

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

Back to the past—forever young: cutting-edge biochemical and microbiological tools for cultural heritage conservation

This is the author's manuscript

Original Citation:

Availability:

This version is available <http://hdl.handle.net/2318/1675847> since 2019-02-05T16:08:08Z

Published version:

DOI:10.1007/s00253-018-9121-3

Terms of use:

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

1 **Back to the past. Forever young: cutting-edge biochemical and**
2 **microbiological tools for cultural heritage conservation**

3 Roberto Mazzoli¹, Gabriella Giuffrida² and Enrica Pessione¹

4

5 **Affiliations**

6 ¹Università di Torino - Dipartimento di Scienze della Vita e Biologia dei Sistemi, Via Accademia
7 Albertina 13, 10123 Torino, Italy.

8 ²CNR-ISPA, Largo P. Braccini 2, 10095 Grugliasco, Torino, Italy.

9

10

11 ¹To whom correspondence should be addressed: Enrica Pessione,
12 Department of Life Sciences and Systems Biology. Università of Turin. Via Accademia Albertina 13.
13 10123 Torino. Italy.

14 Tel. +39 011 6704644

15 Fax +39 011 6704508

16 E-mail: enrica.pessione@unito.it

17

18

19 ***ABSTRACT***

20 Ancient documents and milestones of human history such as manuscripts and textiles are
21 fragile and during aging undergo chemical, physical and biological deterioration. Among the different
22 causes of damage, also human intervention plays a role since some restoration strategies proved to be
23 transient and/or they generated further damage. Outdoor monuments undergo deterioration since they
24 are exposed to pollution, weathering, microbial attack (giving rise to undesired pigmentation,
25 discoloration or true dissolution, corrosion, and overall decay) as well as men-made damage (i.e.
26 graffiti). This review article reports the best fitting strategies used to restore wall paintings, outdoor
27 monuments, textiles and paper documents to their ancient beauty by employing “soft” bio-based
28 approaches such as viable bacteria or suitable enzymes.

29

30

31 **Key-words:** immobilized enzymes, biocleaning, caseinase, collagenase, viable bacteria, graffiti,
32 bioconsolidation

33

34 **INTRODUCTION**

35 Artworks may undergo a number of degradation and deterioration events, which widely vary
36 depending on the specific artifact and the environment and conditions of conservation. These
37 parameters may be extremely different if we consider, for instance, a book conserved at controlled
38 temperature and humidity in a library or in a museum, or a stone statue or a cathedral, which are
39 constantly exposed to weathering, pollution, microbial colonization (extensively reviewed in Mazzoli
40 et al. 2018), vandalism acts, etc... It is worth reminding that damaging of artworks is sometimes the
41 effect of previous restoration interventions which underwent deterioration themselves during time, as
42 in the case of glues applied to consolidate wall paintings or ancient textiles (Beutel et al. 2002; Ferrari
43 et al. 2017).

44 Cleaning and/or restoration of artworks by biotechnological approach has been performed by
45 using enzymes or microorganisms or a combination of both strategies, depending on the specific
46 artifact and issue (Figs. 1, 2; Table 1). Bio-based methods have a number of advantages over more
47 consolidated techniques for artwork restoration (such as those using non-aqueous solvents,
48 bleaching and mechanical treatments), because of their lower impact on the environment, reduced
49 toxicity for operators and higher selectivity and safety for artworks themselves (Barbabetola et al.
50 2016). Enzymes are generally characterized by extremely high substrate specificity which allows high
51 selective choice depending on the “damaging material” (e.g. proteins, polysaccharides, lipids) to
52 treat/remove. Moreover, enzymes can be chosen whose catalytic activity is optimal at the most
53 suitable pH/T ranges for treating a certain artwork thus reducing the application time (Germinario et
54 al. 2017). Enzymes have been used in aqueous formulations, with or without a gel as sorbent, and in
55 ionic liquids (Hrdlickova Kuckova et al. 2014). On the other hand, the use of enzymes may be limiting
56 because of the relatively high amounts required, their relatively high cost, the need for controlled
57 application conditions (e.g. pH and T) and for skilled operators (Barbabetola et al. 2016). Therefore,
58 the use of microorganisms has sometimes been preferred, for instance when very resistant or complex
59 deposit materials (i.e. mixture of heterogeneous substances) or very extended surfaces (e.g. the
60 surface of a cathedral) needed to be removed/treated. In addition, the use of living microorganisms is
61 necessary in the case of complex phenomena such as calcium carbonate deposition for the
62 bioconsolidation of stone material (Dhami et al. 2014). As compared to enzymatic strategies, the use
63 of living microorganisms for biocleaning of artworks is certainly less expensive and may require less
64 controlled environmental conditions, although it is generally less selective (Webster and May 2006).

65

66 *Biocleaning-biorestitution of paper documents and textiles*

67 One of the most common causes of biodeterioration of ancient papers (e.g. books, documents)
68 and textiles that are preserved in museums, libraries and archives are glues employed, with specific
69 variations and modifications, to manufacturing or consolidating/restoring these artifacts
70 (Barbabetola et al. 2016; Ferrari et al. 2017). In the case of paper artworks, glues have been used in
71 manufacturing, such as for bonding and lining of prints, drawings, documents, which were mounted
72 (partly or completely) on secondary support by means of glue spots, as well as for restoration
73 (Barbabetola et al. 2016). As regards historical or ethnographic textiles, glues have mainly been used
74 for restoration purposes, e.g. to fasten them to textiles or to solid (paper or wood) supports (Ahmed
75 and Kolisis 2011). In the past, glues of either vegetal (i.e. starch) or animal (i.e. collagen and/or
76 casein) origin have been used for these purposes (Barbabetola et al. 2016; Ferrari et al. 2017). Both
77 animal and vegetal glues are made up of natural polymers, that is mainly proteins (i.e. collagen
78 derived from the bones, skins, tendons and cartilage of mammals or fish swimming bladder) or
79 polysaccharides (i.e. amylose and amylopectin derived from different plants, such as potato, rice,
80 corn or wheat), respectively (Barbabetola et al. 2016; Ferrari et al. 2017). Through aging, these glues
81 undergo stiffening and thickening which may in turn generate distortions, tensions and discoloration,
82 or form intricate layers that are very recalcitrant to being removed (Blüher et al. 1995; Gostling 1989).
83 In the case of animal glues, humidity, temperature, UV radiation and pollutants can generate protein
84 cross-linking and/or hydrolysis/oxidation of peptide bonds, while microbial metabolism produces
85 acid molecules and pigmented spots (Barbabetola et al. 2016). Starch glue has been commonly used
86 for ancient textile restoration (Ahmed and Kolisis 2011; Whaap 2007). After aging, starch paste is
87 generally found in shrunk, cracked, rigid and brittle form, which cannot provide enough adhesion for
88 effective support. In this form, it can cause heavy damage to ancient textiles because of concomitant
89 embrittlement, hardening, yellowness and acidity of the latter. Furthermore, starch may be a source
90 of contamination by amylolytic fungi and bacteria that contribute to textile decay over time, especially
91 when a suitable degree of humidity supporting microbial growth is present.

92 Consequently, cleaning of glue residues is often a priority in the restoration of ancient paper
93 or textile artworks. Current mechanical and chemical methods display serious drawbacks mainly
94 related to aggressiveness towards material and/or toxicity for the restorers and/or the environment.
95 Humidification (also called wet-cleaning) has been used to swell starch paste, however it generally
96 needs long treatments which are unsuitable for paper or textile artifacts and is often insufficient for
97 aged or hardened glues. Bio-based methods, i.e. the use of enzymes or microorganisms, have been
98 shown to be a very efficient alternative in a number of cases (Ahmed and Kolisis 2011; Barbabetola
99 et al. 2016; Ferrari et al. 2017).

100 Enzymes are certainly the most frequently used method for the treatment of glue-damaged
101 paper (Banik et al. 2003; Corbi et al. 2005; DeSantis 1983; Sandrine 2002) or textiles (mainly linen,
102 silk and cotton fabrics, so far) (Ahmed and Kolisis 2011; Ciatti et al. 2010) and several successful
103 examples have been reported in the literature. For instance, trypsin has been used for detaching a
104 compact block of leaves (Wendelbo 1976). Amylases and proteases have been employed for
105 detaching graphics from their backings (De la Chapelle 2003; Segal and Cooper 1977). Very recently
106 enzyme extracts with protease activity isolated from marine invertebrates have been used to remove
107 aged/altered protein glue layers from the velinatura (Japanese paper bonded by animal glue) of
108 ancient oil on canvas or from polychrome wood (Palla et al. 2016). This proved to be a cutting-edge
109 strategy also useful to bio-clean fragile artworks such as wax sculptures. An additional advantage of
110 these marine invertebrate-derived extracts is their antimicrobial activity useful to control
111 bacteria/fungi growth (Palla and Barresi 2017). It is worth noting that application of enzymes in
112 solution is not always suitable for paper or textile artworks, since it may involve artifact flooding with
113 excess water which favors mold and fungi colonization and growth and thus causes further damage
114 to the artifact (Ahmed and Kolisis 2011). Actually, water-dissolved α -amylase preparations have been
115 applied locally either in solution (Ahmed and Kolisis 2011) (Fig. 1a-c; Table 1) or as poultice (Bott
116 1990; Chapman 1986; Shibayama and Eastop 1996) for the bio-restoration of starch-glue treated
117 textiles. In general, the use of immobilized enzymes is preferable for these applications. Theoretically,
118 all enzymes can be immobilized. However, it is worth reminding that the immobilization yield and
119 the enzyme efficiency should be determined for each specific enzyme and immobilization strategy,
120 to limit the loss of enzyme and catalytic activity. A ready-to-use poultice of amylolytic enzymes,
121 called Albertina Kompresse, was developed by an Austrian group for removing non-swellable starch-
122 based glue from graphic artworks of albums of the “Albertina” graphic collection in Vienna (Schwarz
123 et al. 1999). Phytigel™ was used for lowering and controlling water content in enzyme solutions
124 (Iannucelli and Sotgiu 2009) used for cleaning -etchings depicting the China of Clemente VIII,
125 dating 1598. Gellan hydrogel-immobilized α -amylases have been developed for removing starch
126 paste from ancient paper documents (Mazzuca et al. 2014). A gellan-immobilized bacterial α -amylase
127 has been recently used to clean a wool shroud dating back to the Coptic period from a starch glue that
128 had been used in the 1950s to temporary consolidate the textile (Ferrari et al. 2017) (Fig. 1d-h; Table
129 1). After selection of the suitable enzyme (among those commercially available) and optimization of
130 the conditions for enzyme immobilization, the cleaning of the back of the two fragments (about 4 m²
131 of textile) composing the tunic was completed in 160 h of work (Ferrari et al. 2017). A recent study
132 (Barbabetola et al. 2016) has described the first attempt to bio-cleaning ancient paper from animal
133 glue by using living bacteria (Table 1). To this aim, non-pathogenic, non-spore-forming and non-

134 cellulolytic *Ochrobactrum sp.* TNS15E was used after immobilization on agar gel. This bacterial pack
135 was used to remove glue layers from paper documents dating back to the 17-18th century. Four-hour
136 treatment was sufficient to clean the cellulose fibers from glue, as confirmed by both colorimetric
137 and scanning electron microscopy (SEM) analyses.

138 Apart from paper/textile biocleaning from glue, it is worth reminding the case of aged drying-
139 oil stains on both ancient paper and textiles. During drying and aging, double bonds of unsaturated
140 fatty acids are oxidized by oxygen in the air, giving rise to multiple products which include
141 nonhomogeneous polymeric network of triacylglycerides which may be hard to being removed
142 (Ahmed et al. 2010; Blüher et al. 1997). Lipases such as that of *Candida cylindracea* have been used
143 to clean such aged drying-oily stains from paper documents (Blüher et al. 1997) or textiles such in
144 the case of a coptic tunic (Ahmed et al. 2010) (Table 1).

145

146 [Biorestitution/biocleaning of stone artworks](#)

147 Durable stone (e.g. marble and/or limestone) has been used for the construction of a multitude
148 of artworks and monuments all along the human history and all over the world including the Egyptian
149 Pyramids, the Greek and Roman temples and theaters, the European Cathedrals and the Taj Mahal in
150 India. Unfortunately, all of them have been suffering from progressive deterioration caused by both
151 biotic and abiotic agents (Dhami et al. 2014). These numerous factors have led to stone dissolution,
152 staining or color alteration, surface alteration, bio-corrosion and transformations into smaller sized
153 crystals, etc... (Chand and Cameotra 2011). In the recent decades, microbial biofilm production,
154 deposition of organic (such as residual hydrocarbons and other organic pollutants in dust) and
155 inorganic compounds (formation of nitrate and sulfate alterations such as the black crusts) have been
156 among the main deterioration events (Antonioli et al. 2005; Di Pippo et al. 2009; Fernandes 2006;
157 Warscheid and Braams 2000). Actually, limestone mainly consists of the most stable polymorph of
158 calcium carbonate, i.e. calcite, (with only a small content of aragonite) but is very porous and
159 hydrophilic. This makes limestone very susceptible to water flush (especially acid rain),
160 environmental pollutants and physical, chemical and biological (e.g. microorganisms) weathering
161 (Dhami et al. 2014). Therefore, survival of many cultural and historical assets is in threat. One of
162 such examples is the cave of Lascaux in southwest France which is considered the best conserved
163 prehistorical example of human wall painting art (they are also named the paleolytic Cappella
164 Sistina). In this site, infection of *Fusarium sp.* and other molds have deteriorated the floor and banks
165 of the main chamber (Rosenbaum 2006), but also autotrophic organisms such as green algae have
166 produced green pigments because of the intense illumination and improved CO₂ availability related

167 to visitors (Bastian et al. 2010). Martin-Sanchez and co-workers (2012) have extensively studied the
168 effectiveness of biocides in the cave biocleaning. Fungicides had been intensively applied to treating
169 the cave since 2001 that were essentially targeted to remove *Fusarium sp* but obtained little success.
170 In 2008, a new biocide treatment was planned due to black stains that appeared on the cave surfaces.
171 DGGE analysis on these stains showed the presence of *Ochroconis lascauxensis*. This result
172 demonstrated the ineffectiveness of the previous biocide treatments on the long time which appeared
173 to favor colonization by other fungal strains and therefore increase fungal diversity. Later, i.e. in
174 2010, fungal communities were quite different from those detected in 2008, since the main identified
175 strain was a yeast belonging to the *Herpotrichiellaceae* family. It is clear that careful preliminary
176 study on the possible advantages and disadvantages of applying biocides in subterranean
177 environments is required (Martin-Sanchez et al. 2012).

178 Many attempts have been made to fix such structural damages by application of traditional
179 conservative treatments such as organic and inorganic chemicals (Lazzarini and Laurenzi Tabasso
180 1986). However, these agents have been most often low effective, in spite of their aggressiveness
181 (which, on the other side, has led the concomitant risk of further damaging the artwork). Moreover,
182 these strategies involve the use of high amounts of solvents, which are finally discarded in the
183 environment creating problems of sustainability (Dhami et al. 2014). Alternatively, physical
184 treatments such as laser cleaning have been used, but at significantly higher costs (Germinario et al.
185 2017). Furthermore, all these treatments have short duration effects thus requiring repeated
186 interventions with relevant economic issues for public and private conservation agencies. Overall,
187 conventional treatment methods have therefore proved to be unsatisfactory.

188 The shortcomings of conventional strategies have encouraged research in new conservation
189 and remediation strategies based on biological methods (Fernandes 2006). As for the treatment of
190 other type of artifacts, bio-based restoration approaches for stone materials are characterized by lower
191 cost, toxicity and aggressiveness towards the artworks (Germinario et al. 2017). As described in the
192 following sections, bio-based methods have been used to remove different degradation products from
193 stone monuments, wall paintings, and marble statues (Germinario et al. 2017), including deposits of
194 environmental pollutants (Margesin et al. 2011) and synthetic polymers present in adhesives
195 (Giordano et al. 2018) as well as in paints used by graffiti writers (Sanmartin et al. 2014) (Fig. 2;
196 Table 1). In addition, biocleaning has been performed on stone artworks suffering from inaccurate or
197 aged restoration intervention (Beutel et al. 2002; Antonioli et al. 2005) (Fig. 2; Table 1).

198

199 Removal of sulfate and nitrate alterations

200 One of the most important causes of decay of calcareous stones is the conversion of calcium
201 carbonate into calcium sulfate (gypsum) mainly caused by acid rains (i.e. containing significant
202 amounts of sulfuric and nitric acid) (Ranalli et al. 1997). For instance, the genesis of “gypsum crusts”
203 on the surface of such porous material can engender following fractures of the underlying stone.
204 When calcium sulfate salts are accumulated together with atmospheric particles (pollen, dust, spores,
205 small particles of smog) the so called “black crusts” are formed (Fig. 2a). For the removal of sulfates
206 from artistic stoneworks, procedures based on the use of sulfate-reducing bacteria have been reported.
207 Different bacterial strains of the genus *Desulfovibrio* (e.g. *D. desulfuricans* and *D. vulgaris*) (either
208 pure or in mixed cultures) have been applied under anaerobic conditions to marble samples directly
209 or after adhesion to a sepiolite matrix (Ranalli et al. 1997). The use of sepiolite promoted sulfate
210 removal on both simulated samples and real marble statue artifacts. On the latter, 81 % sulfate
211 removal was obtained after 36 h treatment (Ranalli et al. 1997). Actually, *D. desulfuricans* (Ranalli
212 et al. 1997) and *D. vulgaris* (Ranalli et al. 1997; Cappitelli et al. 2007a; Alfano et al. 2011) have been
213 widely employed in restoration/removal of sulfate crusts from other artifacts (Table 1). Use of
214 biotechnological cleaning on durable stone monuments can sometimes comply with multiple types
215 of deterioration such as described by Cappitelli et al. (2007b). In this study, the sulfate-reducing
216 bacterium *D. vulgaris subsp. vulgaris* ATCC 29579 was employed to remove the black crust found
217 on marble of the Milan Cathedral (Italy). Compared to chemical cleaning (i.e. ammonium carbonate-
218 EDTA) strategy, the microbial-catalyzed approach resulted in more homogeneous removal of the
219 deposits and higher preservation of the original surface (Cappitelli et al. 2007b) (Table 1). Both
220 chemical and biological treatments converted gypsum (i.e. calcium sulfate) to calcite (i.e. calcium
221 carbonate), allowing consolidation. However, the chemical strategy also formed undesirable sodium
222 sulfate while the use of *D. vulgaris* did not (Cappitelli et al. 2007b). Nonetheless, biological removal
223 of sulfates may require quite long application periods, depending on the thickness of the crust. A
224 recent study has demonstrated that this period can be greatly shortened and general efficiency of
225 biocleaning can be significantly improved by combining the use of sulfate-reducing bacteria with a
226 non-biological strategy, e.g. the use of a non-ionic detergent (Troiano et al. 2013) (Fig. 2a, b; Table
227 1). This combined strategy shortened application times of about 38-70 % depending on the specific
228 artifact to be cleaned (Troiano et al. 2013).

229 Another consequence of acid rains (and of the action of living microorganisms) is the deposit
230 of calcium nitrate salts on stone buildings and wall paintings (Dhami et al. 2014). Here, again,
231 pollution increases the presence of various nitrogen oxides in the atmosphere that in turn may react
232 with rain water and form nitrous and, more abundantly, nitric acid which then reacts with stone and
233 replaces calcium carbonate with calcium nitrate (Dhami et al. 2014). Different strains of

234 *Pseudomonas spp.* have been recently applied for removing calcium nitrate salts from two stone
 235 monuments. Agar-entrapped *Pseudomonas stutzeri* DSMZ 5190 has been used for the biocleaning of
 236 nitrate efflorescence from wall paintings located in the lunettes of the central vault of the Santos
 237 Juanes church in Valencia, Spain (Bosch-Roig et al. 2013) (Fig. 2c-e; Table 1). The chosen strategy
 238 proved to be extremely efficient allowing to remove 92 % of the precipitates in 90 minutes.
 239 *Pseudomonas pseudocaligenes* KF707 has been used to remove nitrate salts from the tuff stone
 240 surfaces of the 12th century Matera Cathedral, Italy (Alfano et al. 2011) (Table 1). Here, carbogel-
 241 entrapped bacteria were applied to the Cathedral walls and allowed quick removal of the surface
 242 nitrate deposits, since 55 % of the nitrate salts were “cleaned” after 24 h.

243

244 Bioconsolidation

245 Apart from interventions aimed at removing superficial deposits and/or crust from stone
 246 monuments, the use of calcifying bacteria offers a chance to consolidate decayed building structures
 247 and materials. This application, sometimes also called microbial geotechnology (intending microbial-
 248 based technology for civil structures) actually mimics nature since many carbonate rocks have been
 249 cemented by carbonate precipitation induced by microorganisms during Earth geological cycles. This
 250 relatively novel and environmental-friendly technology has been studied for at least 20 years and has
 251 already been used for protecting and/or restoring different decayed construction materials/artifacts
 252 (Dhami et al. 2012; 2013).

253 Calcium carbonate precipitation is a chemical process (described by equation 1) which is
 254 influenced by four main factors, i.e. calcium concentration, amount of dissolved inorganic carbon
 255 (DIC), availability of nucleation sites and pH (Hammes and Verstraete 2002).



257
$$\text{Eq. 2} \quad K_{SP, \text{Calcite}, 25^\circ\text{C}} = [\text{Ca}^{2+}] [\text{CO}_3^{2-}] = 4.8 \times 10^{-9}$$

258 Calcium carbonate precipitation occurs when the product of concentrations of Ca^{2+} and CO_3^{2-} is
 259 higher than the solubility product (K_{SP}) of calcium carbonate (Eq. 2). However, the amounts of CO_3^{2-}
 260 in a given system depends on both the amount of DIC (which in turn depends on several parameters
 261 such as temperature and partial pressure of carbon dioxide) and pH. Because of the high number of
 262 parameters that may contribute to control calcium carbonate precipitation, different bacteria, isolated
 263 from different habitats, are able to create local micro-environments that induce such phenomenon
 264 (Hamilton 2003) (Fig. 3). The four main groups of microorganisms that may influence calcification
 265 are: (i) photosynthetic organisms such as cyanobacteria and algae, (ii) sulfate reducing bacteria

266 responsible for dissimilatory reduction of sulfates, (iii) organisms utilizing organic acids, and (iv)
267 organisms that are involved in nitrogen cycle either by ammonification of amino acids/nitrate
268 reduction or hydrolysis of urea (Stocks-Fischer et al. 1999; Hammes and Verstraete 2002; Jargeat et
269 al. 2003) (For an exhaustive review please refer to the study of Dhami et al. 2014). The precipitation
270 of carbonates by bacteria through urea hydrolysis is the most straightforward and easily controlled
271 mechanism of microbial induced calcium carbonate precipitation since it produces high amounts of
272 carbonates and an alkaline environment (Dhami et al. 2014). Boquet et al. (1973) firstly demonstrated
273 the precipitation of calcium carbonate by soil bacteria under laboratory conditions (Fig. 3). At that
274 time, several *Bacillus spp.* and *Pseudomonas aeruginosa* were shown to form calcite crystals. In
275 1990, Adolphe et al. patented the concept of using calcifying microorganisms to treat artificial
276 surfaces and founded the “Calcite Bioconcept” company. However, the first *in situ* application of
277 bioconsolidation was carried out in Thouars (France) on the tower of the Saint Médard Church by
278 using *Bacillus cereus* only in 1993 (Le Metayer-Levrel et al. 1999) (Table 1). Although this
279 application was judged as successful, some drawbacks were: the need to regularly repeat the treatment
280 (for instance, each 10 years); the presence of natural pigments in the nutritional medium of *B. cereus*
281 which co-precipitated with calcium carbonate thus giving the new stone layer a light persistent
282 coloring; the formation of endospores and a thin biofilm of *Bacillus sp.* For these reasons, Rodriguez-
283 Navarro et al. (2003) proposed to replace *Bacillus sp.* with a Gram-negative, non-pathogenic soil
284 bacterium, i.e. *Myxococcus xanthus*. Tiano et al. (1999) studied the effect of *Micrococcus spp.* and
285 *Bacillus subtilis* on Pietra di Lecce bioclastic limestone. Variations in the kind of bacteria used and
286 the methods for bacterial cell delivery to the stone surface have been tested by different Authors with
287 variable success (Daskalakis et al. 2014; Dhami et al. 2014; Helmi et al. 2016; Micallef et al. 2016).
288 Recently, the use of indigenous calcifying bacteria for re-inoculation of stone monuments has been
289 proposed as an alternative strategy for bioconsolidation (Jroundi et al. 2017). However,
290 microbiologically driven calcification remains more complex than chemical methods, since microbial
291 activity depends on many factors such as temperature, pH, concentrations of donors and acceptors of
292 electrons and concentration and diffusion rates of nutrients and catabolites. Hence, the use of
293 microbial calcification at large scales has not been always encouraged since it may be hard to manage
294 (Dhami et at. 2014). Also the cost of media required for bacterial growth may be a significant
295 economic limit of this approach (Achal et al. 2009; 2010).

296

297 Biorestitution of wall paintings

298 As previously mentioned, also stone artworks may suffer from inaccurate or aged restoration
299 strategies. This was the case of two wall paintings covered by animal glue layers during restoration
300 interventions which have been restored using different bio-based approaches as described by Beutel
301 et al. (2002) and Antonioli et al. (2005), respectively (Table 1; Fig. 2). The first study concerns
302 medieval wall paintings called “Falcon hunt-meeting of the living and the dead” located in St.
303 Alexander church in Wildeshausen, Germany. These paintings were suffering from severe peeling
304 off from the roughcast surface (Beutel et al. 2002) (Table 1). Actually, this is a common problem of
305 wall paintings in medieval churches of the Northern Europe since they have long been treated by
306 application of casein layers to stabilize them (Beutel et al. 2002). As for other glue-like matrices,
307 aging in addition to climate effects causes progressive hardening and stiffening of casein layers thus
308 causing an even more drastic peeling off of the painted parts from the surface (Beutel et al. 2002).
309 This problem was fixed by removing aged casein layers through application of a selected microbial
310 serine-protease (Alcalase 2.5 DX-L). The enzyme was covalently immobilized onto an epoxide-
311 functionalized cellulose acetate membrane (Beutel et al. 2002). By 2D fluorescence monitoring of
312 the tryptophan exposed by casein hydrolysis, it could be estimated that 30-minute treatment was
313 sufficient for substantial removal of the casein layers from the mural painting.

314 The case of “Conversion of S. Eufisio and battle” fresco by Spinello Aretino at the monumental
315 Cemetery of Pisa (Italy) was even more complicated (Fig. 2f, g; Table 1). Because of weathering and
316 other environmental aging, this fresco needed to be restored and for this purpose it was removed from
317 the wall surface by using the tear-off technique in the 1980s. Firstly, the fresco was covered with a
318 gauze that was stucked by applying an animal glue (mixed with high concentrations of formaldehyde,
319 as antimicrobial agent). Once the glue had been hardened, the fresco was detached from the wall.
320 Unfortunately, the fresco was then forgotten until 2000s in a storeroom, so that, traditional application
321 of protease mixtures were unable to remove the gauze (Antonioli et al. 2005). Because of the long
322 time of storage, it is likely that the presence of formaldehyde had promoted the formation of a resistant
323 net of cross-linked proteinaceous material, which was very recalcitrant to protease catalysis.
324 However, the selection of a bacterial strain (i.e. *Pseudomonas stutzeri*) able to grow on chips of
325 insoluble glue harvested from the “clothed” fresco (and hence possessing the right collagenase
326 enzymes), finally helped to solve the problem. Actually, it was then possible to use this bacterial
327 strain (i.e. cotton strips impregnated with live *Pseudomonas stutzeri* were applied) directly on the
328 fresco and completely degrade the glue layer and remove the cloth from the fresco after only 12 hour
329 treatment (Fig. 2f, g).

330

331 *Biocleaning of graffiti and synthetic adhesives*

332 Stone monuments are not only aggressed by atmospheric events or microorganisms but,
333 unfortunately, also by vandalism acts, including painting by unauthorized graffiti. Graffiti materials
334 have a complex chemical composition that comprises synthetic polymers such as acrylics, alkyds and
335 nitrocellulose, and several additives (Germinario et al. 2017). Quick removal of graffiti is an
336 important issue, since the fresher the graffiti, the easier is their removal. Here again, bio-based
337 (through enzymes or microorganisms) removal of synthetic materials is an emerging strategy which
338 has already shown good results in a number of cases.

339 One of the first examples of acrylic material removal by means of bio-based approach has
340 been described by Bellucci et al. (1999). Here, a lipase has been used to eliminate aged acrylic
341 ParaloidB72 resin from a 15th century tempera painting on panel and a 19th century oil painting on
342 canvas. In both artworks, the presence of surface layers containing ParaloidB72 was the result of
343 previous restoration interventions occurred in the early 1970s and in the 1980s, respectively (Bellucci
344 et al. 1999). Cleaning was likely achieved via hydrolysis of the ester groups of the acrylate and
345 methacrylate units contained in the synthetic resin leading to free carboxylic acid groups. This
346 reaction therefore generated more hydrophilic products which facilitated acrylic resin removal by
347 aqueous cleaning systems (Bellucci et al. 1999). Bio-based removal of acrylic materials is particularly
348 advantageous for treating painting where the use of traditional methods, e.g. organic solvents, would
349 likely remove also original paint layers. However, the same approach can be employed also to treat
350 stone materials. For instance, Germinario et al. (2017) tested different lipases in oil-in-water micro-
351 emulsions for the removal of acrylic marker pen inks from unglazed ceramic surfaces. Very recently,
352 Palla and co-workers (2017) successfully detached a canvas layer glued by the same acrylic
353 ParaloidB72 resin to a mosaic sample by applying a gelled (3% Klucel G) enzymatic solution with
354 esterase activity. The bio-removal was very fast, only 40 minutes at room temperature (Palla et al.
355 2017). The same research group also reported the removal of adhesive-tape glue residues present on
356 specific areas of an acrylic paint on canvas. They used a microemulsion of Velvesil Plus® (a
357 surfactant used in the cosmetic field) containing an esterase derived by a marine organism that proved
358 to be active even at a temperature lower than 30°C (whereas most commercially available enzymes
359 have an optimum temperature of 37°C) (Palla et al. 2016). These authors demonstrated that the
360 enzymatic solution can be merged into the Velvesil Plus® gel (without any negative effect on enzyme
361 activity) and easily applied to remove the undesired layers. The contact between enzyme and the layer
362 to be removed was obtained by gently moving the microemulsion, by a soft-brush for 5 minutes
363 (Giordano et al. 2018). As regards the use of living microorganisms, *D. desulfuricans* has proved able

364 to degrade nitrocellulose-based paints (Giacomucci et al. 2012), while the use of different bacterial
365 strains has been tested for the bio-cleaning of acrylic polymers used in the restoration field (Troiano
366 et al. 2014).

367 **CONCLUSION**

368 This report demonstrates that it is possible to face cultural heritage damage due to aging,
369 weathering, pollution or wrong restoration interventions by using bacteria or purified enzymes
370 suitably immobilized to contain the risk of employing aqueous solutions. Among the wide range of
371 enzymes commercially available those displaying a good catalytic activity at low temperatures (lower
372 than 30°C) are very promising since they can be applied also to fragile items. The microbial world as
373 well as the marine environment seem to be good candidates to be explored for finding such enzymes.
374 This field is promising also to find, in the future, a solution to contain microbial deterioration (Mazzoli
375 et al. 2018) thus avoiding the use of acids, solvents and surfactants (dangerous for the artworks, the
376 art restorers and the environment) for instance by using enzyme- or bacteriocin-mediated bacterial
377 competition. In this case, the safety and effectiveness of the microorganisms employed is mandatory
378 and the need for control and analysis before and after treatments strongly recommended.
379 Interdisciplinary approaches and collaborations between art conservators and biotechnologists,
380 biochemists and microbiologists is the essential requisite to preserve objects that state the immense
381 creativity of artists and the high value of human history.

382

383 **COMPLIANCE WITH ETHICAL STANDARDS**

384 Funding: this work was financially supported by “Ricerca Locale-ex 60%” of the Turin University.

385 Conflict of interest: all the Authors declare that they do not have any conflict of interest.

386 Ethical approval: this article does not contain any studies with human participants or animals
387 performed by any of the authors.

388

389 **REFERENCES**

390 Achal V, Mukherjee A, Basu PC, Reddy MS (2009) Lactose mother liquor as an alternative nutrient
391 source for microbial concrete production by *Sporosarcina pasteurii*. J Ind Microbiol Biotechnol
392 36:433-438.

393 Achal V, Mukherjee A, Reddy MS (2010). Biocalcification by *Sporosarcina pasteurii* using Corn
394 steep liquor as nutrient source. J Ind Biotechnol 6:170-174.

- 395 Adolphe JP, Loubière JF, Paradas J, Soleilhavoup F (1990) Procédé de traitement biologique d'une
396 surface artificielle. European Patent 90400G97.0. (after French patent 8903517, 1989), (France)
- 397 Ahmed HE, Gremos SS, Kolisis FN (2010) Enzymatic Removal of the Oily Dirt from a Coptic Tunic
398 using the Enzyme Lipase. *Journal of Textile and Apparel, Technology and Management* 6 (3)
- 399 Ahmed HE, Kolisis FN (2011) An investigation into the removal of starch paste adhesive from
400 historical textiles by using the enzyme of alpha-amylase. *J Cult Herit* 12:169-179.
- 401 Alfano G, Lustrato G, Belli C, Zanardini E, Cappitelli F, Mello E., Sorlini C, Ranalli G (2011) The
402 bioremoval of nitrate and sulfate alterations on artistic stone work: the case-study of Matera Cathedral
403 after six years from the treatment. *Int Biodeterior Biodegrad* 65:1004-1011.
- 404 Antonioli P, Zapparoli G, Abbruscato P, Sorlini C, Ranalli G, Righetti PG (2005) Art-loving bugs:
405 the resurrection of Spinello Aretino from Pisa's cemetery. *Proteomics* 5:2453-2459.
- 406 Banik G, Cremonesi P, De La Chapelle A, Montalbano L (2003) *Nuove Metodologie nel Restauro
407 del Materiale Cartaceo*. Il Prato, Padova, Italy.
- 408 Barbabietola N, Tasso F, Alisi C, Marconi P, Perito B, Pasquariello G, Sprocati AR (2016) A safe
409 microbe-based procedure for a gentle removal of aged animal glues from ancient paper. *Int
410 Biodeterior Biodegrad* 109:53-60.
- 411 Bastian F, Jurado V, Nováková A, Alabouvette C, Saiz-Jimenez C (2010) The microbiology of
412 Lascaux cave. *Microbiology* 156:644-652.
- 413 Bellucci R, Cremonesi P, Pignagnoli G (1999) A preliminary note on the use of enzymes in
414 conservation: the removal of aged acrylic resin coatings with lipase. *Stud Conserv* 44:278-281.
- 415 Beutel S, Klein K, Knobbe G, Königfeld P, Petersen K, Ulber R, Scheper T (2002) Controlled
416 enzymatic removal of damaging casein layers on medieval wall paintings. *Biotechnol Bioeng* 80:13-
417 21.
- 418 Blüher A, Grube A, Bornscheuer U, Banik G (1997) A reappraisal of the enzyme lipase for removing
419 drying-oil stains on paper. *The Paper Conservator* 21: 37-47.
- 420 Blüher A, Haller U, Banik G, Thobois E (1995) The application of carbopol poultices on paper
421 objects. *Restaur Int J Preserv Libr Arch Mater* 16:234-247.
- 422 Boquet E, Boronat A, Ramos-Cormenzana A (1973). Production of calcite (calcium carbonate)
423 crystals by soil bacteria is a general phenomenon. *Nature* 246:527-529.

- 424 Bosch-Roig P, Regidor Ros JL, Estellés RM (2013) Biocleaning of nitrate alterations on wall
425 paintings by *Pseudomonas stutzeri*. *Int Biodeterior Biodegrad* 84:266-274.
- 426 Bott G (1990) Amylase for starch removal from a set of 17th century embroidered panels. *Conservator*
427 14: 23-29.
- 428 Cappitelli F, Principi P, Pedrazzani R, Toniolo L, Sorlini C (2007a) Bacterial and fungal deterioration
429 of the Milan Cathedral marble treated with protective synthetic resins. *Sci Total Environ* 385:172-
430 181.
- 431 Cappitelli F, Toniolo L, Sansonetti A, Gulotta D, Ranalli G, Zanardini E, Sorlini C (2007b)
432 Advantages of using microbial technology over traditional chemical technology in removal of black
433 crusts from stone surfaces of historical monuments. *Appl Environ Microbiol* 73:5671-5675.
- 434 Chand T, Cameotra SS (2011) Geomicrobiology of heritage monuments and artworks: mechanisms
435 of biodeterioration, bioconservation strategies and applied molecular approaches In: *Bioremediation:*
436 *Biotechnology, Engineering and Environmental Management*, Nova Science Publishers, New York,
437 NY, pp. 233-266.
- 438 Chapman V (1986) Amylase in a viscous medium—textile applications. *Conservator* 10:7-11.
- 439 Ciatti M, Conti S, Fineschi C, Nelson J, K, Pini S (2010) Il ricamo in oro su disegno di Raffaellino
440 del Garbo. Aspetti storico-stilistici, tecnici, minimo intervento e conservazione preventiva. *OPD*
441 *Restauro* 22:81-116.
- 442 Corbi M, Di Franco ML, Severi M, Santarelli ML, Filetici P (2005) Biotecnologie applicate alla
443 rimozione di colle d'amido nel restauro della carta e del libro. *Proceedings of the 3rd IGIIC National*
444 *Congress -Lo Stato dell'Arte*, 98-101.
- 445 Daskalakis MI, Magoulas A, Kotoulas G, Katsikis I, Bakolas A, Karageorgis AP, Mavridou A, Doulia
446 D, Rigas F (2014) *Cupriavidus metallidurans* biomineralization ability and its application as a
447 bioconsolidation enhancer for ornamental marble stone. *Appl Microbiol Biotechnol* 98:6871-6883.
- 448 De La Chapelle A (2003) Utilizzo degli enzimi nel restauro delle opere grafiche policrome. In:
449 Cremonesi P (ed) *Materiali tradizionali ed innovativi nella pulitura dei dipinti e delle opere policrome*
450 *mobili. Atti del Primo Congresso Internazionale Colore e Conservazione - materiali e metodi nel*
451 *restauro delle opere policrome mobili, Piazzola sul Brenta (PD), Italy.*
- 452 De Santis P (1983) Some observation on the use of enzymes in paper conservation. *J Am Inst Conserv*
453 1:7-27.

- 454 Dhama NK, Mukherjee A, Reddy MS (2012) Biofilm and microbial applications in biomineralized
455 concrete. In: Jong Seto (ed) *Advanced Topics in Biomineralization*, InTech, New York, NY, pp. 137-
456 164.
- 457 Dhama NK, Mukherjee A, Reddy MS (2013) Biomineralization of calcium carbonates and their
458 engineered applications: a review. *Front Microbiol* 4:314.
- 459 Dhama NK, Reddy MS, Mukherjee A (2014) Application of calcifying bacteria for remediation of
460 stones and cultural heritages. *Front Microbiol* 5:304.
- 461 Dick J, De Windt W, De Graef B, Saveyn H, Van der Meeren P, De Belie N, Verstraete W (2006)
462 Bio-deposition of a calcium carbonate layer on degraded limestone by *Bacillus species*.
463 *Biodegradation* 17:357-367.
- 464 Di Pippo F, Bohn A, Congestri R, De Philippis R, Albertano P (2009) Capsular polysaccharides of
465 cultured phototrophic biofilms. *Biofouling* 25:495-504.
- 466 Fernandes P (2006) Applied microbiology and biotechnology in the conservation of stone cultural
467 heritage materials. *Appl Microbiol Biotechnol* 73:291-296.
- 468 Ferrari M, Mazzoli R, Morales S, Fedi M, Liccioli L, Piccirillo A, Cavaleri T, Oliva C, Gallo P, Borla
469 M, Cardinali M, Pessione E (2017) Enzymatic laundry for old clothes: immobilized alpha-amylase
470 from *Bacillus sp.* for the biocleaning of an ancient Coptic tunic. *Appl Microbiol Biotechnol* 101:7041-
471 7052.
- 472 Germinario G, van der Werf ID, Palazzo G, Ros JLR, Montes-Estelles RM, Sabbatini L (2017)
473 Bioremoval of marker pen inks by exploiting lipase hydrolysis. *Prog Org Coat* 110:162-171.
- 474 Giacomucci L, Toja F, Sanmartín P, Toniolo L, Prieto B, Villa F, Cappitelli F (2012) Degradation of
475 nitrocellulose-based paint by *Desulfovibrio desulfuricans* ATCC13541, *Biodegradation* 23:705-716.
- 476 Giordano A, Rotolo V, Di Carlo E, Palla F (2018) Novel esterases for cultural heritage. X AIAR
477 National Congress, Torino, Italy, February 14-17, 2018.
- 478 Gioventù E, Lorenzi PF, Villa F, Sorlini C, Rizzi M, Cagnini A, Griffo A, Cappitelli, F. (2011)
479 Comparing the bioremoval of black crusts on colored artistic lithotypes of the Cathedral of Florence
480 with chemical and laser treatment. *Int Biodeterior Biodegrad* 65:832-839.
- 481 Gostling K (1989) Bookbinders and adhesives: part 2. *New Bookbind* 9:30-39.
- 482 Hamilton WA (2003). Microbially influenced corrosion as a model system for the study of metal
483 microbe interactions: a unifying electron transfer hypothesis. *Biofouling* 19:65–76

- 484 Hammes F, Verstraete W (2002). Key roles of pH and calcium metabolism in microbial carbonate
485 precipitation. *Rev Environ Sci Biotechnol* 1:3–7
- 486 Helmi FM, Elmitwalli HR, Elnagdy SM, El-hagrassy AF (2016). Biomineralization Consolidation of
487 Fresco Wall Paintings Samples by *Bacillus sphaericus*. *Geomicrobiol J* 33:625-629.
- 488 Hrdlickova Kuckova S, Crhova Krizkova M, Pereira CL, Hynek R, Lavrova O, Busani T, Branco LC,
489 Sandu IC (2014) Assessment of green cleaning effectiveness on polychrome surfaces by MALDI-
490 TOF mass spectrometry and microscopic imaging. *Microsc Res Tech* 77:574-585.
- 491 Iannucelli, S., Sotgiu, S., 2009. La pulitura superficiale di opera grafiche a stampa con gel rigidi. In:
492 Progetto Restauro, 49. Il Prato, Padova, pp. 15-24.
- 493 Jargeat P, Rekanalt D, Verner MC, Gay G, Debaud JC, Marmeisse R, Fraissinet-Tachet L. (2003)
494 Characterisation and expression analysis of a nitrate transporter and nitrite reductase genes, two
495 members of a gene cluster for nitrate assimilation from the symbiotic basidiomycete *Hebeloma*
496 *cylindrosporum*. *Curr Genet* 43:199-205.
- 497 Jroundi F, Schiro M, Ruiz-Agudo E, Elert K, Martín-Sánchez I, González-Muñoz MT, Rodriguez-
498 Navarro C (2017) Protection and consolidation of stone heritage by self-inoculation with indigenous
499 carbonatogenic bacterial communities. *Nat Commun* 8:279.
- 500 Lazzarini L, Laurenzi Tabasso M (1986) Il Restauro della Pietra. CEDAM, Padua, Italy.
- 501 Le Metayer-Levrel G, Castanier S, Oriol G, Loubiere JF, Perthuisot JP (1999) Applications of
502 bacterial carbonatogenesis to the protection and regeneration of limestones in buildings and historic
503 patrimony. *Sediment geol* 126:25-34.
- 504 Margesin R, Płaza GA, Kasenbacher S (2011) Characterization of bacterial communities at heavy-
505 metal-contaminated sites. *Chemosphere* 82:1583-1588.
- 506 Martin-Sanchez PM, Nováková A, Bastian F, Alabouvette C, Saiz-Jimenez C (2012). Use of biocides
507 for the control of fungal outbreaks in subterranean environments: the case of the Lascaux Cave in
508 France. *Environ Sci Technol* 46:3762-3770.
- 509 Mazzoli R, Giuffrida MG, Pessione E (2018) Back to the past. Find the guilty: microorganisms
510 involved in the biodeterioration of archeological and historical items. *Appl Microbiol Biotechnol* (In
511 press).

- 512 Mazzuca C, Micheli L Cervelli E, Basoli F, Cencetti C, Coviello T, Iannuccelli S, Sotgiu S, Palleschi
513 A (2014) Cleaning of paper artworks: development of an efficient gel-based material able to remove
514 starch paste. ACS Appl Mater Inter 6: 16519-16528.
- 515 Micallef R, Vella D, Sinagra E, Zammit G (2016) Biocalcifying *Bacillus subtilis* cells effectively
516 consolidate deteriorated Globigerina limestone. J Ind Microbiol Biotechnol 43:941-952.
- 517 Palla F, Barresi G (2017) Biotechnology and Conservation of Cultural Heritage, Springer
518 International Publishing, Switzerland.
- 519 Palla F, Barresi G, Chisesi RM, Cammarata M, Di Carlo E, Drago S, Giordano A, Lombardo G,
520 Rotolo V, Schiavone S, Stampone G, Trapani MR (2017) Innovative and Integrated Strategies: Case
521 Studies. In: Palla F, Barresi G (eds) Biotechnology and Conservation of Cultural Heritage, Springer
522 International Publishing, Switzerland, pp 85-100.
- 523 Palla F, Barresi G, Giordano A, Schiavone S, Trapani MR, Rotolo V, Parisi MG, Cammarata M
524 (2016) Cold-active molecules for a sustainable preservation and restoration of historic-artistic
525 manufacts. Int J Conserv Sci 7: 239-246
- 526 Ranalli G, Chiavarini M, Guidetti V, Marsala F, Matteini M, Zanardini E, Sorlini C (1997) The use
527 of microorganisms for the removal of sulphates on artistic stone works. Int Biodeterior Biodegrad
528 40:255-261.
- 529 Rodriguez-Navarro C, Rodriguez-Gallego M, Ben Chekroun K, Gonzalez-Munoz MT (2003)
530 Conservation of ornamental stone by *Myxococcus xanthus* induced carbonate biomineralization. Appl
531 Env Microbiol 69:2182-2193.
- 532 Rosenbaum MS (2006). Field Meeting Report: Bromfield Sand and Gravel Pit, nr Ludlow,
533 Shropshire, led by Ed Webb, 22nd April 2005. Proceedings of the Shropshire Geological Society
534 11:12-17.
- 535 Sandrine D (2002) Enzyme used for adhesive removal in paper conservation: a literature review. J
536 Soc Arch 2:187-195.
- 537 Sanmartin P, Cappitelli F, Mitchell R (2014) Current methods of graffiti removal: a review, Constr
538 Build Mater 71:363-374.
- 539 Schwarz I, Bluher A, Banik G, Thobois E, Maurer KH (1999) The development of a ready-for-use
540 poultice for local removal of starch paste by enzymatic action. Restaurator 20:225-244.
- 541 Segal J, Cooper D (1977). The use of enzymes to release adhesives. Pap Conservator 2:47-50.

- 542 Shibayama N, Eastop D (1996) Removal of flour paste residues form a painted banner with alpha
543 amylase. *Conservator* 20:53-64.
- 544 Stocks-Fischer S, Galinat JK, Bang SS (1999) Microbiological precipitation of CaCO₃. *Soil Biol*
545 *Biochem* 31:1563-1571.
- 546 Tiano P, Biagiotti L, Mastromei G (1999) Bacterial bio-mediated calcite precipitation for
547 monumental stones conservation: methods of evaluation. *J Microbiol Methods* 36:139-145.
- 548 Troiano F, Gulotta D, Balloi A, Polo A, Toniolo L, Lombardi E, Daffonchio D, Sorlini C, Cappitelli
549 F (2013) Successful combination of chemical and biological treatments for the cleaning of stone
550 artworks. *Int Biodeterior Biodegrad* 85:294-304.
- 551 Troiano F, Vicini S, Gioventù E, Lorenzi PF, Improta CM, Cappitelli F (2014) A methodology to
552 select bacteria able to remove synthetic polymers. *Polym Degrad Stab* 107:321-327.
- 553 Warscheid T, Braams J (2000) Biodeterioration of stone: a review. *Int. Biod. Biodegr.* 46:343-368.
- 554 Webster A, May E (2006) Bioremediation of weathered-building stone surfaces. *Trends Biotechnol*
555 24:255-260.
- 556 Wendelbo O (1976) *The Use of Proteolytic Enzymes in the Restoration of Paper and Papyrus.*
557 University Library of Bergen.
- 558 Whaap F (2007) The treatment of two Coptic tapestry fragments. *V&A Conservation Journal* 55:11-
559 13.
- 560

Figure Legends

Fig. 1. Biocleaning of ancient textiles from starch glue. Pictures refer to biocleaning of a historical carpet dating back to the Ottoman period and exhibited in the museum of the Faculty of Applied Arts, Helwan University, Egypt (a, b, c) (modified from Ahmed and Kolisis 2011) and a coptic tunic dating back to the 5-6th century A.D. and exposed at the Egyptian Museum, Turin, Italy (d, e, f, g, h) (modified from Ferrari et al. 2017). b, c Detail of the carpet before (b) and after (c) the α -amylase treatment. e-h, Details of the Coptic tunic before (e, g) and after (f, h) the α -amylase treatment.

Fig. 2. Biorestitution of stone monuments. a, b, Detail of a marble statue dedicate in 1921 by Lina Arpesani to the poetess Anna Zuccari and located in the Monumental Cemetery of Milan (Italy). The black crusts (a) affecting the statue were cleaned (b) by using sulfate-reducing *Desulfovibrio vulgaris* (modified from Troiano et al. 2013). c, d, e Cleaning of the wall painting in the lunette of the Santos Juanes church, Valencia, Spain from nitrate salt efflorescence by means of agar gel-entrapped *Pseudomonas stutzeri*. Pictures represent the fresco area before (c), during (d) and after biocleaning (modified from Bosch-Roig et al. 2013). f, e biorestitution of the Spinello Aretino fresco “Conversion of S. Efsio and battle” in the Monumental Cemetery of Pisa (Italy). f For animal glue removal. Cotton strips impregnated with live *Pseudomonas stutzeri* were applied leading to fresco biocleaning (g) (modified from Antonioli et al. 2005)

Fig. 3. Calcifying bacteria. Colonies of 6 different strains of *Bacillus sphaericus* and *Bacillus lentus* on agar plates during calcium carbonate deposition are shown (Dick et al. 2006).

Table 1. Some of the most significant examples of biorestitution/biocleaning of artworks described in the present study.

Type of artwork	Specific artwork (specimen)	Historical period of the specimen	Issue	Biorestitution/biocleaning strategy	Reference
Paper	Graphic artworks from albums (Graphic Collection Albertina, Vienna, Austria)	XIX century A.D.	Removal of aged starch glue	Gel-entrapped α -amylase	Schwarz et al. 1999
Paper	Paper documents of the Genoese Republic (Central Institute for Graphic Arts, Rome, Italy)	XVII-XVIII century A.D.	Removal of aged animal glue	Agar-immobilized <i>Ochrobactrum sp.</i> TNS15E	Barbabetola et al. 2016
Textile	Coptic tunic (Greek-Roman Museum, Alexandria, Egypt)		Removal of aged oily stains	Lipase from <i>Candida cylindracea</i>	Ahmed et al. 2010
Textile	Carpet (Museum of the Faculty of Applied Arts, Helwan University, Egypt)	Ottoman period	Removal of aged starch glue	α -amylase from <i>Aspergillus oryzae</i>	Ahmed and Kolis 2011
Textile	Coptic tunic (Egyptian Museum, Turin, Italy)	V-VI century A.D.	Removal of aged starch glue	Gellan immobilized α -amylase from <i>Bacillus sp.</i>	Ferrari et al. 2017
Stone monument	Milan Cathedral (Italy)	XV century A.D.	Removal of black crust	Sulfate-reducing <i>Desulfovibrio vulgaris</i> ATCC 29579	Cappitelli et al. 2007b
Stone monument	Florence Cathedral (Italy)	XV century A.D.	Removal of black crust	Carbogel-entrapped sulfate-reducing <i>Desulfovibrio vulgaris</i> ATCC 29579	Gioventù et al. 2011
Stone monument	Matera Cathedral (Italy)	XII century A.D.	Removal of nitrate and sulphate crusts	Carbogel entrapped nitrate-reducing <i>Pseudomonas pseudoalcaligenes</i> KF707 and sulfate-reducing <i>Desulfovibrio vulgaris</i> ATCC 29579	Alfano et al. 2011
Stone monument	Stone column and marble statue, Monumental Cemetery (Milan, Italy)	XX century A.D.	Removal of black crust	<i>Desulfovibrio vulgaris</i> ATCC 29579 plus non-ionic detergent	Troiano et al. 2013

Stone monument	Saint Médard Church, (Thouard, France)	XII century A.D.	Limestone bioconsolidation	<i>Bacillus cereus</i>	Le Metayer et al. 1999
Wall paintings	Wall paintings of the lunettes of the central vault, Santos Juanes church (Valencia, Spain)	XVII-XVIII century A.D.	Removal of calcium nitrate salt efflorescence	Agar-entrapped <i>Pseudomonas stutzeri</i> DSMZ 5190	Bosch-Roig et al. 2013
Wall paintings	Falcon hunt-Meeting of the living and the dead, St. Alexander church (Wildeshauen, Germany)	XIV century A.D.	Removal of aged casein layers	Covalently immobilized protease (Alcalase 2.5 DX-L)	Beutel et al. 2002
Wall paintings	Conversion of S. Efisio and battle by Spinello Aretino, Monumental Cemetery (Pisa, Italy)	XIV century A.D.	Removal of aged formaldehyde-treated animal glue	Cotton strips impregnated with <i>Pseudomonas stutzeri</i> A29	Antonioli et al. 2005
Painting on panel	The Visitation with St. Joseph, St. Zacharias and Four Angels	XV century A.D.	Removal of acrylic resin	Lipase from <i>Candida cylindracea</i>	Bellucci et al. 1999
Painting on canvas	Portrait of a man	XIX century A.D.	Removal of acrylic resin	Lipase from <i>Candida cylindracea</i>	Bellucci et al. 1999

Fig. 1.

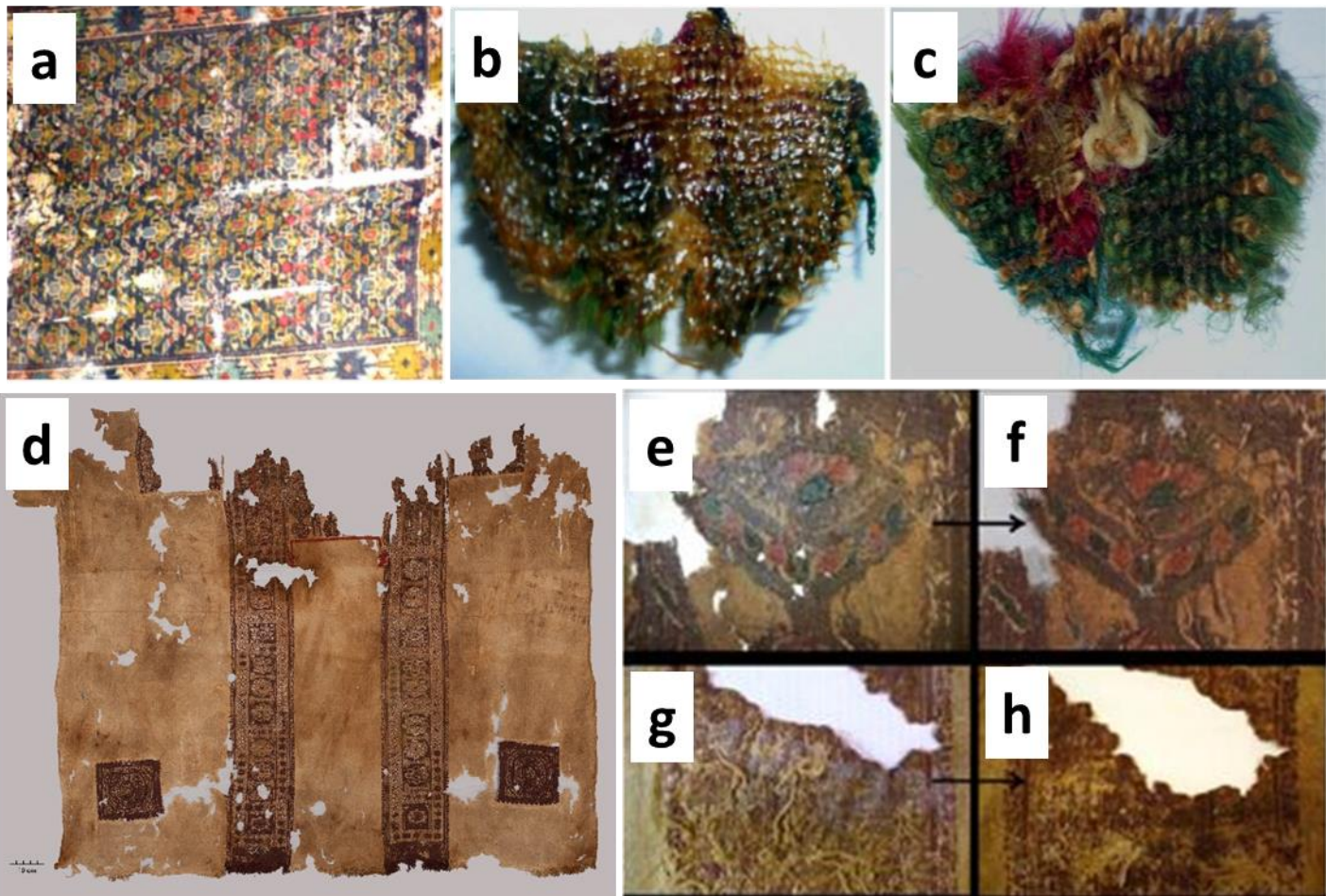


Fig. 2.

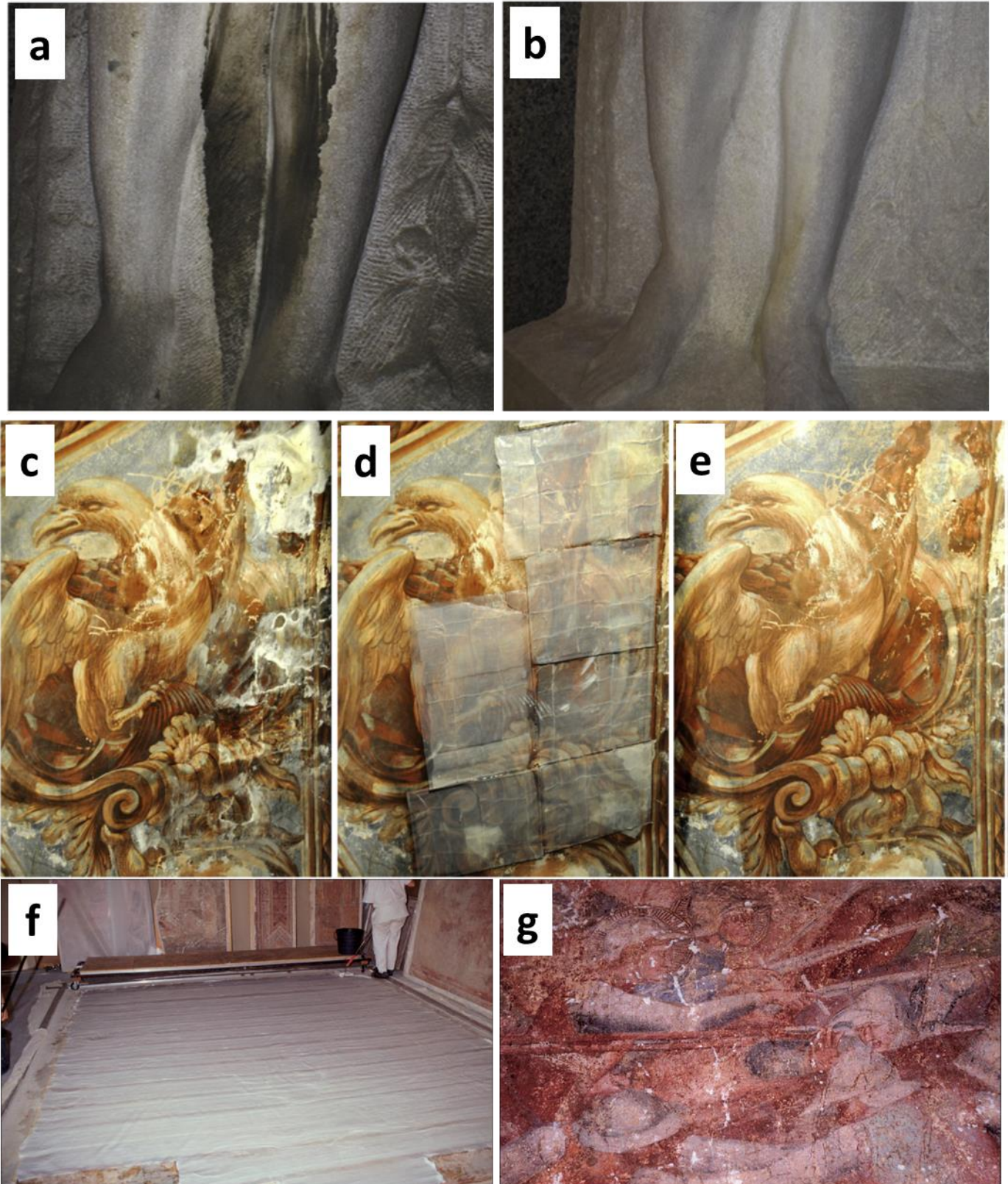
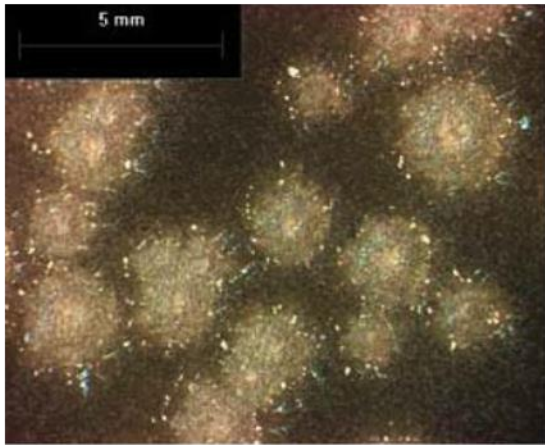
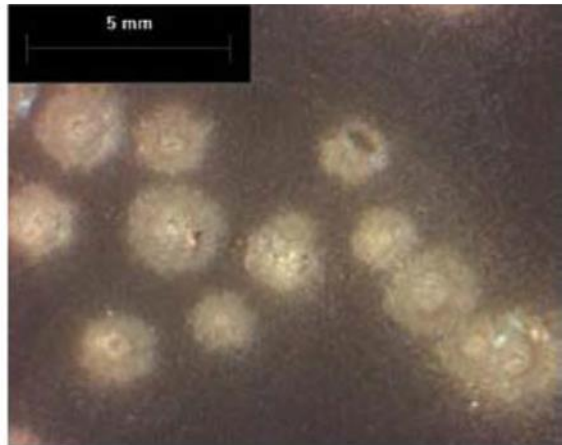


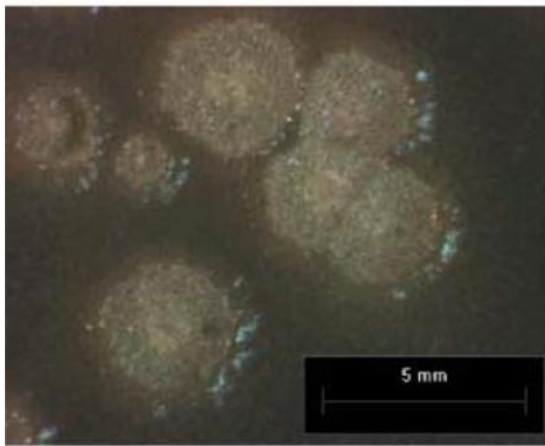
Fig. 3.



55



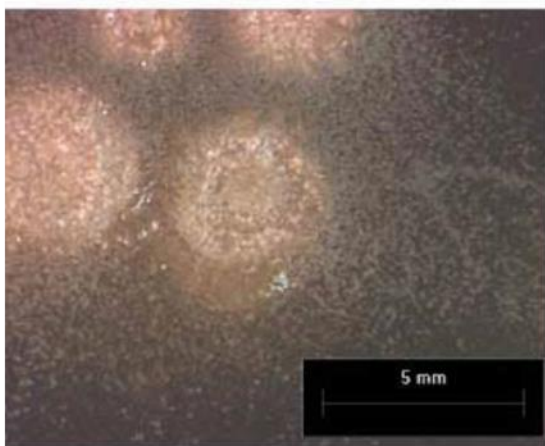
56



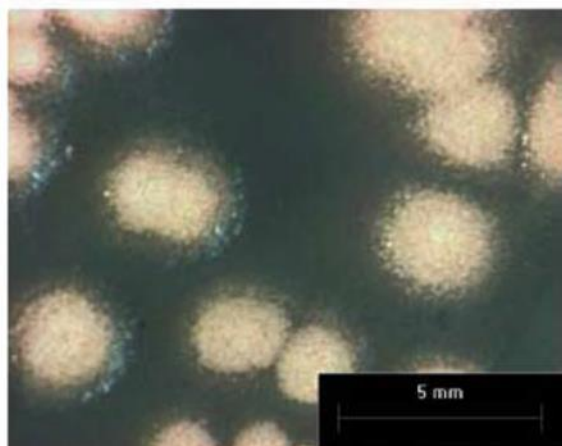
57



58



59



60