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Feasibility of Removing Surface Deposits on Stone Using Biological and Chemical Remediation Methods

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Abstract The study was conducted on alterations found on stone artwork and integrates microbial control and a biotechnological method for the removal of undesirable chemical substances. The Demetra and Cronos sculptures are two of 12 stone statues decorating the courtyard of the Buonconsiglio Castle in Trento (Italy). An initial inspection of the statues revealed putative black crusts and highlighted the microbial contamination causing discoloration. In 2006, the Cultural Heritage Superintendence of Trento commissioned us to study and remove these chemical and biological stains. Stereomicroscopy characterised the stone of the sculptures as oolitic limestone, and infrared analyses confirmed the presence of black crusts. To remove the black crusts, we applied a remediation treatment of sulphate-reducing bacteria, which removes the chemical alteration but preserves the original stone and the patina noble. Using traditional and biomolecular methods, we studied the putative microbial contamination and confirmed the presence of biodeteriogens and chose biocide Biotin N for the removal of the agents causing the discolouration. Denaturing gradient gel electrophoresis fluorescent in situ hybridisation established that Cyanobacteria and green algae genera were responsible for the green staining whereas the black microbial contamination was due to dematiaceous fungi. After the biocide Biotin N treatment, we applied molecular methods and demonstrated that the Cyanobacteria, and most of the green algae and dematiaceous fungi, had been efficiently removed. The reported case study reveals that conservators can benefit from an integrated biotechnological approach aimed at the biocleaning of chemical alterations and the abatement of biodeteriogens.

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Introduction

Many historic, cultural and artistic objects and buildings are made of stone. Like all materials, stone is subject to inexorable deterioration, especially if exposed to the weather. In this regard, a particularly important role is played by pollution, which is the factor most responsible for depositing various chemical substances and biological agents on outdoor stone surfaces. This paper reports our work on two outdoor stone sculptures housed at the Buonconsiglio Castle, Trento, Italy. Before the conservation intervention, visual inspection revealed that most of the sculptures in the castle courtyard show signs of deterioration. On first inspection, the two investigated sculptures presented alterations putatively attributed to chemical agents (possible black crusts) and to microorganisms (putative microbial contamination of different colours).

In the presence of atmospheric pollution and a humid environment, calcareous stone is transformed into hydrated calcium sulphate, namely gypsum ($CaSO_4 \cdot 2H_2O$), which should be removed from surfaces as, because of hygrometric

fluctuations, it undergoes dissolving and crystallising processes that lead to mechanical stress. In areas sheltered from the rain, gypsum embeds mineral and smog particles, leading to the formation of the so-called black crusts that represent chemical alteration and aesthetic disfigurement.

In addition, in the same conditions, many biodeteriogens, organisms capable of causing deterioration, can be found on stone: bacteria, algae, fungi and lichens [4, 24, 35, 37, 41]. Biodeterioration can cause chemical, physical and aesthetic damage [12, 19]. The colour of the microbial contamination depends on the pigments produced by the microorganisms, and an understanding of the complex microbial ecosystem of cultural heritage surfaces is a prerequisite for controlling the growth of microbial species that cause biodeterioration [21, 41].

The importance of an efficient and careful cleaning of stone artwork for the removal of chemical and biological alterations cannot be overstressed. Indeed, before cleaning, it is fundamental to have knowledge of the type of stone and the characterisation of the chemical and biological alterations in order to choose the optimal cleaning intervention. The use of solvents and physical methods (like abrasion) for the removal of chemical alterations can affect the sound stone material and result in low selectivity [10]. In the past few years, much progress has been made using viable cells able to remove sulphates from stone ornamental surfaces [7, 42]. In removing of black crusts, Cappitelli and co-workers [6] demonstrated the advantages of using microbial technologies over traditional chemical technologies: the biological procedure resulted in a more homogeneous removal of the surface deposits without chemically compromising the substrata and preserved the patina noble under the black crust itself. In addition, biocleaning has proven to be more selective than chemicals.

With regard to biodeterioration, microbial abatement is commonly achieved using biocides. As the biocide is a toxic compound, it is very important to identify the biodeteriogens to choose a suitable product that targets only specific biodeteriogen taxa.

In our study, we successfully used, for the first time, an integrated biotechnological system that enables the cleaning of stone cultural heritage affected by chemical and biological agents. It is our belief that this system provides a useful and reliable model for conservation intervention in similar case studies.

Methods

Sculpture Description

The Buonconsiglio Castle, built in the thirteenth century for defence purposes, is the most important secular monument in Trento (Italy). In the second half of the eighteenth century, the courtyard was embellished with 12 statues probably made by Jacob Eberle in 1764. Two of these sculptures were studied in this work: Demetra and Cronos. Visual inspection revealed that the two sculptures presented the following forms of alteration: (1) putative black crusts and (2) green, green–black and black staining putatively attributed to the presence of microorganisms.

Sampling

All the samples were taken by scalpel (sterile for biological analysis) according to the Italian Cultural Heritage Ministry Recommendation [9].

Table 1 reports all the samples, indicating the sculpture from which they were taken, sampling location and description of the alteration. For the chemical analyses, six samples were taken from the Cronos sculpture (1b to 6b) and two from the Demetra (7b and 8b). Samples 4b and 8b were taken from apparently non-altered areas whereas specimens 1b and 2b were putative black microbial contaminations, and samples 3b, 5b, 6b and 7b showed a putative black crust alteration. After the biocleaning, two samples were taken. The sample 3p was collected close to the sampling area of the specimen 3b, and the sample 5p was collected close to the sampling area of the specimen 5b.

In addition, 11 samples affected by putative biological discolouration (green, black and green–black) and black crust were selected to study the microbial community. Four of them were collected from the Demetra sculpture (9b to 13b), and six specimens were taken from the Cronos sculpture (14b to 19b). Three specimens were representative of green microbial contamination (9b, 16b and 17b); three specimens were representative of black microbial contamination (13b, 18b and 19b); samples 11b and 14b were representative of green–black microbial contamination, and sample 12b most likely presented a black crust. In addition, samples 10b and 15b were taken from apparently non-altered areas. After the treatment, three samples were taken. Samples 14p, 17p and 19p were collected close to the sampling area of the specimen 14b, 17b and 19b, respectively.

Chemical Analyses

Stereomicroscope and Cross-Section Observations

Samples were observed by Wild Makroskop M420 stereomicroscope (Heerbrugg, Switzerland), equipped with an Olympus OM1 camera (Chicago, USA). The cross sections were prepared for the 5b and 6b samples and observed using the optical Leitz Ortholux microscope (Wetzlar, Germany).

Table 1 Samples collected: sculpture, location, description and chemical and microbiological analyses carried out

Samples	Sculpture	Location	Putative alteration (symbol)	Type of analysis carried out
1b	Cronos	Child collar	Black microbial contamination (B)	Chemical
2b	Cronos	Arm	Black microbial contamination (B)	
3b	Cronos	Cloak	Black crust (●)	
4b	Cronos	Knee	Non altered sample (○)	
5b	Cronos	Cloak	Black crust (●)	
6b	Cronos	Cloak	Black crust (●)	
7b	Demetra	Shoulder	Black crust (●)	
8b	Demetra	Dress	Non altered sample (○)	
9b	Demetra	Right-hand	Green microbial contamination (G)	Biological
10b	Demetra	Dress	Non altered sample (○)	
11b	Demetra	Left-arm	Green-black microbial contamination (GB)	
12b	Demetra	Left-hand	Black crust (●)	
13b	Demetra	Right-shoulder	Black microbial contamination (B)	
14b	Cronos	Left-elbow	Green-black microbial contamination (GB)	
15b	Cronos	Left-knee	Non altered sample (○)	
16b	Cronos	Breasts	Green microbial contamination (G)	
17b	Cronos	Child abdomen	Green microbial contamination (G)	
18b	Cronos	Left-arm	Black microbial contamination (B)	
19b	Cronos	Left-elbow	Black microbial contamination (B)	
3p	Cronos	Cloak (area of the specimen 3)	-	Chemical
5p	Cronos	Cloak (area of the specimens 5)	-	
19p	Cronos	Left-elbow (area of the specimen 19)	-	Biological
14p	Cronos	Left-elbow (area of the specimen 14)	-	
17p		Child abdomen (area of the specimen 17)	-	

Samples marked with b were collected before the treatment and specimens marked with p after the treatment

Fourier Transform Infrared Spectroscopy

Fourier transform infrared (FTIR) analyses, used to detect both inorganic minerals and organic compounds in the samples, were carried out by a Nicolet Nexus spectrophotometer (Washington, USA) coupled with a Nicolet Continuum Fourier transform infrared spectroscopy microscope equipped with a HgCdTe detector cooled with liquid N_2. Spectra were recorded by a Graseby-Specac diamond cell accessory in transmission mode between 4,000 and 700 cm^{-1}. The FTIR analyses were performed on samples collected both before and after the treatments.

Biocleaning

The remediation treatment using sulphate-reducing bacteria (SRB) was applied for the removal of the chemical alterations due mainly to gypsum. The biomass entrapped in the delivery system was prepared as described by Cappitelli and co-workers [7]: the SRB employed was *Desulfovibrio vulgaris* subsp. *vulgaris* ATCC 29579 maintained in DSMZ 63 medium, and the delivery system was Carbogel (CTS, Vicenza, Italy). Before use in the treatment, the cells were grown in DSMZ 63 medium modified by eliminating any iron source. After centrifugation and prior to mixing with the Carbogel powder, the cell pellet was suspended in deaerated phosphate buffer supplemented with 0.599 gl^{-1} sodium lactate at pH7.0 at a concentration of 10^8 cells per millilitre. All the manipulations described above were done under anaerobic conditions in a glove box. The surfaces to be treated were moistened and covered with tissue paper before any applications began. The biologically treated areas underwent three 12-h applications for a total duration of 36 h. 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 [6].

Biological Analyses

Culturable Community Analyses

Sample powders were inoculated in two cultural media to determine the microbial counts on the Demetra and Cronos surfaces: plate count agar medium (PCA, Merck) for aerobic heterotrophic bacteria with incubation at 30°C for 3–5 days and potato dextrose agar medium (PDA, Merck) for fungi with incubation at 30°C for 3–5 days. The samples collected after the cleaning treatment were also inoculated in PCA and PDA, under the same conditions adopted before the treatment. Quantification of microorganisms grown on PCA and PDA was expressed as colony-forming unit (CFU) per gramme.

Sample powders were also inoculated in Chu and Detmer media [5] to study the Cyanobacteria and Chlorophyta communities, respectively. The samples were incubated for 30 days under natural light at room temperature. The samples collected after the cleaning treatment were also inoculated in Chu and Detmer media under the same conditions adopted before the treatment. Presence or absence of Cyanobacteria and eukaryotic algal growths was recorded.

DNA Extraction

Total DNA was extracted directly from the stone samples as described by [2] but with the following modifications: no lysozyme was added and three cycles −80°C/70°C were performed before the addition of the proteinase K. The Cyanobacteria and eukaryotic algal DNA were also extracted from communities grown, respectively, in Chu and Detmer media, using the same protocol.

Both the microbial communities present on the sculptures before and after the treatment were studied.

Analysis of Bacteria Community

The 16S rRNA gene fragments extracted from altered stone samples were amplified with primers GC-357 F and 907 R with the following chemical conditions: 1× of polymerase chain reaction (PCR) Rxn buffer, 1.5 mM of $MgCl_2$, 0.12 mM of dNTP mix, 0.3 µM of each primer and 1 U of Taq DNA polymerase in 50-µl PCR reaction. The thermal cycling programme included an initial denaturation at 94°C for 4 min followed by ten cycles consisting of denaturation at 94°C for 30 s, annealing at 61°C for 1 min and extension at 72°C for 1 min, 20 cycles consisting of denaturation at 94°C for 30 s, annealing at 56°C for 1 min and extension at 72°C for 1 min and a final extension step at 72°C for 10 min.

Analysis of Fungal Community

The 18S rRNA gene fragments extracted from altered stone samples were amplified by two-step PCRs performed as follows: a first amplification step using the combination of primers NS1 and EF3 [30] with 1× of PCR Rxn buffer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTP mix, 0.2 µM of each primer and 1 U of Taq DNA polymerase in 25-µl PCR reaction and the cycling programme as reported in Oros-Sichler and co-workers [30] except for the 56°C annealing temperature. The first PCR product was used as template for a second amplification step performed with the primers NS2 and GC-clamped NS1. The reaction mixture was identical to first-step PCR except for 0.12 mM of dNTP mix and 0.3 µM of each primer. The cycling programme 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.

Analysis of Culturable Cyanobacterial Community

The 16S rRNA gene fragments extracted from Chu medium cultures were amplified by two-step PCRs performed as follows: a first amplification step using the primers 27 F and 1495 R with 1× of PCR Rxn buffer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTP mix, 0.25 µM of each primer and 2.5 U of Taq DNA polymerase in 50-µl PCR reaction and with a touchdown thermal protocol

consisting in an initial denaturation at 95°C for 1 min and 30 s followed by five cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 4 min, five cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 4 min and 25 cycles of denaturation at 95°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 4 min and a final extension at 72°C for 10 min. The first PCR product was used as template for a second amplification step performed with the primers GC-357 F and 907 R and with 1× of PCR Rxn buffer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTP mix, 0.3 µM of each primer and 1 U of Taq DNA polymerase in 50-µl PCR reaction. The PCR included an initial denaturation at 94°C for 4 min followed by ten cycles consisting of denaturation at 94°C for 30 s, annealing at 61°C for 1 min and extension at 72°C for 1 min, by 20 cycles consisting of denaturation at 94°C for 30 s, annealing at 56°C for 1 min and extension at 72°C for 1 min and a final extension step at 72°C for 10 min.

Analysis of Culturable Eukaryotic Algal Community

The 18S rRNA gene fragments extracted from Detmer medium cultures were amplified by PCR with the primers NS2 and GC-clamped NS1 with 1× of PCR Rxn buffer, 1.2 mM of $MgCl_2$, 0.2 mM of dNTP mix, 0.2 µM of each primer and 0.8 U of Taq DNA polymerase in 50-µl PCR reaction and the cycling programme as reported in Oros-Sichler and co-workers [30] except for the 63°C annealing temperature.

Denaturing Gradient Gel Electrophoresis and Sequencing

The amplicons obtained by amplifying bacterial and eukaryotic DNA were analysed by denaturing gradient gel electrophoresis (DGGE). The DGGE was performed as follows: 8% polyacrylamide (Sigma) gels were prepared according to the method of Muyzer and co-workers [28] using a gradient maker (Amersham Biosciences) according to the manufacturer's guidelines. The DNA fragments were separated by electrophoresis run for 15 h at 90 V performed by D-Code Universal Mutation Detection system (Bio-Rad). The gels were stained by SYBR-Green (Armersham Pharmacia Biotech) and the results observed by GelDoc (Bio-Rad) apparatus. The excised bands were eluted in 50 µl milli-Q water by incubation at 37°C for 3 h and successively overnight at 4°C. In order to compare the results, both samples, collected before and after the treatment, were loaded on the same gel.

The DGGE was performed with the following denaturant gradients: 40–60% for the Bacteria community, 25–40% for the fungal community, 30–60% for the culturable cyanobacterial community and two types of denaturing gradients, namely 35–45% and 25–35%, for the eukaryotic algal community.

All the excised bands were identified by sequencing (Primm, Milan). The sequences were analysed using the BLASTN software (www.ncbi.nlm.nih.gov/BLAST).

Fluorescent In Situ Hybridisation

The abundance of Cyanobacteria within the Bacteria community before the treatment was investigated by fluorescent in situ hybridisation (FISH). Powder samples collected before the treatment were fixed with paraformaldehyde solution (4% in phosphate-buffered solution, PBS, 0.01 M phosphate buffer, 0.0027 M potassium chloride pH7.4 at 25°C) and incubated for 2 h on ice. After two PBS washings, the powdered samples were resuspended in an aliquot of 1/1 ethanol/PBS solution and stored at −20°C. A small amount of sample (25 µl) was pipetted onto silane-coated slides (Sigma-Aldrich), allowed to air dry and then dehydrated with an alcohol series (ethanol solutions 50%, 80% and 96% *V*/*V*). The spots with the samples were treated with 30 µl of hybridisation buffer made with 2 µl (100 ng) of the labelled probes in 28-µl hybridisation buffer. The probes chosen for the hybridisation protocol were as follows: EUB338 fluorescein-labelled, targeting Bacteria; CIV/V 1342, CYA762, CYA361 and CYA664 Texas-red-labelled, targeting Cyanobacteria [22]. Details of the probes (sequences, coverage percentage, annealing temperature and references) are reported at www.probebase.net. The slides were observed by epifluorescence microscope Leica DM 4000 B (Leica Microsystems, Milan, Italy). Images of samples were acquired by CoolSNAP CF (Photometrics Roper Scientific, Rochester, USA), and cells positive after hybridisation were counted.

Statistical Analyses

Quantitative data of FISH images were analysed by one-way factorial analysis of variance (ANOVA). The positive cells were counted from ten digital images for each sample considered, and the means were compared separately for Bacteria and Cyanobacteria by post hoc comparison according to Tukey–Kramer.

Biocidal Treatment

A chemical cleaning was applied to remove the biodeteriogens. The selected chemical was Biotin N (CTS, Italy), a broad-range biocide, soluble in water and constituted by a mixture of tributyltin naphthenate (20% *w*/*v*) and didecyl dimethyl ammonium chloride (35% *w*/*v*). It was applied as a 1.5% aqueous solution, as per manufacturer instructions.

Results

Microscopy Observations and Chemical Analyses

Using the stereomicroscope, the stone of the samples 4b and 8b was characterised as an oolitic limestone with oval or semi-circular grain structure. Despite the specimens being collected from areas that were apparently non-altered by visual inspection, FTIR spectra showed a slight degree of surface sulphatation. The samples 3b, 5b, 6b and 7b were all characterised by the presence of gypsum (3,540, 3,406, 1,685, 1,621, 1,132 and 670 cm^{-1}) and calcite (1,798, 1,429, 875 and 712 cm^{-1}) with a small amount of silicate (1,034, 1,001, 797 and 779 cm^{-1}; Fig. 1). The speculation of the presence of black crusts was therefore confirmed. The sample 3b also contained calcium nitrate (1,384 cm^{-1}). Optical and chemical analyses of the specimens 5b and 6b showed the presence of a thin yellow-ochre inner layer composed of a mixture of calcium oxalate (1,646 and 1,324 cm^{-1}) and gypsum. In addition to calcite, silicates and a small amount of gypsum, FTIR analyses of samples 1b and 2b revealed the presence of a peptidic bond (see the peaks at 1,647 and 1,540 cm^{-1}).

Biocleaning Treatment

The biocleaning procedure was applied to three areas of the Cronos sculpture strongly altered by thick black crust. One area was where samples 5b and 6b were taken; the other areas had very similar features and corresponded to the abdomen region. Cronos' abdomen before, during and after the biocleaning is presented in Fig. 2 (a, b, c): complete removal of the black crust was observed after three applications of 12 h each. FTIR analyses performed on the samples after the treatment confirmed that the gypsum was removed almost com-

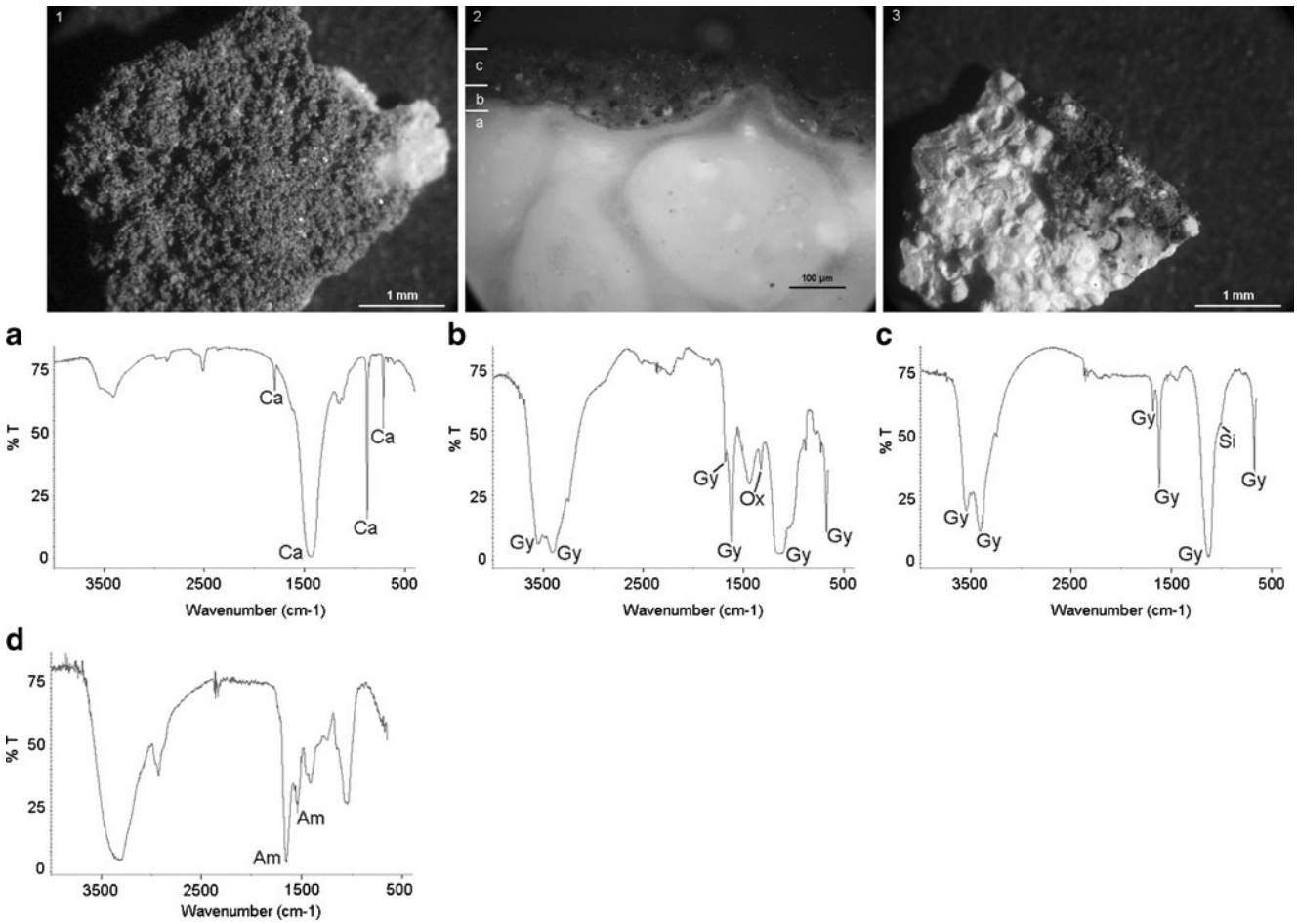

Figure 1 (*1*) Top view of sample 6b under the stereomicroscope. (*2*) Cross section of sample 6b under optical microscopy in reflected light with the presence of the three layers: oolitic limestone (*a*), noble patina (*b*) and black crust (*c*). (*3*) Top view of sample 2b under the stereomicroscope. (*a*) FTIR spectrum of the layer *a* showing the characteristic peaks of calcite (*Ca*). (*b*) FTIR spectrum of layer *b* showing the presence of calcium oxalate (*Ox*) and gypsum (*Gy*). (*c*) FTIR spectrum of layer *c*. The black crust is composed mainly of gypsum with a small amount of silicate (*Si*). (*d*) FTIR spectrum of the black patina on sample 2b. The spectrum shows mainly the presence of organic material of proteinaceous nature (amide I and amide II, *Am*)

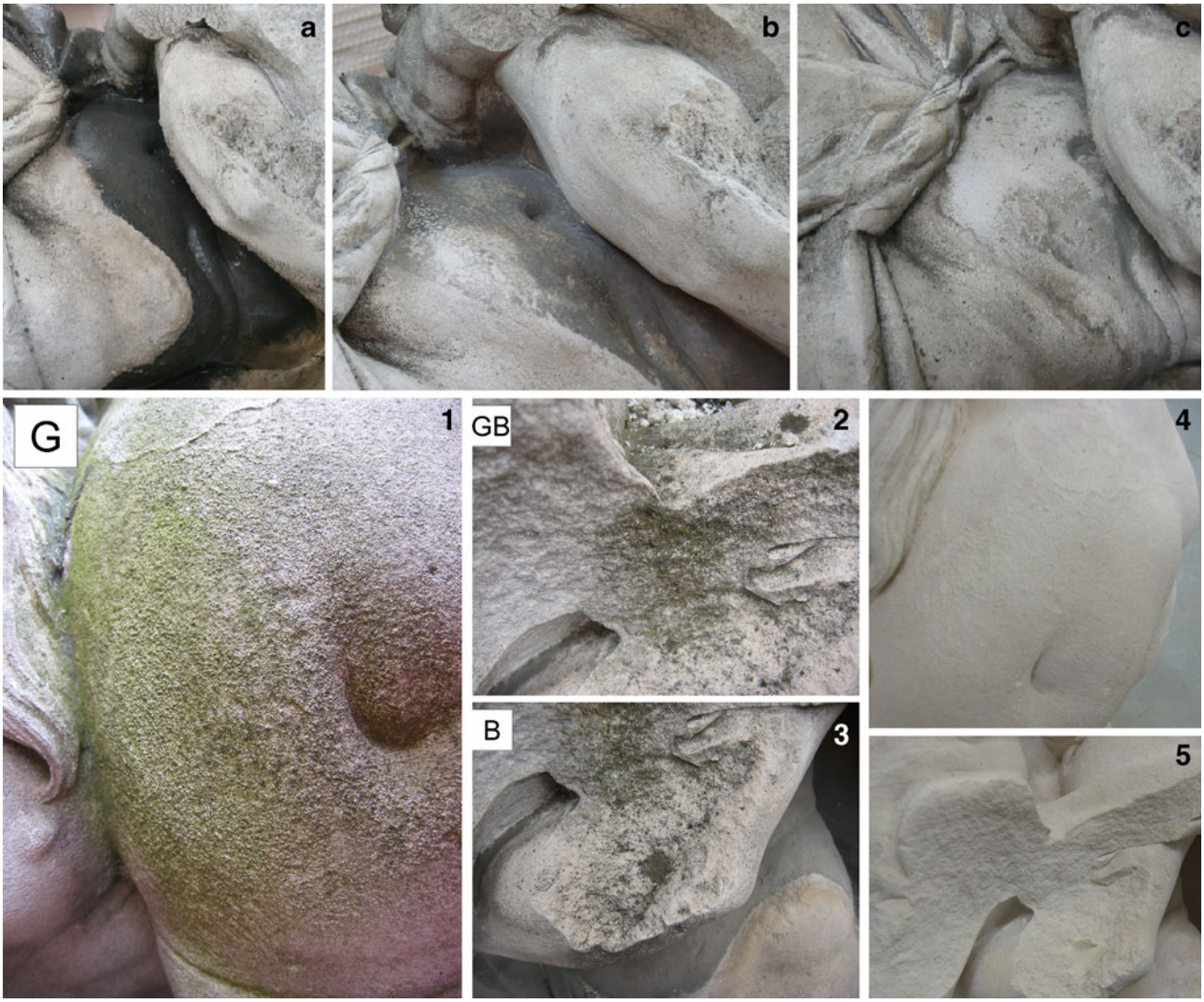

Figure 2 Bioremediation treatment of Cronos statue (*a*, *b*, *c*) and effects of the biocide on the microbial community (*1*, *2*, *3*). (*a*) Untreated surfaces showing a black crust; (*b*) result after the first 12-h application; (*c*) result after three 12-h applications, before the final washing with water and a soft brush. Green, black and green–black microbial contamination before treatment (*1*, *2* and *3*, respectively) and after treatment (*4* and *5*). Chronos child abdomen (*1*, *4*); Cronos left-elbow upper part (*2*, *5*) and lower part (*3*, *5*)

pletely. After the biocleaning process, in the area corresponding to samples 5b and 6b, the presence of a yellow-ochre layer definitively different from the limestone colour was observed, and the presence of calcium oxalate was proven by FTIR spectra.

Biological Analyses

To obtain preliminary information on the microbial community present on the samples before and after the treatments, we performed a study on the culturable community. More detailed information on the total microbial community was obtained by a molecular approach (DGGE, sequencing and FISH).

Culturable Community Analyses

The bacterial and fungal counts evaluated by cultural methods in the samples collected before any treatments were between 5 and 7 log(CFU/g) and between 4 and 6 log (CFU/g), respectively, with the only exception of sample 13b in which neither heterotrophic bacteria nor fungi were found.

In the samples collected after the biocidal treatment, no fungi were found, and the bacterial counts were two to five orders of magnitude lower than those found in the samples collected before the treatment.

Cyanobacteria and eukaryotic algae grew in all the samples except specimens 12b, 13b, 15b and 17b. The

cultural results demonstrated that the microbial communities in the samples were complex and included heterotrophic bacteria, microfungi and algae. Neither Cyanobacteria nor eukaryotic algae grew from the samples collected after treatment.

DGGE and Sequencing

To better characterise the microflora, DGGE analyses coupled with partial sequencing of 16S and 18S rRNA gene fragments were performed. Even though the amplification efficacy differed among the samples, the 16S gene amplification clearly showed a total of 28 bands separated in the DGGE gel, while for the 18S gene amplification nine bands were evidenced. The corresponding sequences were determined. Tables 2 and 3 show the band number with the identification to the closest relative strain and similarity percentage.

FISH

FISH was performed to evaluate the importance of the Cyanobacteria within the total community of Bacteria. Figure 3 shows the mean values of the Bacteria and Cyanobacteria cells positive after hybridisation in several samples. With the sole exception of samples 16b and 17b, most of the cells belonging to the domain Bacteria were also positive to the Cyanobacteria probes. Sample 13b was found to have no Bacteria cells positive to the EUB338 probe (according to cultural analysis results). The ANOVA analysis revealed statistically significant differences in the number of cells positive after hybridisation with probes specific to Bacteria and Cyanobacteria among the samples.

Biocidal Treatment

Figure 2 (1, 2, 3) shows green, green–black and black microbial contamination before the treatment and its complete removal after treatment (4, 5). To confirm the visual observations, DGGE analyses were repeated after the biocidal treatment. Figure 4 shows the taxa identified by DGGE analyses before and after the biocidal treatment, and it is to be noted how the biodiversity of the microbial community was reduced: before the biocidal treatment, 15 taxa were recovered from the DGGE bands, while after treatment only six taxa were retrieved. The biocidal treatment was effective against Cyanobacteria, Betaproteobacteria, Alphaproteobacteria, Actinobacteria, Bacteroidetes, Acidobacteria, Deinococcus-Thermus, Chlorophyta, *Cladosporium*, *Alternaria* and *Geosmithia*. In fact, these taxa were found before the treatment but were not identified after it.

Discussion

An initial visual inspection of the Demetra and Cronos sculptures allowed the detection of two putative alterations: some black crust and some biological discolouration (green, black and green–black). The chemical analyses demonstrated that the putative chemical alterations were indeed black crusts as they were composed mainly by gypsum. Calcium nitrate was also present: in addition to the transformation of calcite to calcium sulphate, it is also possible that calcium nitrate forms due to the interaction of calcite with airborne NO_x in the presence of humidity [32]. Also, the silicate particles are of atmospheric origin and are due to pollution [8]. In addition, peptidic bonds, observed in the FTIR spectra of samples 1b and 2b, were ascribed to the presence of microorganisms, as previously reported [5]. Microscopic observations and chemical analyses demonstrated that the stone is an oolitic limestone. Finally, the cross-section observations and the FTIR spectra showed that some areas affected by black crust were characterised by the presence of three different layers: the substratum (limestone), a thin yellow-ochre inner layer characterised by the presence of calcium oxalate, characteristic of a patina noble [33], and the superficial black crust. In contrast to black crusts, the patina noble is a superficial layer acquired by stone in the course of time and protects the substratum. It is associated with the best-preserved surfaces and should remain intact during cleaning [23].

In this work, the chemical alterations were removed by a biocleaning treatment before the biocidal treatment intervention on the biological contamination that had caused the discolourations; this was to avoid that the biocide acted also on the SRB cells. In the past, a biocleaning treatment by *D. vulgaris* was applied successfully to remove sulphates from marble [6]; in the present study, we applied it, for the first time, on limestone that is more porous than marble. On comparing the results of the chemical analyses performed before and after the biocleaning, it was proven that bioremediation with *D. vulgaris* successfully removes gypsum black crust from the Demetra and Cronos sculptures. After three applications, the black crusts were no longer detectable by visual inspection, and the FTIR analyses proved that the gypsum was almost completely removed. Neither nitrate nor silicate were found in the FTIR spectra after the treatment, implying that these compounds were removed together with the gypsum. In addition, as previously demonstrated for marble [6], both optical evidence and FTIR analysis showed that the noble patina was preserved on the limestone substratum of the sculptures.

The cultural analyses proved that all the four groups investigated (heterotrophic bacteria, fungi, prokaryotic and eukaryotic algae) were present and that in some samples the

Table 2 Identification of partial 16S rRNA gene sequences from DGGE profiles

Bands	Samples											BlastN reference strains				RDP tassonomic classifier	
	9b	10b	11b	12b	13b	14b	15b	16b	17b	18b	19b	Closest relative strain	Strain	Accession number	Similarity (%)	Most probable taxon	Similarity (%)
1, 4	x	x	x	x	x	x		x	x	x	x	*Halospirulina tapeticola*	CCC Baja-95 Cl.2	NR_026510	90	Cyanobacteria	100
2		x		x								*Hymenobacter aerophilus*	I/26Cor1; DSM 13606	NR_025398	90	Adhaeribacter	96
3		x		x	x	x	x	x	x	x	x	*Pseudomonas geniculata*	ATCC 19374	NR_024708	98	*Stenotrophomonas*	100
5						x						*Meiothermus chliarophilus*	ALT-8	NR_026244	83	*Truepera*	100
6		x	x			x						*Loktanella salsilacus*	R-8904	NR_025539	92	Rhodobacteraceae	100
7						x		x	x		x	*Arthrobacter agilis*	DSM 20550	NR_026198	99	*Arthrobacter*	100
8				x								*Arthrobacter nitroguajacolicus*	G2-1	NR_027199	98	*Arthrobacter*	100
9		x	x	x	x	x		x	x		x	*Cryptosporangium minutisporangium*	IFO 15962	NR_024746	90	Actinomycetales	99
11		x				x						*Sphingomonas wittichii*	RW1	NR_027525	86	Sphingomonadaceae	84
14				x	x	x	x	x	x	x		*Methylocystis echinoides*	IMET 10491	NR_025544	91	Rhizobiales	94
15				x		x			x			*Mesorhizobium plurifarium*	LMG 11892	NR_026426	94	Rhizobiales	94
20								x		x		*Sphingomonas aquatilis*	JSS-7	NR_024997	98	*Sphingomonas*	100
21, 22						x				x		*Sporocytophaga myxococcoides*	DSM 11118	NR_025463	89	Flexibacteraceae	99.5±0.5
23								x				*Ralstonia mannitolilytica*	LMG6866	NR_025385	92	Burkholderiales	94
24						x		x	x			*Herbaspirillum frisingense*	GSF30	NR_025353	93	Burkholderiales	97
25								x				*Herbaspirillum chlorophenolicum*	CPW301	NR_024804	93	Oxalobacteraceae	90
26						x		x		x		*Herbaspirillum chlorophenolicum*	CPW301	NR_024804	91	*Oxalobacteraceae*	98
27, 32						x						*Methylobacterium extorquens*	TK 0001	NR_025856	93.5±0.5	Rhizobiales	99.5±0.5
28						x		x		x		*Methylobacterium fujisawaense*	DSM 5686	NR_025374	96	*Methylobacterium*	100
29						x		x		x		*Sphingomonas aquatilis*	JSS-7	NR_024997	94	Sphingomonadaceae	99
30						x				x		*Sphingomonas aquatilis*	JSS-7	NR_024997	91	Alphaproteobacteria	100
31						x		x		x		*Methylobacterium extorquens*	TK 0001	NR_025856	95	Microvirga	89
33						x						*Pseudomonas geniculata*	ATCC 19374	NR_024708	88	Xanthomonadaceae	84
35, 37, 38						x		x		x		*Rubritepida flocculans*	H-8	NR_025221	93	*Belnapia*	91±5.5
36						x						*Rubritepida flocculans*	H-8	NR_025221	92	Acetobacteraceae	100
39											x	*Bacteroides capillosus*	ATCC 29799	NR_025670	82	Acidobacteriaceae	100
41						x						*Ruminococcus flavefaciens*	C94	NR_025931	77	Acidobacteriaceae	97
42					x							*Pseudomonas umsongensis*	Ps 3-10	NR_025227	97	Pseudomonadaceae	100

A cross indicates the presence of the taxon in the sample

Table 3 Identification of partial 18S rRNA gene sequences from DGGE profiles

Bands	Samples											BlastN reference strains			
	9b	10b	11b	12b	13b	14b	15b	16b	17b	18b	19b	Closest relative	Strain	Accession number	Similarity (%)
1, 7		x	x	x	x		x	x	x	x	x	*Geosmithia* sp.	MKA1-b	AM947666	98
11, 13, 14		x	x	x	x		x	x	x	x	x	*Isaria fumosorosea*	IFO 7072	AB086629	99
2, 3, 4, 5, 6, 12, 15, 17, 19, 21		x	x	x	x		x	x	x	x	x	*Verticillium leptobactrum*	IAM 14729	AB214657	99±0.5
16			x		x					x		*Verticillium leptobactrum*	CBS 414.70	EF641846	99
28		x	x				x	x	x	x	x	*Cladosporium* sp.	CPC11224	DQ780937	99
30, 31			x					x		x	x	*Alternaria* sp.	CPCC 480375	EU826479	99
20, 24, 25, 26, 27		x	x	x	x	x	x	x	x	x	x	*Klebsormidium fluitans*	SAG 9.96	AM490839	99.5±0.5
18			x			x				x	x	*Myrmecia astigmatica*	IB T76	Z47208	99
22, 32			x							x	x	*Myrmecia* sp.	H1VF1	AF513369	99
23										x	x	*Friedmannia israeliensis*		M62995	99

A cross indicates the presence of the taxon in the sample

counts were quite high, up to 7 log(CFU/g) for bacteria and up to 6 log(CFU/g) for fungi. However, the microbial abundance in the samples did not seem to be related to the type of alteration. The negative cultural results for samples 13b could be attributed to the small quantity (0.3 mg) of available sample; the other samples ranged from 0.4 to 15 mg. The microbial count on the black crust was comparable to the other samples. It was demonstrated that these alterations host an active microflora able to use accumulated organic pollutants as a carbon and energy source [34]. On the apparently non-altered samples, the counts were up to three orders of magnitude lower for both bacteria and fungi, partly explaining why visual inspection revealed no discolouration.

The sequences were phylogenetically most closely related to the Cyanobacteria, Actinobacteria, Bacteroidetes, Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria, Deinococcus-Thermus and Acidobacteria. One of the most common bacterial groups associated with carbonate stone biodeterioration is Actinobacteria [40]. In fact, Actinobacteria, Bacteroidetes and Alphaproteobacteria were identified on a gothic monument made of oolitic and dolomitic limestone [27], while Actinobacteria, Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and Acidobacteria were found in limestone from a Maya archaeological site in southern Mexico [25]. Proteobacteria, Acidobacteria and Actinobacteria were the major components of microbial communities in coloured colonisations on cave walls with palaeolithic paintings [31, 38]. The bacterial group Deinococcus-Thermus is not commonly associated with limestone; however, it was identified in a cave with palaeolithic paintings by Stomeo and co-workers [36]. Despite many of these taxa being found on stone surfaces and producing discolouration [41], the FISH results demonstrated that the Cyanobacteria generally were dominant among the other prokaryotics belonging to the Bacteria domain: on average, more than 60% of the cells belonging to the Bacteria domain were also positive to the Cyanobacteria probes. Several studies conducted on monuments have proven that Cyanobacteria are among the most deteriorating biological agents dwelling on stone material [39] and are able to form colourful microbial contaminations. Cyanobacteria were identified in all the samples, which were stained by green and green–black microbial discolourations, collected before the treatment; thus, they can be thought to be among the biological agents responsible for the colour of these alterations. However, in samples 16b and 17b, characterised by green microbial contamination, most of the cells belonging to the bacterial domain were not positive to the Cyanobacteria probes. This evidence suggests that the green putative microbial contamination might also be caused by other green-pigmented microorganisms such as green algae. Indeed,

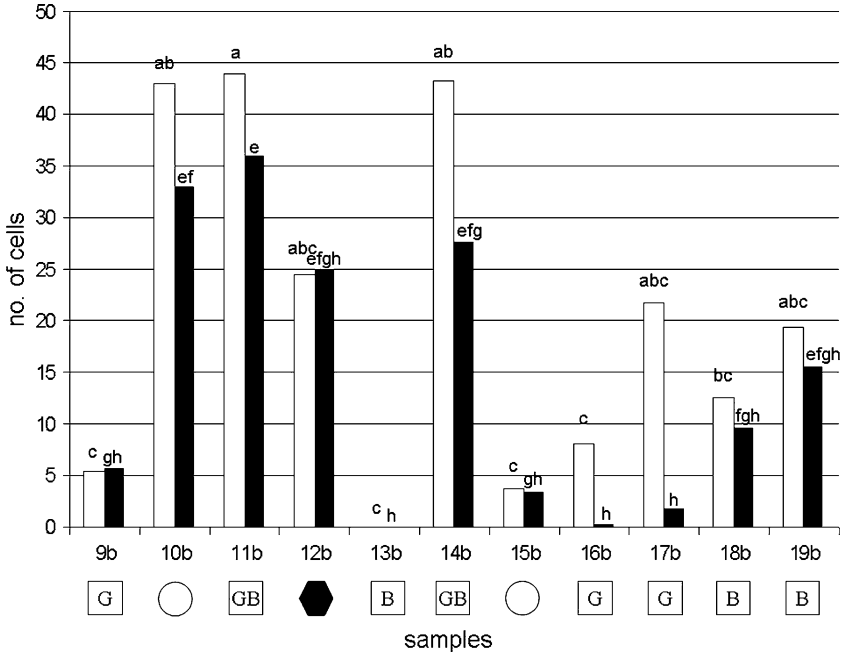

Figure 3 Mean values of cells positive after hybridisation with the Bacteria domain (*white bars*) probe and specific Cyanobacteria (*black bars*) probes in several samples. The values with different *letters* are considered significantly different according to Tukey–Kramer ($p<0.05$)

the sequencing of 18S rRNA gene fragments showed that the eukaryotic community included the algal genera *Myrmecia*, *Klebsormidium* and *Friedmannia*. Note that *Myrmecia* and *Friedmannia* are Chlorophyta green algae and *Klebsormidium* is a Streptophyta green alga characterised by an enhanced freezing tolerance [29], so it probably adapted to the rigorous climate of Trento. Tomaselli and co-workers [39] claimed that Chlorophyta algae are very frequently isolated on monuments, and Gaylarde and colleagues [11] reported that green algae are the first colonisers of outdoor buildings in Latin America. As these were the green algae genera isolated from the green microbial contamination, they, in conjunction with Cyanobacteria, can be considered responsible for the alterations on the Demetra and Cronos surfaces. Photosynthetic microorganisms can directly participate in decay processes, causing aesthetic damage and, subsequently, structural and chemical damage and, indirectly, by supporting the growth of other microorganisms [1, 16].

The sequencing of 18S rRNA gene fragments showed that the eukaryotic community included the fungal genera *Isaria*, *Geosmithia*, *Verticillium*, *Alternaria* and *Cladosporium*. The first, *Isaria*, is an entomogenous genus of fungi. In Spanish caves, the cadavers of arthropods were found covered by *Isaria*, which extended from the insect body to the adjacent soil. Arthropods are vectors of entomogenous fungi and play an important role in fungal dispersion in caves, catacombs and mural paintings [17]. *Geosmithia* spp. were recently found to be regular associates of many insect species that invade the phloem or sapwood of various plant genera [18]. Dematiaceous fungi that are characterised by the production of the black pigment melanin and manifest meristematic growth are considered by several authors the most harmful microorganisms of outdoor stone material [20], causing mechanical damage and aesthetic alterations (black coating) [3, 26, 43]. *Alternaria* and *Cladosporium* are very well-known microcolonial black fungi [43]. Many authors have isolated them on marble and limestone in several environments, including monuments and archaeological sites [13, 36, 43]. The *Verticillium* species are common colonisers of rock substrata [3], and many of them are able to deposit melanin in the cell wall [15]. These mycetes have also been isolated from monuments [14]. For these reasons, *Alternaria*, *Cladosporium* and *Verticillium*, found in black and green–black microbial contamination, can be thought to be the biological agents responsible for black staining. From the above results, we concluded that prokaryotic and eukaryotic algae were responsible for the green microbial contamination and dematiaceous fungi for the black discolouration. Since the biodeteriogen agents were present in all the differently coloured microbial-contaminated sites, it is most likely that the colour of the contamination varied from green to black depending on the abundance of the individual biodeteriogen agent.

The variety of biodeteriogens forced us to choose a broad-range biocide to remove the discolouration. Treatment with the biocide resulted in a decrease of the bacterial load (up to five orders of magnitude), and neither culturable fungi nor culturable prokaryotic and eukaryotic algae grew on the samples collected after the treatment. Indeed, after the treatment, the DGGE results showed that the samples from surfaces that had, prior to treatment, shown green, black and green–black microbial contamination, now showed far fewer bands than before treatment. The

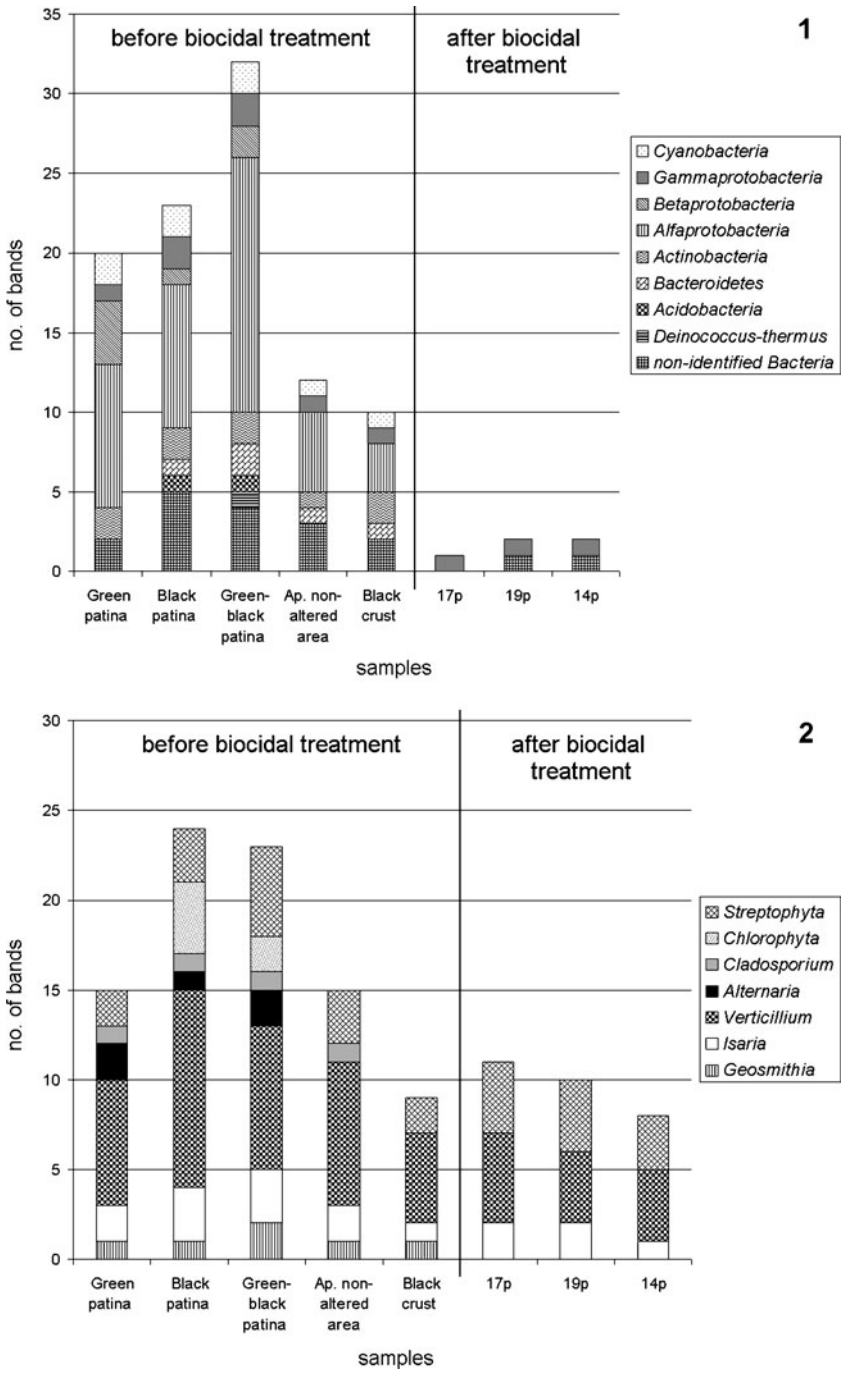

Figure 4 Bacterial taxa (*1*) and fungal and algal taxa (*2*), identified by DGGE before treatment, in the green microbial contamination (samples 9b, 16b and 17b), black microbial contamination (samples 13b, 18b and 19b), green–black microbial contamination (samples 11b and 14b), black crust (sample 12b) and apparently non-altered area (samples 10b and 15b) in comparison to the taxa identified in the samples collected after the treatment: samples 17p, 19p and 14p collected close to the sampling areas of specimens 17b, 19b and 14b, respectively

sequencing of these bands testified to the significant decrease, brought about by the biocide, in the biological diversity of the Bacteria, fungal and algal taxa, a result in accordance with the cultural results.

Importantly, the DGGE results proved that Cyanobacteria (among those responsible for the green microbial contamination) and most of the green algae and dematiaceous (the latter responsible for the black microbial contamination) were not identified in samples collected after the treatment. Among the taxa present on the surface after the treatment (Gammaproteobacteria, *Verticillium*, *Isaria* and Streptophyta), only Streptophyta and *Verticillium* were potential biodeteriogen agents that remained.

Conclusions

The Demetra and Cronos statues presented two concomitant deterioration phenomena found very frequently on outdoor stone artwork: the sulphatation of limestone caused

by air pollution and surface biodeterioration. The first phenomenon caused the thick black crusts in the areas sheltered from rainfall, and the second generated colourful microbial contamination on surfaces. This work shows that an integrated biotechnological approach can provide an important solution to both these problems. In fact, the bioremediation treatment with *D. vulgaris* successfully removed black crust from limestone, and the identification of biodeteriogen agents (Cyanobacteria, green algae and black pigmented fungi) by DGGE allowed the selection of a suitable cleaning treatment for the colourful microbial contamination. However, given the presence of some green algae and melanin-containing fungi after the treatment and the constant exposure of the sculptures to atmospheric pollution, we recommended an ongoing maintenance routine to ensure longer-lasting conservation.

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