum indoor environments.

The aerobiological monitoring that was carried out showed that in winter, the bacterial loads did not exceed the limits either inside or outside the repository; instead the fungal loads always exceeded the threshold values at all the sampling sites (both in the semi-confined and confined environment). During the summer, the microbial load (both bacteria and fungi) exceeded the threshold values at every sampling site, with the exception of the bacterial load outside the repository door. The microbial load outside the repository door during both summer and winter were about one order of magnitude less than that reported by other similar investigations conducted in outdoor environments (Cappitelli et al. 2009). Instead, inside the repository the microbial counts agreed with those reported in investigations conducted in other indoor environments (Cappitelli et al. 2009; Borrego et al. 2012).

In summary, microscopic and viability assays currently demonstrate that biodeterioration does not represent a threat to the conservation of the manuscripts. Nevertheless, to ensure long-lasting conservation of the manuscripts, the marked differences in the presence of microbes between winter and summer (probably due to inadequate controlled environmental conditions), the presence of potential biodeteriogens and an opportunistic human pathogen and the retrieval of microorganisms related to the human body led to the following proposals: i) environmental remediation of the repository; ii) routine monitoring of air and surfaces; and iii) improvement of handling procedures. In order to prevent seasonal fluctuations in thermo-hygrometric conditions, the door and windows of the repository should be insulated and adequate climate control equipment installed. Furthermore the microclimatic parameters and the microbial contamination on the surface of heritage objects, and the surrounding air should be monitored from both the quantitative and qualitative points of view at least twice a year, corresponding to the winter and summer seasons. Finally, from now on, the manuscripts should be handled only using gloves. While in this specific case study the number of microorganisms detected was only small, it is possible that there could be extensive colonization of parchment surfaces by a microbial community that could actively attack collagen. To obtain information about the biodegradation potential of the microbial communities present on objects, molecular techniques could be used. Indeed, bacteria and fungi capable of degrading parchment produce a group of enzymes called collagenases (Talwar & Srivastava 2003). A molecular approach, based on the use or design of primer/probe sets specific for collagenase gene identification could be adopted to evaluate the potential ability of the community to degrade collagen (Tsuruoka et al. 2003; Sadikot et al. 2005). Today, the need for integrated microbiological risk management, supported by well-managed information, is crucial to collection institutions where human and financial resources are often limited. The coupling of the fast detection of viable microbial colonization with more selective molecular techniques has proved effective for a quick and exhaustive inspection of both surfaces and air quality. In this respect, the present work promotes the proposal of guidelines for the correct management of historical documents, in order to preserve them from microbiological attack and, in turn, to ensure long-lasting conservation.

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Successful combination of chemical and biological treatments for the cleaning of stone artworks²

Abstract

The removal of sulfate-based crusts from stone artworks using sulfate-reducing bacteria (SRB) has been proven to be an effective cleaning procedure. However, some concerns still remain: it is a time-consuming process when the crust is thick, and it is not clear how the biotechnological approach can fit within a complex conservation treatment. To address these challenges, the effects of an SRB strain (*Desulfovibrio vulgaris* subsp. *vulgaris* ATCC 29579) coupled with a non-ionic detergent pre-treatment was studied on a stone column affected by black crusts. The coupling of the two treatments removed the black crust without affecting the original sound marble, with 38% reduction in cleaning time. The combined method was later applied to a one-century-old artistic marble statue weathered by sulfate-based crusts and grey deposits. The detergent used alone effectively removed the grey deposit, but not the black crust. However the co-treatment synergy resulted in the complete removal of the black crust layers, with the added advantage, compared to the biocleaning alone, of fewer biological applications and a 70% reduction in total cleaning time, but still retaining all the advantages of the biocleaning approach.

1. Introduction

Stone surfaces on buildings exposed to the urban environment undergo rapid and differentiated deterioration due to chemical and physical attack (Camuffo et al. 1983). Dark grey or black surface layers can be found everywhere on lithoid materials, especially on sculpted elements sheltered from direct rainfall; some alterations derive only from a deposition mechanism, others from the sulfation of marble and soiling deposition acting contemporaneously (Moropoulou et al. 1998; Toniolo et al. 2009). Marble sulfation occurs in the presence of moisture when sulfur dioxide, a major urban atmosphere pollutant, is converted to sulfuric acid, which reacts with marble and other soluble calcareous substrates to form gypsum (Böke et al. 1999). During gypsum crystallization, airborne organic pollutants and carbonaceous particles accumulate on surfaces protected from rainfall and wash-out, and are subsequently trapped in the newly-formed mineral matrix (Moropoulou et al. 1998) to form so-called 'black crust' (El-Metwally and Ramadan 2005). Air pollution also causes the deposition of a variety of organic and inorganic compounds on the surface of historic monuments. Such deposits contain a gypsum component but have greater porosity than black crust because gypsum does not come from the chemical corrosion of the substrate, it comes from an atmospheric deposition mechanism of carbonaceous particles, mixed together with dust, pollen, and spores from the atmosphere (Alessandrini et al. 2002; Toniolo et al. 2009).

A cleaning method for the removal of altered layers must respect the chemical-physical nature of the material of the artwork, and its historical and artistic value, but when there are different weathering forms on a stone surface it is difficult to use a single cleaning procedure (Slaton and Normandin 2005). Indeed, quite often, more than one cleaning method is needed. However, even though it is common in the conservation field to combine cleaning procedures (Bromblet et al. 2003), the cleaning methods themselves can interact with the surface materials and with each other (Sansone et al. 2008).

Biocleaning has been tested, successfully, on many materials including white and colored marbles (Cappitelli et al. 2007; Gioventù et al. 2011) and porous limestone (Polo et al. 2010), and has proved to be more selective than mechanical, chemical and laser techniques as it efficiently removes black crusts without affecting the stone surface (Cappitelli et al. 2007; Gioventù et al. 2011). Despite being a successful treatment, biocleaning alone has been found to be highly time-consuming in the presence of thick and compact crusts, and its compatibility with other cleaning procedures has not, until now, ever been studied (Gioventù et al. 2011).

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The aim of the present study was to assess the effects of a chemical and sulfate-reducing bacteria (SRB) co-treatment for the removal of black crusts and grey deposits. The method was first tested on an old outdoor stone column that had been exposed to a polluted environment for a long time. It was then applied to an old artwork, a funeral monument realized by Lina Arpesani in 1921 in memory of 'Neera', the poetess Anna Zuccari. The marble statue, a naked young woman supporting a big open book on her head, had stood in the courtyard of the Cimitero Monumentale in Milan (Italy) and therefore had long been exposed to outdoor conditions and the polluted urban atmosphere; in 1958 the statue was moved to the Famedio porch of the same cemetery, where it is currently located in a semi-confined environment. Its state of conservation was good and there was no evidence of structural damage. However, visual inspection revealed two main alterations: a putative grey deposit covering all the surface and black crust-like alterations in the areas formerly protected from the direct rain-wash, i.e. under the right arm and on the face.

Considering the putative nature of the weathering forms of the marble statue, two cleaning methods were combined and an assessment made of their interaction and synergic effect: the soft detergent Tween 20 together with a SRB-based biocleaning procedure for the removal of the grey deposit and black crust respectively. Up until then, nothing was known of the interaction between biocleaning and other chemical cleaning procedures like the use of detergents.

2. Materials and methods

2.1 Sampling and material characterization

Sampling was by means of a micro-chisel and a steel scalpel for both the stone column and the statue, and was as little invasive as possible. The samples were selected so as to be representative of the surface before and after the different cleaning procedures (see paragraph 2.2): i) surface with a black crust-like alteration before any treatment (Figure 1a); ii) biologically cleaned surface; iii) chemically cleaned surface; iv) surface after the chemical pre-treatment and prior to the subsequent biological cleaning; v) surface after combined chemical and biological cleaning. Samples taken from the column are coded with the letter 'C', and samples from the statue with 'S' (Table 1).

The stereomicroscope observations were performed with a Leica M205C, equipped with a Leica DFC290 digital camera. For optical microscopy, the samples were prepared in polished cross-sections with bi-component epoxy resin. Optical microscopy was carried out with a Leica DMRE Microscope, equipped with a Leica DFC290 digital camera.

Environmental Scanning Electron Microscopy (ESEM) for morphological observations of the sample fragments and polished cross-sections was performed with an Environmental Scanning Electron Microscope Zeiss EVO 50 EP equipped with an Oxford INCA 200 - Pentafet LZ4 spectrometer in Secondary Electrons mode.

X-ray diffraction (XRD) analyses on powder materials were performed by a Philips PW1830 instrument with Bragg-Brentano and Thin Film geometry, using a Cu anticathode and $K\alpha$ radiation ($\lambda = 1.54058 \text{ \AA}$).

Fourier Transformed Infrared Spectroscopy (FTIR) analyses were carried out with a Thermo Nicolet 6700 instrument using a DTGS detector in the spectral range $4000 - 400 \text{ cm}^{-1}$, coupled with a FTIR Thermo Nicolet Continuum microscope using a MCT detector in the spectral range $4000 - 600 \text{ cm}^{-1}$. Sample fragments were ground in an agate mortar, and analyzed after dispersion in KBr pellets (KBr FTIR grade by Sigma-Aldrich). Micro-fragments were analyzed by means of a compressive diamond cell. Average spectra of 128 scans were recorded for each sample.

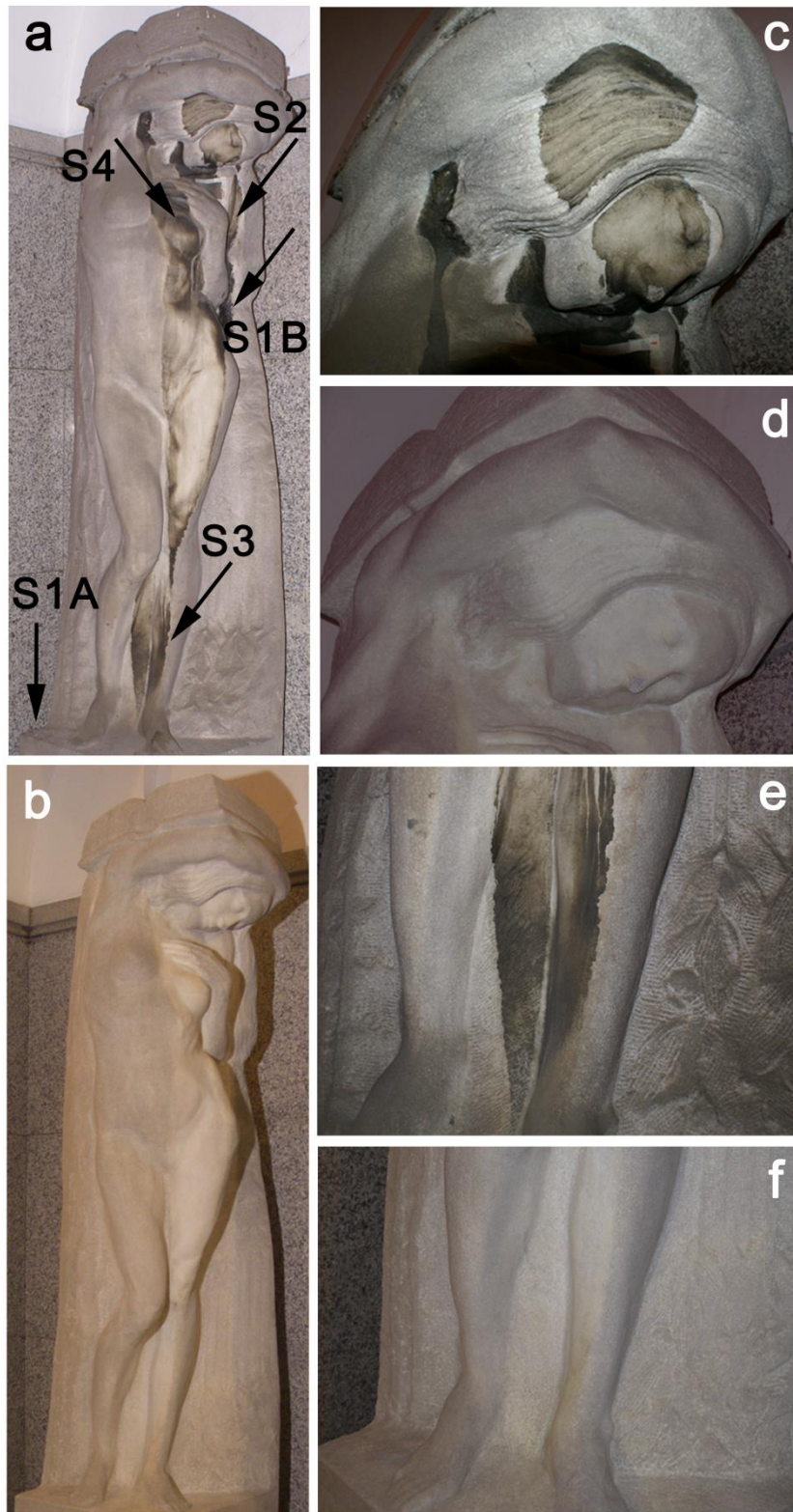


Figure 1. (a) is the marble statue before the cleaning; the arrows indicate the following sampling areas: sample S1A for the characterization of the grey deposit, sample S1B for the characterization of the black crust-like alteration. Samples S2-4 were taken after cleaning tests with different methods: sample S2 after the biological treatment, sample S3 after the combined chemical and biological treatment, and sample S4 after the chemical treatment as control. (b) shows the results after the removal of the grey deposit and the black crusts on the whole statue. (c, e) show the details before the cleaning. (d, f) give the details after the cleaning.

Object	Sample code	Treatment
Column	C1	Surface before any treatment for the characterization of the black crust-like alteration
	C2	biological treatment alone
	C3A	Combined chemical and biological treatment, taken after the chemical pre- treatment and before the biological treatment
	C3B	Combined chemical and biological treatment, taken after the biological treatment
	C4	Chemical treatment alone as control
Statue	S1A	Surface before any treatment for the characterization of the putative grey deposit
	S1B	Surface before any treatment for the characterization of the black crust-like alteration
	S2	Biological treatment alone
	S3	Combined chemical and biological treatment
	S4	Chemical treatment alone as control

Table 1. Samples taken from the column and from the statue. Samples taken from the column are coded with the letter ‘C’, and samples from the statue with ‘S’.

2.2 Cleaning procedures

Three different treatments were applied on both the column and statue: (i) chemical treatment alone, (ii) biological cleaning alone, and (iii) the combined chemical-biological treatment.

i) The chemical treatment was a wet poultice prepared with a suspension of Arbocel in a 1.5% solution of the non-ionic detergent Tween 20 (Sigma-Aldrich, St. Louis, MO) in distilled water. Three applications of 22 hours each were performed to investigate its role in the bioremoval of the black crust on both stone column and statue.

For the statue only, the chemical treatment was performed alone also to remove the grey deposit. In this case, two applications of 17 hours each were performed.

ii) The biological cleaning treatment was performed using the aerotolerant sulfate reducing bacterium *Desulfovibrio vulgaris* subsp. *vulgaris* ATCC 29579, which had previously been used to remove black alterations due to gypsum (Cappitelli et al. 2007). Growth conditions and culture preparation were as reported in Cappitelli et al. (2006). Briefly, the cells were grown in DSMZ 63 medium and incubated at 30°C for 4 days under anaerobic conditions. Cultures were filtered on a Rapida A Perfecte cellulose filter (Cartiera di Cordenons, Vicenza, Italy) with an 8-µm particle size range that allowed the bacteria to pass through, but trapped the iron sulfide precipitates.

After centrifugation, the cell pellet was resuspended in anoxic phosphate buffer at pH 7.0 (KH_2PO_4 , $0.408 \text{ g}\cdot\text{l}^{-1}$; K_2HPO_4 , $0.522 \text{ g}\cdot\text{l}^{-1}$) added with $0.599 \text{ g}\cdot\text{l}^{-1}$ sodium lactate, to reach a cell density of around 10^8 cells/ml. This cell concentration was previously shown to be effective for the removal of sulfates on altered stone (Cappitelli et al. 2006). All the manipulations described above were done under anaerobic conditions in a glove box.

The biomass was entrapped in an Arbocel polymeric matrix (CTS, Vicenza, Italy) as the delivery system (Cappitelli et al. 2006). The treatment procedure was as previously reported in Cappitelli et al. (2007). Briefly, the surfaces to be treated were moistened with the phosphate buffer and covered with tissue paper before any applications began. The biological cleaning system was covered with a polyvinylchloride film (Silplast, Italy) to reduce undesirable evaporation. After the treatment, removal of the bioformulate was accomplished by removing the tissue paper and subsequently washing the area with distilled water. Applications of 22 hours were applied on the altered surface of both stone column and statue up to the complete removal of black crust. No treatment with only the delivery system (without entrapped microorganisms) was carried out as this had never removed black crust in previous experiments (Cappitelli et al. 2006; Cappitelli et al. 2007; Polo et al. 2010; Gioventù et al. 2011).

iii) The combined chemical-biological treatment consisted of two steps. First, a chemical pre-treatment consisting of a wet poultice prepared with 1.5% solution of Tween 20 in Arbocel as reported above. This poultice was applied to the black crust for two hours, and covered by a polyvinylchloride film. The site was then rinsed and the residual poultice washed off with distilled water while gently wiping with a small brush. The second step, the biological treatment performed as reported above, consisted of 22 hour-applications up to the complete removal of black crust.

3. Results

3.1 Column

3.1.1 Chemical characterization before treatment

The preliminary stereomicroscopic observation of sample C1 showed the presence of a diffused and rather compact black colored alteration covering most of the stone surface (Figure 2a). After optical observation of the polished cross-section (Figure 2b), the sample stratigraphy appeared to be composed of a single crust layer (layer 2) overlapping the stone substrate (layer 1). The alteration continued along the entire section, with an irregular shape and thickness varying from less than $50 \mu\text{m}$ to almost $200 \mu\text{m}$. A few red particles embedded in a generally very dark crust matrix were also observed. The mineralogical and compositional characterization of the alteration after XRD and FTIR analysis confirmed the carbonatic nature of the substrate, which was composed of calcite. The alteration composition included gypsum (FTIR peaks at $3543\text{-}3405$, $1685\text{-}1621$, $1142\text{-}1117$, $670\text{-}602 \text{ cm}^{-1}$) as main component, together with nitrates (FTIR peak at 1384 cm^{-1}), silicates and quartz (FTIR peaks at 1005 , $797\text{-}779 \text{ cm}^{-1}$), calcite (FTIR peaks at 1427 , 875 , 713 cm^{-1}). ESEM-EDX results highlighted a dramatic weathering of the stone substrate (Figure 2c) but no sulfation, according to the EDX spectrum of this area (Figure 2d).

Intergranular corrosion was observed along the borders of the calcite grains and the mineral matrix appeared to be deeply disaggregated. Voids and discontinuities were present between the substrate and the black alteration layer (layer 2). The latter was rather heterogeneous and its composition mainly included Si, S, and Ca, together with Al, Fe and Mg (Figure 2e). Some residual traces of a thin discontinuous F1-enriched layer between the substrate and the crust were also identified. The overall data indicated that the superficial dark alteration on the stone surface was a black crust.

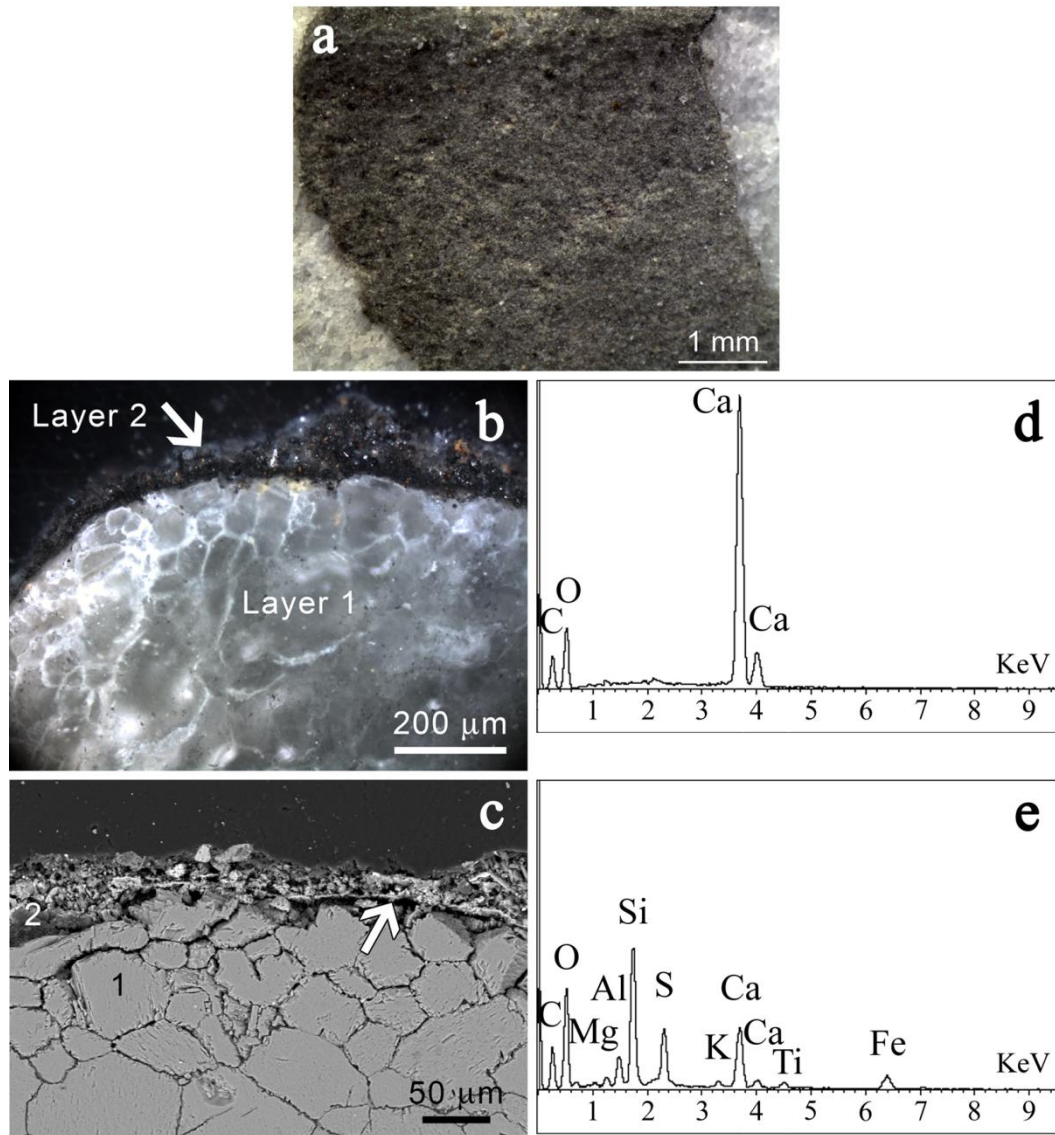


Figure 2 - Characterization of stone column (sample C1). (a) stereomicroscopic documentation of the sample collected before any treatment, showing a diffused and rather compact black colored superficial crust. (b) optical microscopy of the polished cross section of the stone substrate (layer 1) covered by a continuous crust (layer 2). (c) ESEM observation of the sample showing a particularly disaggregated stone substrate. (d-e) EDX spectra of layer 1 and layer 2, respectively.

3.1.2 Characterization of the surfaces after chemical pre-treatment and chemical cleaning

The use of Tween 20, both as 2 h pre-treatment (sample C3A) and as multiple cleaning applications of 22 h (sample C4), gave the same results: no significant cleaning effects on the superficial crust (Figure 3). The stereomicroscopic observations of the sample surfaces (Figures 3a-b) showed no morphological differences from the untreated surface and the dark appearance of the crust was preserved. The same applied when the polished cross section was considered (Figures 3c-d). The crust continued along the entire section, maximum thickness around 200 μm , and several red coloured particles were embedded in the crust. SEM-EDX analysis of the section confirmed the high degree of granular disaggregation of the stone substrate (Figures 3e-f), and leaching of the uppermost grains along the stone/crust interface was evident. The crust appeared highly heterogeneous and several carbon particles trapped in the newly formed mineral matrix

could be identified by their peculiar spherical morphology. The crust's overall composition was mainly based on the presence of S, Si and Ca, and reflected that of the untreated crust.

3.1.3 Characterization after biological and combined cleaning

On visual inspection, the removal of black crust was complete after, respectively, five and three biological applications for the biological cleaning alone (sample C2) and the combined chemical-biological treatment (sample C3B).

The final cleaning results of the two biological methods are comparable, see figure 4. The biological cleaning proved to be highly effective in crust removal. The stereomicroscopic observation of the cleaned surfaces showed that the stone substrate was completely exposed and only a few very small dark spots from the previous crust could be observed (Figures 4a-b). The successful cleaning effect was confirmed by the cross section observation. The substrate layer was the only one still visible, whilst the presence of the crust layer previously overlapping the stone material was no longer detectable (Figures 4c-d). As far as the state of conservation of the substrate was concerned, the biological treatment seemed to have not significantly increased the already high degree of granular disaggregation of the stone (Figures 4e-f).

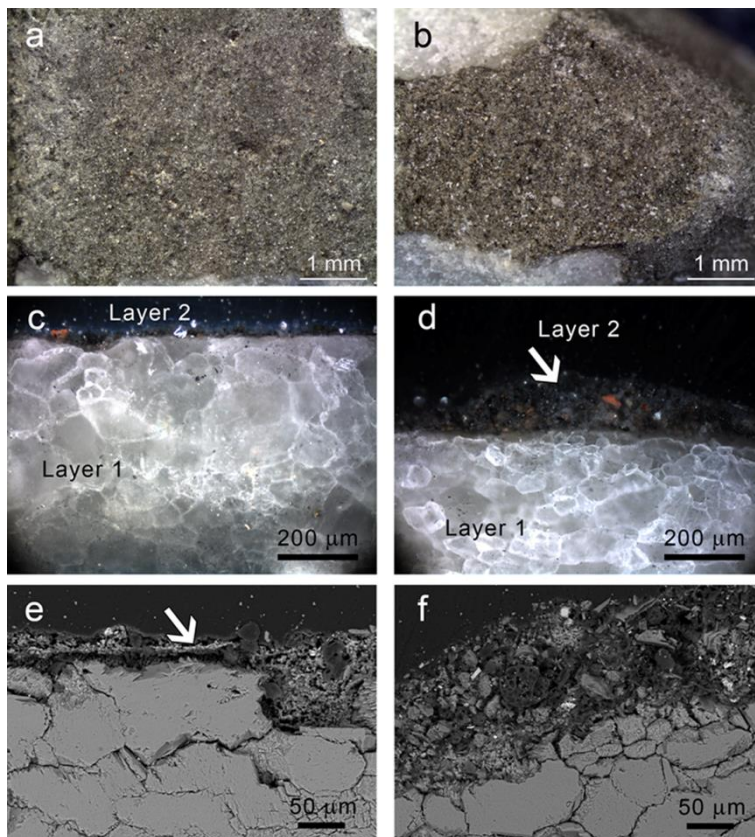


Figure 3. Stone column: comparison of the results of the chemical pre-treatment cleaning (left column, sample C3A) and the chemical cleaning alone (right column, sample C4). (a-b) stereomicroscopic documentation. (c-d) optical microscopy on polished cross sections. (e-f) ESEM observation of the polished cross sections. The same magnification has been used for each couple of images to allow a proper comparison of the cleaning efficacy.

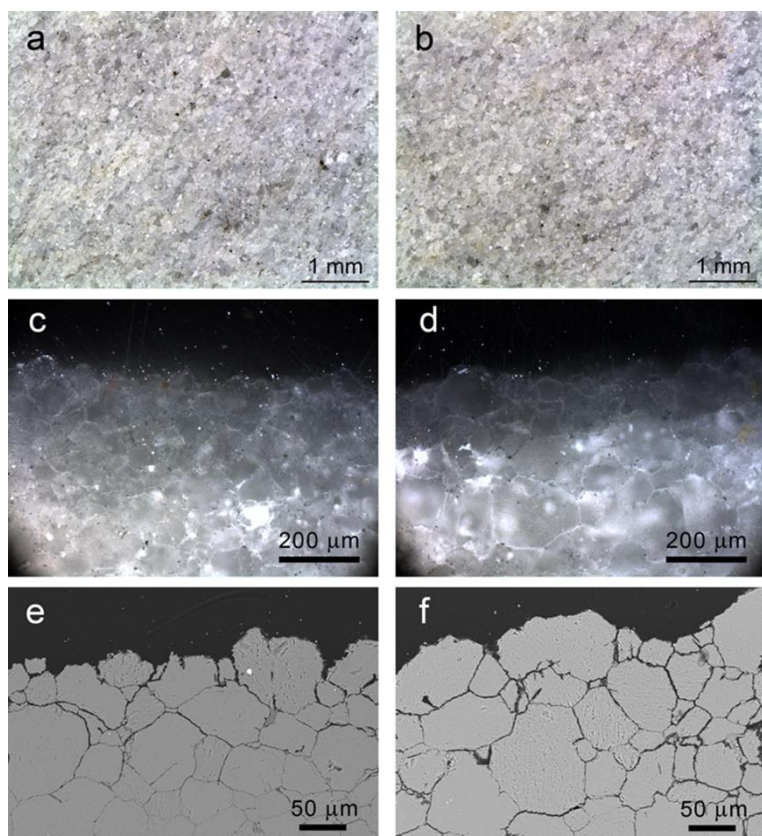


Figure 4. Stone column: comparison of the results of the biological cleaning alone (left column, C2) and the combined chemical and biological cleaning (right column, C3B). (a-b) stereomicroscopic documentation. (c-d) optical microscopy on polished cross sections. (e-f) ESEM observation of the polished cross sections. The same magnification has been used for each couple of images to allow a proper comparison of the cleaning efficacy.

3.2 Statue

3.2.1 Characterization of the surface before the treatments

Chemical, mineralogical and morphological analyses were conducted for the characterization of both the putative grey deposit (sample S1A) and the black crust-like alteration (sample S1B). On stereomicroscope observation, sample S1A appeared very compact and close fitting to the marble surface. Optical microscopy observation of the polished cross section revealed only one layer overlying the marble substrate. This layer was very compact and had an irregular thickness and shape; it contained particles different in size and color. FTIR and XRD analyses showed that it had a heterogeneous composition; in fact, it contained calcite (1425 , 875 and 712 cm^{-1}), nitrates (1385 cm^{-1}), gypsum (3544 - 3406 , 1682 - 1621 , 1145 - 1119 , and 670 - 603 cm^{-1}), silicates and quartz (797 and 778 cm^{-1} respectively). The chemical, mineralogical and morphological analyses confirmed that it was a grey deposit.

A preliminary visual and stereomicroscope examination of the zone interested by black crust-like alteration before any surface treatment (sample S1B) revealed a non-homogeneous distribution of compact texture and embedded black coloured particles of different size. Optical microscopy observations of the cross section showed a stratigraphic structure, with a thickness of up to 500 μm , made of four well-defined layers, of variable composition, thickness and color (Figure 5a): an inner, and sound, stone material (layer 1); a grey layer of irregular thickness with embedded black and orange particles (layer 2); a thin (around 20 μm), irregular white layer (layer 3); a black external layer, particularly irregular in thickness (layer 4). ESEM-EDX analysis of the different layers (Figure 5b) showed that the innermost layer (layer 1) had a carbonatic composition with calcium prevailing and a small amount of sulfur (Figure 5c). Layer 2 (Figure 5d) was characterized by more sulfur, almost equaling the calcium. Moreover, the layer 2 microstructure appeared far less compact and homogeneous than layer 1. In layer 3, the sulfur content slightly exceeded the

calcium, and there was the unexpected presence of barium (Figure 5e). The external layer (layer 4) had the same composition as layer 2 but the sulfur prevailed slightly over calcium (Figure 5f). Moreover, the ESEM morphological observation of the sample S1B external surface (corresponding to the outer part of layer 4) showed that the crust was made of well-defined rhombohedral gypsum crystals and carbonaceous particles (Figure 5g). FTIR and XRD analyses of the sample S1B showed gypsum (peaks at 3547-3405, 1685-1621, 1143-1117, and 670-602 cm^{-1}) with traces of calcite (1422, 876 and 712 cm^{-1}). The overall data indicated that the superficial dark, crust-like alteration on the stone surface was indeed a black crust.

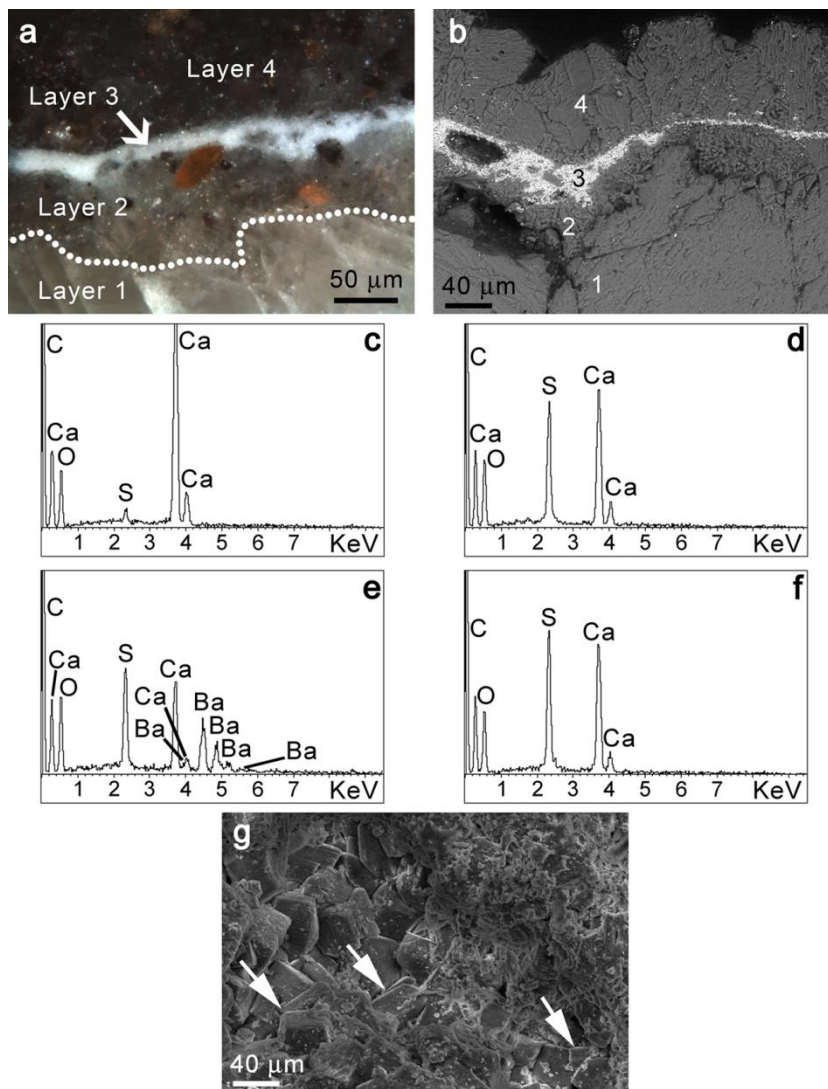


Figure 5. Polished cross section of sample S1B before the treatments. (a) optical microscopy. Layer 1, sound stone; layer 2, grey layer with embedded black and orange particles; layer 3, white layer; layer 4, black external layer. (b) ESEM observation. Numbers indicate the different layers. (c-f) EDX spectra of layers 1, 2, 3 and 4 respectively. (g) ESEM observation of the surface. Arrows indicate the rhombohedral gypsum crystals.

3.2.2 Characterization of the surface after the treatments

Chemical and morphological analyses (stereomicroscope and optical microscopy observations, ESEM and FTIR), conducted to characterize the grey deposit after the chemical treatment, proved its removal. No traces were found on the surface (Figure 1b).

As regards the areas interested by black crust, chemical and morphological inspection of the sampled surfaces after treatments showed different results, depending on the treatment employed.

For the biological treatment alone, it took seven applications to remove the black crust, though there some residual traces were still visible after the treatment (Figure 6a). Optical microscopy

revealed that, for the cross-section, all the black crust layers originally overlying the marble were completely removed, and there was no damage to the stone substrate (Figure 6b). ESEM-EDX analysis revealed no residual S and Ba traces on the areas treated with the biological treatment (Figure 6c); indeed, no gypsum crystals were found on the surface after the biological treatment (Figure 6d), and only calcite was detected by FTIR analysis.

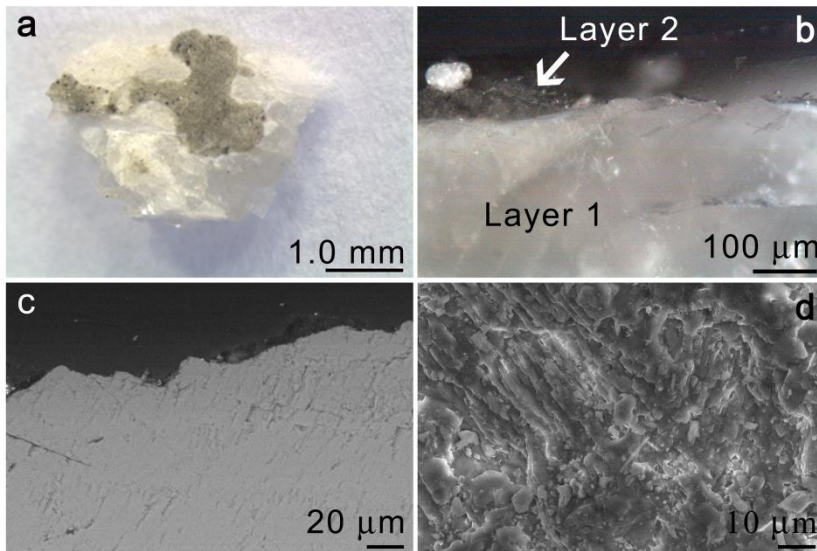


Figure 6. Sample S2 after the biological treatment. (a) Stereomicroscopy observation. (b) optical microscopy observation of the polished cross section. (c) ESEM observation of polished cross section. (d) ESEM observation of treated surfaces. The numbers show the different layers.

Stereomicroscopy revealed that the area treated exclusively by the chemical treatment, the control, resulted in the black crust being only partially removed (Figure 7a) as black remains were still present on the surface after three applications of the chemical poultice. The cross section optical observation of sample S4 revealed a very limited effectiveness of the chemical treatment alone, as the final stratigraphy was almost comparable to the one of the untreated samples; furthermore, in only a few areas the layers overlapping the marble substrate (layers 2 to 4) were partially removed (Figure 7b). ESEM-EDX analysis of the sample S4 cross section, performed to characterize the alteration layers still present after chemical treatment as control, confirmed that all the layers were characterized by the same composition of the corresponding layers of the untreated area (Figure 7c). ESEM observation of the surfaces showed that the rhombohedral gypsum crystals were still present on the surface treated with Tween 20 (Figure 7d). FTIR analysis confirmed the presence of gypsum on the chemically treated area, though less than in the untreated area.

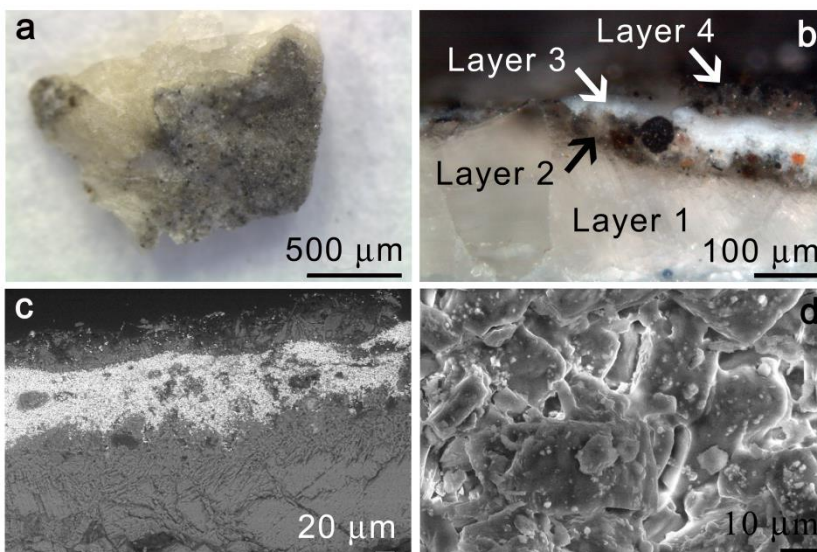


Figure 7. Sample S4 after the chemical treatment. (a) stereomicroscopy observation. (b) optical microscopy observation of the polished cross section. (c) ESEM observation of polished cross section. (d) ESEM observation of treated surface. The numbers show the different layers.

Stereomicroscopy inspection of the area subjected to the combined cleaning method showed that the black crust was still present after the chemical treatment, but after two biological applications it was completely removed (Figure 8a). Optical microscopy observation of the cross section of the areas treated with the combined chemical and biological treatment was characterized exclusively by a sound-stone basal layer, while the overlying layers (layers 2, 3 and 4) were absent, there being only rare and small alteration residuals (Figure 8b), confirming the stereomicroscopy observations of the surface. ESEM observations of the polished cross section of the areas treated with the combined chemical-biological treatments showed that both gypsum and silicate were almost completely removed, no residual S and Ba traces were found (Figure 8c). Furthermore, there were no gypsum crystals on the surface after the combined chemical-biological treatments (Figure 8d), only calcite being detected with ESEM observations of the surface. According to the obtained results, it was decided to proceed with the cleaning of the whole statue for the removal of both the grey deposits and the black crust. Figures 1d and 1f show two details of the cleaned areas, the face and legs, which can be compared with the same untreated zones (Figure 1c and 1e).

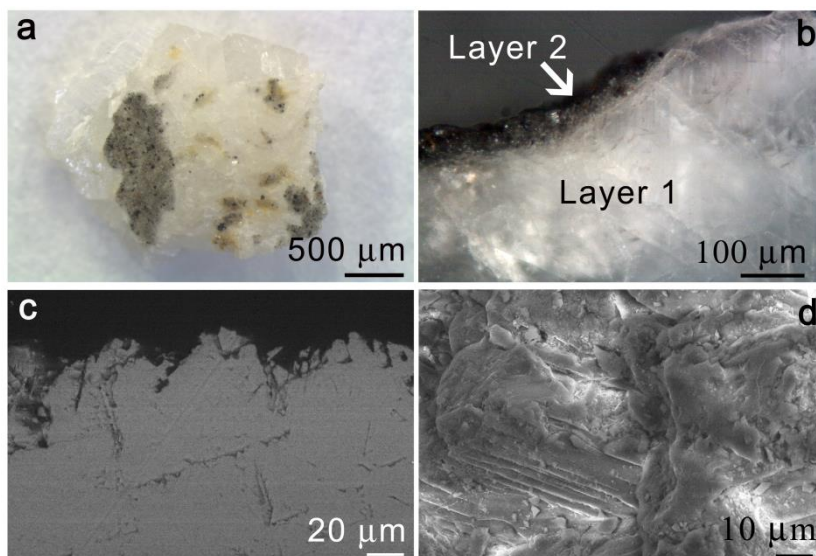


Figure 8. Sample S3 after the combined chemical and biological treatment. (a) stereomicroscopy observation. (b) optical microscopy observation of the polished cross section. (c) ESEM observation of polished cross section. (d) ESEM observation of treated surface. The numbers show the different layers.

4. Discussion

Using chemical, mineralogical and microscopy analyses, the stone of the column was characterized as marble. The intergranular corrosion and the disaggregation of the mineral matrix was due to calcite dissolution caused by exposure to the polluted urban environment. In fact, the synergic effect of water (in liquid or vapour phase) and acid pollutants is known to cause damage to carbonatic stones (Siegesmund and Snethlage, 2011). According to the chemical and mineralogical analyses, the crusts of the stone column can be considered as a sulfatic black crust. The Al, Fe and Mg detected inside the crusts were associated with soil-dust particles and environmental contaminants usually embedded in the crust's matrix, while the residual traces of the Fl-enriched layer between the substrate and the crust most probably derive from some previous conservative treatments of the stone (Pedrazzani et al. 2006; Toniolo et al. 2009). The chemical treatment using Tween 20 alone was applied to investigate its role in black crust removal. The chemical pre-treatment of the surface and the chemical cleaning alone had no significant effect on the superficial crust, confirming previous findings reporting that Tween 20 has no specific activity towards the sulfate component of the crust (Doebley et al. 1991; Zeng, 2000). Instead, the biological cleaning, both alone and combined in the chemical-biological

treatment, was able to selectively remove the sulfatic layer even in the presence of a deeply decayed carbonatic substrate, and without worsening the already weathered stone. This fact also suggested the potential of using the biotechnological approach within a more complex conservation treatment. Importantly, a total of 110 h were needed for the complete removal of black crust by biological cleaning alone, while only 68 h were required by the combined chemical-biological treatment, an advantage of a 38% reduction in cleaning time and a significant reduction in the number of applications.

According to the chemical analysis, Lina Arpesani's funeral monument presented two forms of alteration: a grey deposit covering all the surface, which compromised the marble statue's appearance, and black crusts of varying thickness in zones protected from wash-out. Therefore, a complex conservation treatment was needed. On the basis of the satisfactory results achieved on the stone column, we tested the simultaneous action of the cleaning methods for black crust and grey deposit removal on the statue.

The features of the grey deposit are typically connected with the dry deposition (impaction, gravitational setting) of aerosol particles (Torfs and Van Grieken, 1997; Toniolo et al. 2009), different from the wet deposit and sulfation phenomena in an acid environment that causes black crust. A soft chemical treatment using the non-ionic detergent Tween 20 was adopted for the removal of the grey deposit. Neutral or alkaline detergents, used alone or in water solution, are often used for the removal of deposits covering buildings exposed to a polluted atmosphere (Castello, 2008); after two 17 hour-applications the grey deposit was completely removed, as was apparent on visual inspection.

As regards the black crust, ESEM revealed the morphology to be rather atypical with respect to the lamellar or acicular morphologies usually described in the literature (Toniolo et al. 2009; Török et al. 2011). The stratigraphy consisted of 4 layers: layer 1 corresponded to sound marble; its main composition was Ca with minor amounts of sulfur, indicating good overall conservation of the stone substrate. Layers 2 and 4 were mainly Ca and S, though in different ratios; the chemical composition of these layers was confirmed as typical of black crusts (Sarmiento et al. 2008). Between layers 2 and 4 there was an intermediate discontinuous layer (layer 3) of Ca, S and Ba, corresponding to a barium sulfate layer, most probably deriving from an undocumented conservation treatment. Indeed, the barium hydroxide technique is well known in the literature (Matteini, 1991) and was performed in the past to desulfate and consolidate stone surfaces. Barium sulfate (BaSO_4) was meant to act as a protective layer, being less soluble than CaSO_4 even in an acid environment (Price, 1984; Hansen et al. 2003), and thus reducing the solubilization-crystallization sulfate cycles and the risk of both acid attack and penetration of soluble salts (Hansen et al. 2003; Giorgi et al. 2010). At ESEM, layer 3 appeared discontinuous, probably because of the uneven barium sulfate distribution; in fact, below and above layer 3 there were differently formed sulfated layers (layers 2 and 4 respectively). Silicate particles were also found in the black crust structure, presumably of atmospheric origin and most likely ash released by industrial sources (Maravelaki-Kalaitzaki, 2005; Kramar and Mirtič, 2008).

Both the biological cleaning alone, and the biological method coupled with the soft chemical pre-treatment using Tween 20, were used for the selective removal of the black crust. Also the chemical treatment using the non-ionic detergent was applied alone to investigate its role in black crust removal. Each method's contribution to black crust removal was evaluated.

After seven 22-hour applications of the biological treatment alone, chemical and morphological analyses revealed that the surface was homogeneous and satisfactorily cleaned. Indeed, the cross-section observation demonstrated that all the layers originally overlying the marble were completely removed. ESEM analysis of the sample S2 cross-section demonstrated that the bacteria also reduced the sulfate bound to barium, the barium sulfate being completely removed by the microbiological method. Indeed, sulfate-reducing bacteria can use barite as a sulfate source for anaerobic respiration (Bolze et al. 1974), and *D. vulgaris* has been shown to use BaSO_4 as a sulfate source (McCready et al. 1980).

On our artwork under study, layer 4 most probably originated from an external deposition process, which rendered it less compact and more porous than the black crust that derives from direct sulfation of the stone substrate (Schifter et al. 2001; Ozga et al. 2011). Chemical and morphological analyses confirmed the chemical poultice's effectiveness in removing the less coherent particulate matter embedded in the crust (little reduction in the thickness of layer 4). Note that barium sulfate was not removed by the chemical treatment as it was still present on sample S4 after the chemical treatment.

Despite the fact that the advantages and effectiveness of the biocleaning method have already been well documented (Cappitelli et al. 2006; Cappitelli et al. 2007; Polo et al. 2010; Gioventù et al. 2011), and reiterated also in this work, nobody, till now, has studied the interaction between biocleaning and chemical cleaning treatments. In the present study the chemical pre-treatment consisted of a single application aimed at softening the black crust, past results having shown that chemical treatment removed only the less compact part of the black crust. Tween 20 is commonly used in microbiological laboratories to avoid the binding of bacteria (Courtney and Hasty, 1991) and, being a mild detergent, it does not affect the structural features of membrane proteins (le Maire et al. 2000; Schuck et al. 2003).

The chemical pre-treatment was then followed by the biological procedure. In terms of cleaning effectiveness, electron microscope observations, elemental analysis and infrared spectroscopy showed that the combined methods achieved results comparable to those of the biological treatment alone. Using Tween 20 and SRB practical advantages were achieved: a very significant reduction in the number of applications (from seven to two), and a remarkable 70% reduction in the total cleaning time with respect to the biological approach. Despite being a successful treatment, biocleaning alone has been demonstrated to be a highly time-consuming process in the presence of thick and compact crusts (Gioventù et al. 2011). The chemical treatment did not interfere in any way with the biological method, but it did make the biological sulfate removal quicker, likely because it softened the crust, reducing the cleaning time, a parameter conservators must consider when choosing a restoration approach (Gaspar et al. 2003). In addition, when the surface state of conservation is powdery and incoherent, prolonged contact with water can further exacerbate the degradation (Slaton and Normandir, 2005). In such cases, the synergic combination of chemical and biological methods reduces this hazard.

In conclusion, to obtain homogeneous and satisfactory cleaning results, we suggest, for appropriate cases, chemical pre-treatment with the soft non-ionic detergent Tween 20, followed by safe and non-invasive biological treatment for a quick removal of black crust.

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A methodology to select bacteria able to remove synthetic polymers³

Abstract

Synthetic polymers have often been used for the protection and consolidation of cultural heritage. Although it was generally thought that synthetic polymers were not susceptible to deterioration, there are now many papers in the scientific literature claiming the opposite. The degradation of synthetic polymers can be due to chemical, physical and biological factors. At present, the traditional way for removing a degraded synthetic polymer is the use of mixtures of solvents that pose some health risks. This work proposes a method to select bacteria able to remove synthetic polymers from cultural heritage surfaces. The ability of five bacteria to attack Paraloid B72, the most commonly used polymer in conservation treatments, was evaluated by optical and scanning electron microscopy observations, weight loss measurements, Fourier transform infrared spectroscopy and differential scanning calorimetric analysis. Although none of the bacteria were able to attack Paraloid B72, the methodology developed can be applied to select other bacteria with this ability. Therefore the results offer insightful guidance to a better design of bioremoval experiments of synthetic resins used in conservation.

1. Introduction

Man-made polymers used to restore art objects have become increasingly important and abundant in museum collections (Cappitelli et al. 2006). Indeed, with the aim of preserving artefacts from further chemical-physical deterioration, synthetic polymers have been widely employed as adhesives, consolidants and protective coatings to treat cultural heritage objects (Favaro et al. 2006; Miliani et al. 2002). Since the 1950s polyacrylates and polymethacrylates, such as Paraloid B72 (PB 72), have been among the polymer-based products frequently used in stone conservation (Cappitelli et al. 2004).

One of the reasons for introducing synthetic polymers in conservation treatments was the expectation that these materials would be less prone to chemical, physical and biological deterioration than natural organic products (Cappitelli and Sorlini 2008). Unfortunately, as the years went by, all the drawbacks of applying such conservation treatment became more evident (Favaro et al. 2006). Polymer deterioration can modify both the physical properties of the polymer as well as the chemical structure, e.g. via cross-linking or reduction in molecular weight due to chain scission (Cappitelli et al. 2005; Chiantore and Lazzari 2001). Chemical decay also leads to the formation of oxidized species, quite often producing the yellowing of treated stone surfaces (Melo et al. 1999), and physical changes induce polymer stiffening and brittleness, often resulting in polymer cracking, detachment from the heritage substratum and worsening of mechanical properties (Favaro et al. 2005). Although often effective, the application of a synthetic coating on heritage surfaces is usually irreversible, sometimes accelerating, in the long term, the deterioration of the monument or object (Torraca 1976). Even though it is generally thought that synthetic resins are more resistant to microbial attack than natural organic products, there are many articles in the scientific literature claiming the contrary (Cappitelli et al. 2007b; Milanesi et al. 2009; Giacomucci et al. 2011). Meristematic fungi are the first cause of biodeterioration on outdoor stone consolidated or protected with aged synthetic resins (Cappitelli et al. 2007a). Moreover, also in indoor environments, synthetic polymers can act as a growth substrate for microorganisms, especially when applied, but not completely removed, during previous or inadequate attempts to restore an artefact (Lustrato et al. 2012).

Chemical stability and solubility of synthetic polymers in solvents commonly used in the conservation field are two essential requirements for the reversibility of treatment with synthetic

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polymers (Favaro et al. 2006; AIC 2000). The removal of aged polymeric film from works of art is usually achieved by surfactants and organic solvents (acetone, xylenes, toluene, alcohols), or mixtures of them (Lomax and Fisher 1990; Granowski 2006; Ranalli et al. 2000). However exposure to the organic solvents used as cleaning agents in the removal of dirt, stains, overpaints, old varnishes or coatings (e.g. the acrylic resins), can represent a health risk for workers (Langworth et al. 2001). In fact, cleaning agents are potentially toxic, and are often primary irritants of the skin, eyes and mucous membranes (Anundi et al. 2000). It is also likely that a small part of the solvent used to remove the polymer matrix could be retained in the underlying layer, thus increasing the potential risk to human health (Bonini et al. 2007). Solvent application can also result in an undesired spreading of the dissolved materials within the porous structure of the artwork (Carretti et al. 2005). Moreover, cross-linked polymers are very difficult to remove with solvents, although the polymer can be swollen in the solvent and then mechanically removed. After the cross-linking process, it is not possible to employ the same solvent used to apply the undegraded polymer (Lomax and Fisher 1990). Recently, microemulsions and micellar solutions have proved effective for the removal of naturally or artificially aged polymeric coatings (Carretti et al. 2005; Grassi et al. 2009; Carretti et al. 2007). In contrast with organic solvents, the reduced organic solvent content of microemulsions and micellar solutions lowers the environmental impact of these systems (Grassi et al. 2009) and limits the redistribution of solubilized hydrophobic material (Carretti et al. 2005). The surfactant content of these systems ranges between less than 1% and c.a. 3-4%, and the surfactant residues after cleaning can be removed from the treated surfaces through accurate washing with water (Carretti et al. 2005; Grassi et al. 2009; Carretti et al. 2007). Also cleaning artwork with gels has increased enormously in recent decades. Aqueous, nonaqueous, and mixed gels have been used to remove varnish and overpaint from paint surfaces, and to remove stains from stone. Sequestration of solvents in gel matrices minimizes the deleterious effects of using liquids for cleaning surfaces, and introduces advantages. Moreover, to ensure their complete removal (after the cleaning action) so that no damage occurs to the surface (Carretti et al. 2010), recent significant updates have focused on minimizing or avoiding gel residues after cleaning through the use of peelable gels and chemical gels (Natali et al. 2011; Domingues et al. 2013; Pizzorusso et al. 2012).

Biotechnology represents an attractive and sustainable alternative to traditional cleaning in the conservation of cultural heritage materials (Fernandes 2006). Biocleaning methodologies are easily performed, applied and controlled and do not need the presence of skilled and trained personnel (Gioventù and Lorenzi 2013). Furthermore, in the case of the biocleaning of a fresco (Ranalli et al. 2005) the cost of the biological cleaning using viable bacterial cells was assessed as much lower than that of other conventional methods, making this biotechnology not only very interesting but also very cost competitive. Until now, the bioremediation of undesired organic matter on artwork using living microorganisms has mainly been focused on the removal of casein and animal glues on frescoes (Lustrato et al. 2012; Ranalli et al. 2005).

The aim of this study was to set up a biological methodology using bacteria for the removal of the naturally-aged synthetic resin Paraloid B72. The removal ability of five bacterial strains to use Paraloid B72 as sole carbon and energy source was evaluated. Since calorimetric (Menczel and Prime 2009; Stassi et al. 1998) infrared spectroscopy (Klöpffer 1984; Cappitelli et al. 2007b) and optical and scanning electron microscopy (SEM) (Cappitelli et al. 2007b) have proved to be very useful techniques in both polymer science and microbiological investigations, we applied these techniques to evaluate naturally aged Paraloid B72 susceptibility to bacteria.

2. Material and methods

2.1 Biodegradation assay

Four-year dried Paraloid B72 (PB 72), originally solubilized in 15% ethyl-acetate, was supplied by the Opificio delle Pietre Dure (Florence). The PB 72 was kept in a can under ambient conditions until it was used for the experiments reported in this paper. The resin, a dry layer of 0.5 cm

thickness, was cut into seventeen 1×1 cm coupons. The coupons were not sterilized to maintain the chemical and physical characteristics of the material.

Pseudomonas aeruginosa (PA01), *P. stutzeri* (ATCC 23856), *P. putida* (DeFENS collection, isolated from wastewater treatment plant), *Escherichia coli* (ATCC 25404) and *Bacillus licheniformis* (DeFENS collection, isolate from a biodeteriorated acrylic painting on canvas by a contemporary artist) were the test bacteria used as inocula for biodegradation tests. The bacteria were grown in Trypticasein Soy Broth (TSB, Merck) overnight at 30°C. The cultures were then aseptically centrifuged three times and the supernatant liquids discarded to avoid any medium residue. The bacterial pellets obtained from each culture were diluted in sterile mineral solution (NaCl 5 g/l and K₂HPO₄ 2.5 g/l) to a density of 10⁶ cell/ml. The mineral solution, which did not provide any source of carbon, was used supplemented with coupons of PB 72 in presence of and without (negative control) the microbial inoculum. In addition, a positive control was included adding 5% (v/v) TSB as carbon source to the mineral solutions with bacteria. The coupons were placed in Petri dishes and inoculated with 25 ml mineral solution supplied with 3% (v/v) microbial inoculum. The Petri dishes were then set to incubate in a climatic chamber at 30 ± 2 °C and 95 ± 5 % relative humidity, for 30 days. Each experiment was performed in triplicate. These incubation conditions are not representative of on-site conditions but were used here to favour the bacterial degradation, and therefore make the selection of the most promising bacterium easier. Every ten days after incubation, the microbial activity inside the Petri dishes was checked (data not shown). At the end of the incubation period, polymer solubility was studied with weight loss measurements, polymer surface change with stereomicroscope and scanning electron microscopy observations, and chemical modifications using differential scanning calorimetry (DSC) and Fourier transform infrared spectroscopy (FTIR).

2.2 Polymer characterization techniques

The polymer weight loss induced by the microbial degradation was determined gravimetrically, soaking 0.5 g of the sample in 10 ml of acetone. The specimen was put into the solvent, at constant temperature, and its weight was measured at different times by extracting the sample from the acetone and by removing the solvent wetting the surface with filter paper. The procedure was repeated several times to determine the weight loss. The weight generally decreases over a period of time, depending on the solubility of the macromolecular chains. The evaluation of surface change of the specimens was performed using a stereomicroscope (Wild Heergrugg - Switzerland) and a Field Emission Scanning Electron Microscope (SEM) Zeiss Supra VP40, after metallization of the inoculated and untreated specimens to obtain good conductivity. SEM image acquisition was carried out in Secondary Electron Imaging (SE). The glass transition temperature (T_g) of the polymers, and its variation during microbial degradation, was evaluated using a Mettler Toledo differential scanning calorimeter (Model DSC 1 STAR^c System) with a heating rate of 20°C/min from -50°C to 150°C. To eliminate any effects deriving from earlier thermal histories, a second heating cycle was always carried out, heating the sample to 150°C at 20°C/min and then rapidly cooling it to -50°C.

Chemical modifications were evaluated by FTIR analysis on a FTIR Bruker Vertex 70 system. The samples were analysed using Platinum attenuated total reflectance (ATR): a single reflection diamond ATR sampling accessory. Spectra were achieved, between 4000 and 400 cm⁻¹, by the accumulation of 32 scans with the Fourier transformation method, and resolution of 4 cm⁻¹. Spectral acquisitions and data treatments were performed with OPUS software (Version 7.0, Bruker Optics, Inc.).

3. Results and Discussion

The potential ability of five bacterial species to degrade a naturally-aged synthetic resin commonly used in conservation treatment was investigated using chemical and morphological analyses. A wide variety of methods is available to measure polymeric material biodegradability (Pagga 1997).

In this work, a number of techniques for bulk and surface characterization were coupled, namely weight loss, stereomicroscope and SEM observations, infrared and DSC analyses. Indeed, all the tested polymers were characterized before and after incubation, thus making it possible to see any changes due to the medium (negative control) and incubation.

The biological degradation of synthetic polymers is due to the ability of selected heterotrophic microorganisms to utilize such compounds as carbon and energy source, or in co-metabolism (Cappitelli et al. 2005). Fungi, especially black fungi, are known to heavily attack aged synthetic acrylics; thus, acrylics, instead of preventing damage, could accelerate both physical and aesthetic decay processes due to the presence of hyphae or pigment production (Cappitelli et al. 2007a). In any bioremediation application in conservation, bacteria are to be preferred to filamentous and black meristematic fungi, despite the fact that the latter are known to grow on synthetic polymers. For instance, it is reported in the literature that an isolated *Cladosporium* sp., a black fungus, was able to attack a freshly dried poly-isobutylmethacrylate used to consolidate marble in the Milan Cathedral (Cappitelli et al. 2007b). However, for bioremediation purposes black fungi are an unsuitable choice as they are among the most dangerous microorganisms to stone that is often protected and consolidated with synthetic polymers (Cappitelli and Sorlini 2008; Giacomucci et al. 2011; Cappitelli et al. 2007a). Black meristematic fungi together with filamentous fungi often cause chromatic changes due to pigment excretion, and physical disruption due to penetration into sound material, leading to severe damage of the cultural heritage surface (Polo et al. 2010; Leznicka et al. 1991).

As a consequence, five species, belonging to the genera of *Pseudomonas*, *Escherichia* and *Bacillus*, were selected for the bioremoval of the synthetic resin PB 72. Among the bacteria, *Pseudomonas* spp. are the most versatile microbial species involved in polymer degradation (Mueller 2006). Species belonging to the *Pseudomonas* genus are able to catalyse the hydrolysis of the ester bonds in both aliphatic and aromatic polyesters, leading to a decrease in chain length and finally ending up in water soluble intermediates that can be transported into microbial cells and metabolized (Herzog et al. 2006). It is reported in the literature that some strains of *P. aeruginosa* are able to degrade polyester polyurethane, and to use it as sole carbon and energy source (Kay et al. 1991). Another species involved in polymer degradation is *Escherichia coli*, due to the presence of membrane-bound esterase enzymes (Pacaud 1982). *Bacillus* spp. were isolated from cinematographic films made of cellulose acetate stored in the Spanish Archive in Madrid (Abrusci et al. 2005); cellulose acetate is an ester of the cellulose and its biodegradation is a process mediated by the cooperative action of many enzymes (Sakai et al. 1996).

3.1 Gravimetric determination of weight loss

Weight loss measurement is a widely used method in polymer degradation tests, due to its simplicity and wide adaptability, although it provides no direct proof of biodegradation (Shah et al. 2008). Potential weight loss of the coupons, induced by biodegradation, was determined gravimetrically, soaking the specimens in acetone as PB 72 is commonly soluble in this solvent. The experiment was performed on both untreated and treated samples. A slight increase in solubility was detected in coupons inoculated with bacteria, as well as in those soaked in an abiotic mineral solution; comparison was made with the solubility of untreated samples. No significant difference was observed between the solubility of the control and the specimens inoculated with different bacteria. Usually, the variation in the solubility of a synthetic polymer is mainly related to the molecular weight variation, as polymer solubility increases when molecular weight decreases (Bugani et al. 2008). The total or partial insolubilization of acrylic coatings has been reported as a long-term ageing effect for acrylic resins used in the conservation of works of art. This insolubility has been ascribed to the cross-linking of polymeric material (Melo et al. 1999; Popescu et al. 2011). The results show that 4 years of natural aging did not influence the solubility of the synthetic resin, which remained soluble in acetone. The literature reports that microbial metabolism can significantly increase polymer solubility (Gu and Gu 2005). However,

we found that the bacteria selected for our biodegradation tests did not affect the solubility of the synthetic resin, the solubility of both the inoculated specimens and the controls in mineral solution being the same.

Thus gravimetric data should be interpreted with caution as weight loss can be influenced by processes dominated by chemical hydrolysis and polymer breakage during exposure, especially when elevated temperature and humidity are imposed (Gu and Gu 2005). Moreover, a decrease in polymer mass is usually justified by the depolymerisation reactions that occur during degradation, but evaporation of residual solvent, used for polymer deposition, or water absorbed by the polymer during casting, must be considered (Camaiti et al. 2011). Consequently, the gravimetric technique is always associated with other techniques (Lucas et al. 2008).

3.2 Stereomicroscopic and SEM observations of the surface

The evaluation of acrylic resin biodegradability is based on physical property changes (surface modifications, formation of holes and cracks, biofilm formation on surfaces etc.) (Shah et al. 2008; Tokiwa et al. 2009). Surface changes do not prove the presence of a biodegradation process in terms of metabolism, but they can be used as a first indication of any microbial colonization (Shah et al. 2008).

Stereomicroscopy observations in this investigation showed surface swelling on both the inoculated samples and the control (Figures 1a and 1b, respectively). Thus, the changes on the specimen surfaces could have been due to both the mineral solution and bacterial growth. Given that the magnification is higher than stereomicroscope observations, SEM investigations are often used to characterize surface changes in plastic materials due to biological degradation (Leznicka et al. 1991; Setua et al. 2000; Gu et al. 1997). SEM images showed clusters of cells lying on the surface of the samples, especially in the case of *E. coli* (Figure 2), which could be due to an active colonization of the surface, mediated by the bacterium (Cappitelli et al. 2006). The growth of microorganisms on a polymeric surface is not a sufficient condition to prove an assimilation of the carbon contained in the polymer, though it does represent a necessary condition for biodegradation (Motta et al. 2009). As a consequence, physical and chemical analyses were performed to prove the biodegradation effectiveness of the five selected species.

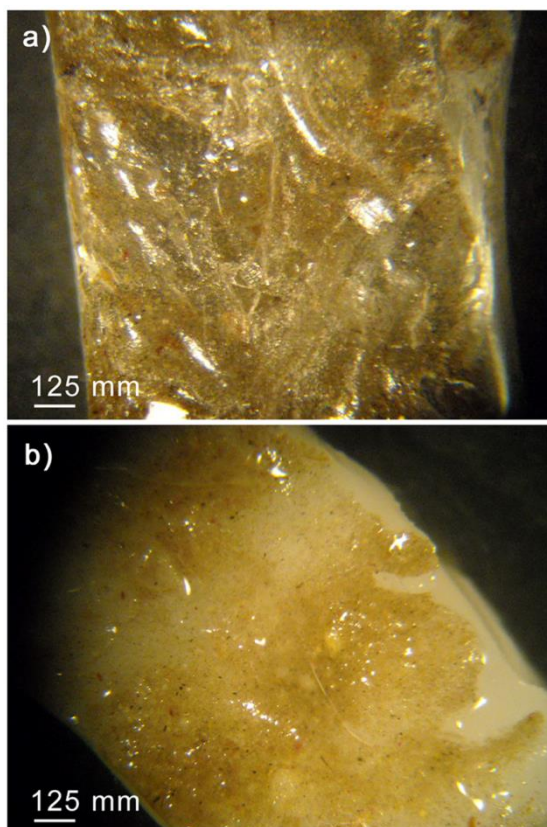


Figure 1. a) Stereomicroscope observation of a coupon of PB 72 in ethyl acetate 15 %: a) before inoculation with *E. coli*; b) after 30 days of incubation with *E. coli*.

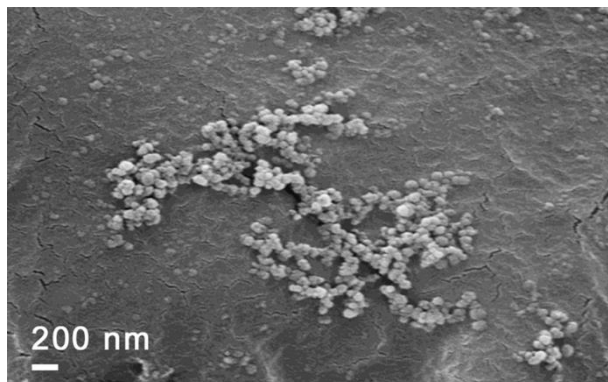


Figure 2. SEM observation of a coupon of PB 72 in ethyl acetate 15% after 30 days of incubation with *E. coli*.

3.3 Differential Scanning Calorimetric (DSC) analysis

DSC is a useful technique for the estimation of different thermal properties of materials, and to study the thermal transition of synthetic polymers, such as glass transition, crystallisation and/or melting point (Lucas et al. 2008); all changes that take place when a polymer is heated or cooled. The DSC curves of the untreated and treated samples are reported in Figure 3; the T_g values obtained by DSC are reported in Table 1.

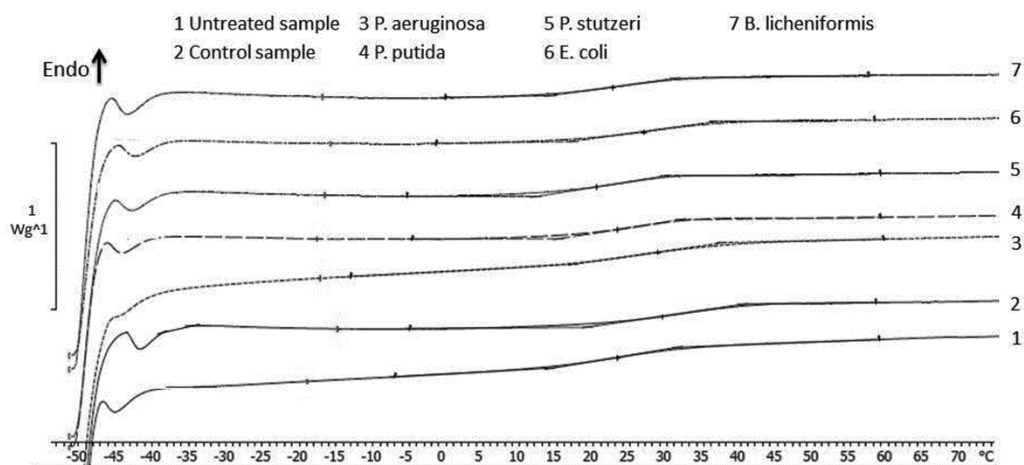


Figure 3. DSC curves (from -50° to 150°) with heating rate $20^{\circ}\text{C}/\text{min}$ of the synthetic resin before and after biodegradation assays.

Sample	T _g (°C)
Untreated sample	11-15
Control sample	20
<i>P. aeruginosa</i>	18
<i>P. putida</i>	16
<i>P. stutzeri</i>	13
<i>E. coli</i>	18
<i>B. licheniformis</i>	15

Table 1. Glass transition temperature (T_g) of the synthetic resin before and after biodegradation assays.

PB 72 has a T_g of 41-42 °C (Princi et al. 2005). The untreated sample, the samples inoculated with bacteria and the control showed lower T_g values than the T_g value of PB 72. The shifting of T_g to lower temperatures could be related to a decrease in the stability of the polymer during degradation (Popescu et al. 2011). Indeed, a decrease in T_g value can be associated with a molecular weight decrease (Vicini et al. 2004). Keeping this in mind, the T_g shift of our samples, compared to the T_g of unaged PB 72, could thus be due to a lower molecular weight and shorter chains of the synthetic polymer under analysis, the result of the natural ageing process of the investigated synthetic resin, confirming the observation that all the tested samples were soluble in the organic solvent. Natural weathering of PB 72 could affect its chemical and thermal properties (Bracci and Melo 2003), favouring biological attack (Iovino et al. 2008). Certainly, the chemical and physical properties of plastics influence the biodegradation mechanism. Note that the surface conditions, the first order structures (chemical structure, molecular weight and molecular weight distribution) and the high order structures (glass transition temperature, melting temperature, crystallinity and crystal structure) of polymers play important roles in biodegradation processes (Tokiwa et al. 2009). Molecular weight is also important for biodegradability because it determines many physical properties of the polymer; increasing a polymer's molecular weight decreases its degradability (Tokiwa et al. 2009).

No difference in the T_g values (all values are in the same temperature range) was detected between the untreated sample and the sample inoculated with the five bacteria. Also this analysis indicates that the selected bacteria did not prove effective in the use of the acrylic resin as carbon and energy source.

3.4 Fourier Transform Infrared Spectroscopy (FTIR)

FTIR is frequently used to reveal chemical modifications of the polymer structure, and for monitoring chemical changes in polymeric film (Lucas et al. 2008). It allows the detection of oxidation reactions, as well as the elimination of small molecules due to decomposition or depolymerisation reactions (Camaiti et al. 2011).

In the FTIR spectrum of Paraloid B72 a large band at about 1730 cm^{-1} , characteristic of the carbonyl stretching vibration, could be observed. This peak consisted of two components, at 1742 and 1722 cm^{-1} , related to the presence of the two ester carbonyl groups (Benedetti et al. 2000). These peaks were present in both the untreated sample and the samples inoculated with the different microbial species, confirming the synthetic nature of the samples, made of Paraloid B72. The infrared spectra of naturally aged PB 72 (untreated) showed only a slight decrease and a very limited broadening of the carbonyl peak compared with the fresh, non-aged Paraloid (Figure 4a), suggesting good stability towards decomposition of ester groups in the present aging conditions (room conditions). Only chain scission occurred, as was evident by the decrease in T_g and the good solubility that was maintained.

When an acrylic resin undergoes biodeterioration phenomena, it is subjected to polymer chain scission and ester bond cleavage, resulting in the formation of oxidation products (Hasan et al. 2007). The literature reports that fungal growth on synthetic polymers leads to decreased peak intensity between 1710-1715 cm^{-1} (corresponding to the carbonyl group)-and 830-880 cm^{-1} (corresponding to $-\text{C}=\text{C}-$). Moreover, the presence of a peptidic bond in the FTIR spectrum (peaks at 1648 and 1546 cm^{-1}), ascribed to some proteinaceous material related to fungal growth, distinguishes a biodeteriorated synthetic resin from the same non-biodeteriorated polymer (Cappitelli et al. 2004; Cappitelli et al. 2005; Cappitelli et al. 2007a; Cappitelli et al. 2007b). After incubation, a comparison of the spectra of inoculated samples revealed that the untreated sample and the control showed no significant differences in peak intensity of any of the investigated microorganisms (Figure 4b). No proteinaceous material was detectable as no peak intensity differences in the treated and untreated samples were observed.

It is reported in the literature (Chiantore et al. 2001; Lazzari et al. 2000) that the stability of acrylic resins appears to be controlled by the reactivity of alkyl side groups. In all the resins the

molecular weight distribution change due to chain scissions and/or coupling of macroradicals is the first degradative event. In both acrylic and methacrylic resins, where all or the majority of the alkyl side groups are short (as in PB 72 -ethyl methacrylate/methyl acrylate-), chain scissions prevail over cross-linking and no insoluble fractions are formed. In contrast, when the side chain is longer, as in PB 66 (with high percentage of butyl methacrylate), where relatively longer esters are present, cross-linking occurs, evidenced by the solubility decrease and FTIR spectra. The high cross-linking rate of these polymers during the first phases of ageing is attributed to oxidation of the butyl groups, which gives rise to unstable secondary hydroperoxides, immediately photolysed to alkoxy radicals. Owing to high mobility of the side chains, such radicals rapidly decay via bimolecular combinations.

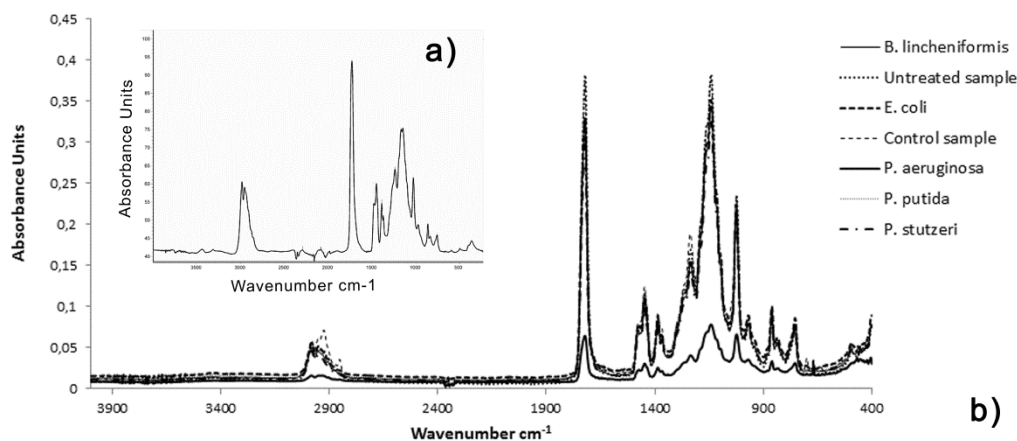


Figure 4. (a) FTIR spectrum of fresh, non-aged Paraloid B72 (Institute of Chemistry, University of Tartu, Estonia http://tera.chem.ut.ee/IR_spectra/index.php); (b) FTIR-ATR spectra of PB 72 in ethyl acetate 15% before and after biodegradation assays.

Paraloid B72 showed good stability towards oxidation, which reflects the good stability of their main components, the methacrylic units EMA. Degradation takes place through the initial oxidation of the acrylic units, i.e. MA. It is worth noting that in PB 72 the balance between scission reactions and macromolecular couplings is such that the polymers do not become insoluble. This evidence is confirmed in our samples, where the side groups of the co-monomers are short ($-C_2H_5$ and $-CH_3$); they maintain their solubility after the natural aging and the biodegradation.

Conclusions

The traditional cleaning techniques that, until now, have been used for the removal of synthetic polymers can be effective but lack in selectivity. Conversely, recently biotechnology has been proved promising for a selective and environmental/health safe approach in the cultural heritage field (Lustrato et al. 2012; Ranalli et al. 2000; Bellucci et al. 1999). Consequently, every study that relates to the use of viable microorganisms for bioremoval is an advance in the cultural heritage conservation field.

In the current study, despite the presence of cell clusters over the surface of the sample inoculated with *E. coli*, observations from the solubility test, DSC and FTIR analysis and the stereomicroscope and SEM excluded that the selected bacteria were able to attack the polymer. A possible explanation for the inability of the selected bacteria to remove the four-year old synthetic resin is that this polymer is not sufficiently aged, as shown by IR. The behaviour of freshly dried Paraloid B72 may be completely different from that of the same resin when aged. This fact was proved by subjecting freshly dried and aged synthetic resins, including PB 72, to biodeterioration treatment according to ASTM G21- 96(2002) "Standard Practice for

Determining Resistance of Synthetic Polymeric Materials to Fungi” (Villa et al. 2009; Cappitelli 2010).

Nevertheless, combining the physical and chemical techniques proved effective, revealing changes in both the surface and the bulk of the synthetic resin. Therefore, the proposed methodology will be applied in future research aimed at the selection of a bacterial strain for the bioremoval of Paraloid B72 and other synthetic polymers.

A prospective strategy for the selection of bacteria for biocleaning purposes could be their isolation from varnish, paint or polymer manufacturing industrial wastewater and sludge (Chen et al. 2007; Arutchelvan et al. 2005). In addition, the successful use of a yeast lipase from *Candida cylindracea* to remove aged Paraloid B72 from two paintings is reported in the literature (Bellucci et al. 1999). Moreover, a yeast isolated from a bronze statue treated with Incralac (an ethyl methacrylate and butyl acrylate copolymer) was found to accelerate the deterioration of the coating itself, as determined by scanning electron microscopy and electrochemical impedance spectroscopy (McNamara et al. 2004). Consequently, another suggested strategy could be the use of viable cells of yeasts as biocleaning agents; yeasts are considered safe microorganisms for both operators and the environment, due to low production of toxic metabolites, and suitable for biocleaning purposes (Saleem et al. 2008).

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Conclusions

The conservation of both historic and modern art objects – a unique and invaluable legacy for the mankind – calls the attention of archivists, curators, restorers and conservators. Organic and inorganic artworks, indeed, undergo a continuous process of deterioration due to the natural aging process and the attack of biological, chemical and physical agents. It is also worth mentioning the great effect of environmental parameters on the preservation of exhibits and outdoor heritage. In this respect, biotechnology, based upon non-invasive sampling and molecular-based techniques, provides information on microbial deteriogens and gives tools to succeed in preventing microbial colonization. On the basis of the work related to the seven parchment manuscripts case-study, it is possible to conclude that:

- ✓ Biotechnology is effective for an exhaustive investigation of surface-associated communities on ancient documents and for investigating the air quality of the repository where the documents are stored.
- ✓ At present, biodeterioration does not represent a threat to the conservation of the manuscripts; however, due to the presence of microorganisms related to the human body and of potential biodeteriogens, an improvement of the handling procedures and an environmental remediation of the repository should be undertaken.
- ✓ A marked difference in the presence of airborne microbes between winter and summer, detected inside the repository, suggests: an environmental remediation of the repository, an insulation of the environment, and a routine monitoring of air in terms of microbial load and environmental parameters such as temperature and relative humidity.
- ✓ The knowledge about the biodeterioration of parchment has been improved, suggesting to collection institutions some general guidelines for the correct management of their legacy.

Biotechnology does not deal only with the use of molecular-based techniques to identify biodeteriogens, but also with the development of new bio-based methodologies contributing to the biocleaning of weathered artworks. Microorganisms, in fact, have two different roles in the cultural heritage conservation, acting both as biodeteriogens and as biocleaners: two sides of the same coin. Biocleaning using SRB has been proved promising for a selective and environmental/health safe approach in the removal of black crusts from stone monuments; however some concerns still remain. Cleaning the funeral monument of the poetess Neera with bacterial agents has shown that:

- ✓ Biocleaning using SRB for the removal of black crusts from weathered stone monuments fits within a complex conservation treatment using the soft detergent Tween 20, resulting in homogeneous and satisfactory removal of black crusts.
- ✓ The disadvantage in terms of time of biocleaning of black crusts has been overcome, proving that the biological method coupled with the soft chemical pre-treatment using Tween 20 reduces of 70% the application times, a crucial factor taken in account by conservators when choosing a cleaning method.
- ✓ The synergic combination of chemical and biological methods reduces the hazard of a prolonged contact with water of surfaces with a poor state of conservation (e.g. powdery and incoherent surfaces).

The success of biotechnology in the preservation and restoration of stone cultural heritage materials requires strong interaction and dialogue between scientists and conservators. Due to the indiscriminate use of synthetic polymers in the past to protect stone artworks, and the susceptibility to biodegradation of synthetic polymers, a fact well known in the scientific literature, the removal of synthetic molecules by microorganisms in relation to cultural heritage conservation has been presented in this thesis, leading to:

- ✓ Set-up a methodology useful for future researches aimed at the selection of bacterial or yeast strains for biocleaning of synthetic polymers from stone, implementing the bioremediation techniques.
- ✓ Highlight that biotechnology, in conjunction with other techniques based on physics and/or chemistry, can contribute to develop novel techniques to preserve our historical artworks.

The introduction of molecular-based techniques, applied on identification of biodeteriogens of cultural heritage materials, represents an advanced and useful tool for investigating the flora responsible for the artwork deterioration. Important biotechnological applications on artwork preservation are emerging and advances in biotechnology predict further innovation, offering new means for preserving our legacy. Also biocleaning technologies, applied on restoration of cultural heritage materials, are an attractive and sustainable alternative to traditional cleaning methods, opening new horizons in art preservation. Biocleaning, thanks to its recent developments and applications in many areas, is increasingly attracting interest of conservators, forever asking microbiologists how advances in biotechnology could contribute to the conservation of heritage. However, the contribution of the biotechnology in the preservation and restoration of culturally relevant materials has still a long road ahead; consequently, each study that relates with the use of biotechnology both in artwork preservation and biocleaning is an advance in the cultural heritage conservation field.

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