

Article

Black on White: Microbial Growth Darkens the External Marble of Florence Cathedral

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Abstract: Weathering processes seriously affect the durability of outdoor marble monuments. In urban environments, a very common deterioration phenomenon is the dark discoloration or blackening of marble. This paper describes a multidisciplinary study on the state of conservation of white marbles of the Florence Cathedral and the microbial community involved in their deterioration. The study is focused on the widespread dark discoloration of marble analyzed in two differently exposed sites of the Cathedral. It aims to provide information useful for future interventions to control the microbial growth. By chemical and petrographic analysis, in situ and ex situ microscopy, and cultivation and identification of microorganisms, it was found that (i) the darkening is mainly due to the growth of black fungi and dark cyanobacteria and (ii) the state of conservation of marble and the growth pattern of microorganisms seems to be linked to the microclimatic conditions, in particular to solar radiation exposure. This is the first report on the lithobiontic community inhabiting the Florence Cathedral marbles, with a more detailed investigation of the culturable mycobiota.

Keywords: marble decay; biodeterioration; dark discoloration; stone microbiota; black fungi; cultural heritage conservation



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1. Introduction

The preservation of stone-built cultural heritage is a major issue in modern societies to be pursued through conservation strategies for its transmission to future generations and the protection of its authenticity [1–3]. Outdoor stone monuments are exposed to several natural abiotic, biotic, and anthropogenic weathering factors. The mineralogy, chemical composition, porosity, surface roughness, water uptake, and state of conservation of the lithotypes as well as the macro- and micro-environmental conditions are key factors affecting the so-called stone bioreceptivity and influencing microbial colonization and growth [3–6]. Although stone represents a harsh habitat to live on/inside, complex and diverse lithobiontic communities develop on rocks coping with large variations in environmental factors such as temperature, water and nutrient availability, electrolyte concentration, and solar radiation [7,8]. Microbial communities usually grow as biofilms adhering to the surface or penetrating inside stone [9,10]. They cause deterioration manifested mainly by aesthetical damages, such as colored patinas or discolorations, but also by severe structural modifications of stones due to biogeophysical and biogeochemical processes that act synergically [2,11].

In urban environments, outdoor monumental and artistic stones are also exposed to air pollution, mainly caused by fossil fuel combustion, which affects chemical weathering,

bioreceptivity, and biodeterioration of stones [10,12–14]. A wide range of airborne hydrocarbons and fatty acids settles on stone surfaces [15] and can be utilized by heterotrophic microorganisms for their growth, so that, according to some authors [16], the rock surface in such environments should no longer be regarded as oligotrophic.

Among the natural stones used as constructive and decorative materials since ancient times (e.g., since about 4700 BC in Egypt), marble plays a relevant role, due to its bright white color and translucence. Marble is a metamorphic rock consisting prevalently of calcite or dolomite, and despite its hardness, it is subject to weathering processes that can seriously affect its durability. In outdoor conditions, marble can be damaged by solar irradiation and consequent temperature variations, rain washing, salt crystallization, and atmospheric pollution [17]. In particular, marble is extremely susceptible to acid attack caused by atmospheric compounds such as carbon, sulfur, and nitrogen oxides [18]. Furthermore, outdoor marble monuments can be colonized by several kinds of microorganisms such as bacteria and cyanobacteria, algae, lichens, filamentous, and meristematic fungi, which can cause biodeterioration [5,10,19–21]. Some studies highlight how marble surfaces display the highest degree of microbial growth among monumental stones [22]. Documented biodeterioration phenomena on ancient marble monuments include the production of organic acids by fungi and lichens that chelate metallic cations and dissolve calcite, biomineralization, biopitting, and discolorations [13].

Despite the widespread presence of discolorations on marble, the scientific literature on this subject is limited. Since marble is one of the most used stones by architects and artists of all times [17], its discolorations are worthy of being studied [23], and their specific causes should be clearly identified. The dark discoloration or blackening is often ascribed to the formation of calcium sulfate or gypsum, which contributes to black crusts formation [13,24], but it can be also caused by the colonization of cyanobacteria [25,26] as well as of the so-called black or dematiaceous fungi [27–30].

To plan appropriate conservation measures aimed to control the microbial growth and protect the heritage stone, a correct analysis and diagnosis of each specific biodeterioration aspect is essential [31]. This requires the knowledge of the rock characteristics and the resident microbial community, as well as the interactions between microorganisms and stone [32]. Although biodeterioration phenomena are rarely ascribable to a single microbial component, most studies focus on single taxonomic groups and not on the whole communities that colonize stone surfaces, not considering some of the inter-taxa interactions that may be responsible for deteriorative changes [33].

The city of Florence, known as the cradle of the Renaissance, houses the world's largest concentration of universally renowned artistic and architectural masterpieces. The Historic Centre of Florence is a unique artistic realization, an exceptional testimony of both a medieval city and of a Renaissance one, and a living archive of both European and Italian culture. For these reasons, it was registered in the list of UNESCO World Heritage Sites in 1982. The Cathedral of Santa Maria del Fiore (SMFC; Figure 1) is the most emblematic monument and symbol of Florence. It is an impressive building located in Piazza del Duomo, the beating heart of the city, standing tall over the city, and its conservation is a main issue of worldwide concern. Nonetheless, the exterior of the Cathedral, mainly covered with Apuan marble, shows extended forms of decay, macroscopically visible, consisting of deposits, discolorations, patinas, crusts, erosion, mechanical damage, and granular disaggregation [3,34]. In recent years, the Opera di Santa Maria del Fiore (OSMF)—the institution actively engaged in the protection of the monuments of the complex of Santa Maria del Fiore—started a maintenance program for the cleaning and restoring of all the external façades of the Cathedral. Such interventions, especially those aimed at removing biological patinas, would be better planned with a previous knowledge of the inhabitant lithobiontic community.

The OSMF expressed interest in testing innovative methods to remove patinas through on-site trials on selected external areas, not accessible to visitors, that did not undergo recent restoration interventions. In these areas, marble surfaces showed extended dark

discolorations as the main deterioration phenomenon. Therefore, we first investigated the cause of the darkening, then the interaction of microbial communities with marble. We thus had the unique opportunity to carry out a multidisciplinary study on the state of conservation of the SMFC external white marbles and the lithobiontic community involved in their deterioration. The results will be essential to plan adequate interventions to control the microbial colonization.

2. Site Description

The Historic Centre of Florence is an area (latitude 43°46′22″ N; longitude 11°15′25″ E; 50 m at sea level) characterized by a temperate macro-bioclimate with the sub-Mediterranean bioclimatic variant [35]. The average temperature (T), calculated in the period 2010–2020, is about 16.7 °C, with a monthly minimum average of 1.3 °C (February 2012) and a monthly maximum average of 40.5 °C (July 2015). The average winter temperature is about 9.3 °C, while the mean temperature in summer is about 25 °C. The average yearly value of precipitation, mostly occurring in autumn and spring, is 799 mm, and the average relative humidity (RH) is around 61.9%, with maximum peaks of 89–97% in winter. The RH of Florence never dropped below 40% during the reported period. In the climatic zone of SMFC, persistent winds blow all year, mainly from the southeast and northwest at about 1.3–1.6 m/s wind speed [36].

The Cathedral of Santa Maria del Fiore (Figure 1) was built on the site of the previous early-Christian cathedral dedicated to Santa Reparata. Its construction was commissioned by the city council at the end of the 13th century as a symbol of richness and power of the city and lasted from 1296 to 1434. When it was completed, in the 15th century, it was the largest church in the world; today, it is the third-largest church in the world. The first design, in Gothic style, was by the Italian architect Arnolfo di Cambio; successively, other architects, among them Giotto, Andrea Pisano, and Francesco Talenti, tended to its construction, imparting the different styles that we can observe in the current structure. The dome was erected between 1418 and 1434 on the ingenious project of Filippo Brunelleschi. The neo-Gothic façade, on the elaborate design of Emilio de Fabris, was completed only in 1884. The size of the Cathedral, which shows a Latin cross plan, is enormous (8300 m² total area); the exterior part is covered with polychrome stone panels consisting of white marble, serpentinite, and red limestone [37–39] coming from Florence’s surrounding quarries (Figure 1).



Figure 1. The Cathedral of Santa Maria del Fiore and the study sites. (a) The façade; (b) the external gallery running around the apses of the SE exposed façade; (c) the external gallery on the NW exposed façade; (d) a detail of the marble surface of the inner face of the openwork parapet of the SE exposed gallery; (e) a detail of the openwork parapet at the NW site showing a widespread dark patina.

The white marbles mainly come from the Apuan Alps, in the Carrara district [37], which represents the world's most important and largest (more than one hundred active quarries) mining area. The Carrara marbles, used since the Ancient Rome time [40,41], represent the best-known stone materials used by architects and artists around the world, from the temple of Apollo Palatinus in Rome to the Marble Arch in London, the Harvard Medical School Building in Boston, and the Buddhist Temple in Singapore [41]. Due to its technical properties, this marble is considered suitable for any type of use, excellent for sculptures—so much that Michelangelo wanted it for his *Pietà* and *David*.

In general, marbles, although having the same chemical and mineralogical composition, can show different microstructural characteristics which affect their behavior when subjected to weathering. The microstructural characteristics of Apuan marbles vary depending not only on the different extraction site but also on different areas of the same quarry, according to the complex tectono-metamorphic history that they have undergone [42]. Moreover, in the case of the Cathedral of Florence, Apuan marbles of different provenance were used for the original construction (common white marble quarried from Carrara quarries—the marble studied in the present paper) and the recent 19th century façade (common white marble from Seravezza, Lucca) [37]; the latter displays a more advanced state of decay in comparison to those used in the 15th to 18th centuries [37,43].

3. Materials and Methods

3.1. Sampling

The study was conducted in two differently exposed sites located in the external gallery running around the apses on the upper part of SMFC (Figure 1b,c) at a height of 36 m above ground. They are northwest (NW) and southeast (SE) exposed and characterized by different light/shadow durations (SE area is illuminated longer by direct sun than NW area). The balcony is accessible by an internal staircase. The last intervention on these areas dates back to 1952 and consisted of substitutions of deteriorated marble slabs (B. Agostini, personal communication).

Most in situ observations and sampling were conducted in the fall of 2019. Microscopical observations and microbiological sampling were carried out in different areas of the vertical inner surface of the gallery's openwork parapet (Figure 1d,e).

To perform mineralogical, petrographic, and ex situ microscopic analyses, small fragments already detached from areas with evident forms of degradation (such as fractures and cracks) were collected. The fragments were SMF1, SFMF2, and SMF8 from the NW area, and SMF6, SMF7, and SMF9 from the SE area (Figure S1).

For microbiological analysis, superficial particulate was gently scraped from marble with a sterile spatula (micro-invasive method) and collected into sterile tubes. Three samples of about tens of mg were taken from a surface of about 1000 cm² at both the NW and SE sites. Samples were immediately brought to the laboratory and processed.

3.2. In Situ and Ex Situ Microscopy

Biological growth on selected areas was firstly ascertained by naked eye and then was observed under a hand lens (9× magnification) and a portable light microscope Scalar DG-2A (Tecmet 2000, Corsico, Italy) equipped with an optical zoom 25–200× and an image capturing system.

The biological growth on and below the surface was examined under transmitted and reflected light (RLM) microscope and scanning electron (SEM) microscope.

The samples were observed under the stereomicroscope Leica DMS300 (Leica Microsystems, Wetzlar, Germany) and the particulate suspensions (Section 3.4) under the ZEISS Axiolab 5 (Zeiss, Oberkochen, Germany) microscope equipped with the video camera AxioCam 208 color (Zeiss, Oberkochen, Germany).

The RLM observations were carried out using the Zeiss Axio Skope.A1 (Zeiss, Oberkochen, Germany) microscope equipped with a video camera (5 megapixel resolution and image analysis software AxioVision) on cross sections (2.5 cm diameter, 1.5 cm

height) from both NW and SE areas. The sections were obtained by cutting the samples with a diamond saw. They were stained using the PAS kit (Periodic Acid-Schiff, Sigma Aldrich, St. Louis, MO, USA) to visualize the biological component within the lithic substrate. Since PAS stains carbohydrates, it is used to detect cells as well as extracellular polymeric substances (EPS), and consequently biofilm, on/inside marble.

The petrographic observation was carried out on ultrathin sections (thickness 12–15 μm), using the same Zeiss Axio Scope.A1 (Zeiss, Oberkochen, Germany) microscope with polarized light.

Scanning electron microscopy (SEM) observations were carried out using the Zeiss EVOMA15 microscope (Zeiss, Oberkochen, Germany). The samples were previously coated with both carbon and gold using the Quorum Q150R ES sputter coater (Quorum Technologies, Laughton, UK).

3.3. Chemical Analysis

The surface of the marble samples was scraped and analyzed using the Fourier Transform Infrared Spectroscopy (FT-IR) through ATR mode with a Spectrum 100 FTIR spectrometer (Perkin-Elmer Inc., Norwalk, CT, USA) equipped with a Universal ATR accessory. The acquisition was carried out at room temperature, in the spectral range between 4000 and 350 cm^{-1} , repeating 4 scans with resolution of 4 cm^{-1} . The data were acquired and processed using the Spectrum 100 software.

3.4. Cultivation, Morphological Analysis and Identification of Microorganisms and Lichens

To cultivate green algae and cyanobacteria, aliquots (10 mg) of the collected marble powder were immersed in the liquid nutrient medium BG-11 prepared according to Rippka et al. [44] and adjusted only with 5 mL/L NaNO_3 instead of 15 mL in order to allow the growing of nitrogen fixing cyanobacteria, as well. After 2 months, the main phototrophic biodiversity was observed under a Zeiss Axio Scope A1 microscope (Zeiss, Oberkochen, Germany) equipped with a Zeiss Axio Cam ICc3 (Zeiss, Oberkochen, Germany), and the morphological characterization was executed according to Komarek et al. [45] and Bourrelly [46].

In a previous work [3], we performed various lab tests to find suitable conditions for a high recovery of fungal colonies from SMFC marble. Such conditions will be applied in future evaluations of biocide treatments. Among the nutrient media used, Malt Extract Agar (MEA) gave the best results, with a viable titer up to 1.5×10^4 CFU/g at the NW site and an apparently high biodiversity. MEA was also modified by adding 0.1% and 1% marble powder, and 1% aqueous marble extract and no significant differences were observed in viable titer [3]. Consequently, we used MEA at the subsequent sampling. To cultivate fungi, 10 mg of marble particulate, obtained by mixing three samplings from each area, were suspended in 1 mL of Phosphate Buffered Saline (PBS; 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4 , g/L 0.24 KH_2PO_4 , pH 7.4) added with 0.001% Tween 80 and vortexed. Then, 0.1 mL of suspension were plated in quadruplicate on MEA (Oxoid) supplemented with 10 $\mu\text{g}/\text{mL}$ of chloramphenicol to prevent bacterial growth and then incubated at 30 $^\circ\text{C}$ for at least seven days. Viable titer was calculated as mean value of the number of Colony Formant Units (CFUs) per gram of marble particulate. Morphological analysis of colonies was conducted by observing them under the stereomicroscope Olympus SZX9 (Olympus, Tokyo, Japan). Colonies with the same morphological characteristics were grouped in the same morphotype. For each morphotype, at least one strain was re-isolated on MEA and then stored on slant. Identification of fungal morphotypes was based on rDNA analysis. Genomic DNA of isolated strains was extracted using Wizard[®] Genomic DNA Purification kit (Promega, Madison, WI, USA) following the yeast protocol and modifying the cell lysis step as follows: about 1 cm^2 mycelium from an isolated colony was cut, suspended in 500 μL physiological solution, and centrifuged. The pellet was then suspended in 47.5 mM EDTA + 0.5 mg/mL zymolyase (Sunrise Science Products, Knoxville, TN, USA) and incubated at 37 $^\circ\text{C}$ for 45 min. Amplification of the internal transcribed spacer (ITS) region of rRNA

genes was performed using primers ITS1D (5'-GTTTCCGTAGGTGAACCTGC-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), and PCR BIO Taq Polymerase (PCRBioSystem, London, UK). PCR conditions consisted of an initial denaturation step at 95 °C for 2 min followed by 30 cycles at 95 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 5 min. The Sanger sequencing was performed by Bio-Fab Research s.r.l. (Rome, Italy). To identify the isolates, the nucleotide sequences were analyzed by BLAST (Basic Local Alignment Search Tool) using the National Center of Biotechnology Information (NCBI) database [47]. Newly generated ITS rDNA sequences were deposited at the NCBI database under the accession numbers from MW361278 to MW361325.

Lichens were identified directly in situ using the portable stereomicroscope. The identification key of Clauzade et al. [48] was used as a reference. Nomenclature follows Nimis [49].

3.5. Carbonate Solubilization Test

The potential ability of the isolated fungi to solubilize calcite was screened on CaCO₃ glucose agar medium (glucose 1%, CaCO₃ 0.5%, agar 1.5%; pH adjusted to 8.0 with 1 M HCl). Fungal mycelium was inoculated into the center of the Petri dishes (± 1 cm² agar blocks) and incubated at 30 °C for 8 weeks. CaCO₃ dissolution ability was evaluated by the presence of a clear zone around the colony.

4. Results

4.1. In Situ Observations

The marble surfaces of both NW and SE areas display a dark discoloration with a higher surface coverage on the NW area (Figure 1d,e).

In situ observations with the stereomicroscope revealed the presence of two patterns of biological colonization: (i) around the grains of the marble but not covering them (Figure 2a), (ii) forming black little spots that cover the marble surface (Figure 2b) and growing up to form patinas more developed at the NW site (Figure 2c). The total effect at the naked eye observation is a strong alteration of the marble color that appears dark gray with widespread black spots (Figures 1 and 2). The dark colonization around grains and the black spots would resemble the growth pattern of cyanobacteria and black fungi (Figure 2a,b). Some lichens preferentially grow on biofilm black spots; the latter seem to behave as pioneer microorganisms, somehow helping the succession to the lichens even if they are able to grow also in polluted atmospheres (Figure 2d).

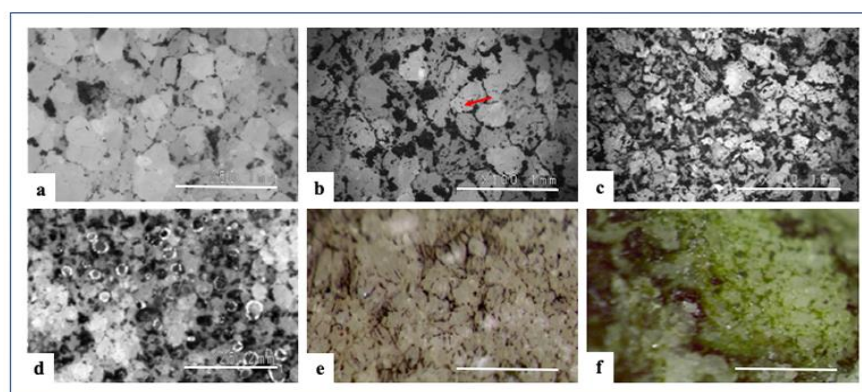


Figure 2. Microscopic observation of the surface of the SMFC external white marbles: (a–d) in situ observations; (e,f) ex situ observations of marble fragments. (a,b) Microbial colonization around the marble grains at the SE (a) and NW sites (b), little black spots presumably composed of black fungi (arrow) are visible over the marble grains in (b). (c) Colonization by a more developed biofilm forming a patina over the marble at the NW site. (d) Thalli of the lichen *Myriolecis dispersa* growing preferentially on biofilm black spots at the SE site. (e) Growth around grains resembling that of cyanobacteria on a marble sample. (f) Growth of algae and dark colonies on a marble sample. The scale bar is 1 mm in (a–f) and 2 mm in (d).

4.2. Chemical and Petrographic Analyses

The studied marbles mainly consist of calcite [3]. Accordingly, the obtained FT-IR spectra (Supplementary Figure S2) are characterized by the stretching band around 1420 cm^{-1} and by the absorbance at 871 cm^{-1} and 712 cm^{-1} [50]. In almost all the spectra, also visible is the absorbance at 1033 cm^{-1} (silicates) and the characteristic sharp peaks of gypsum (bending vibrations at 1796 and 1641 cm^{-1} and at 1115 cm^{-1}) whose content is very low. In Supplementary Figure S2, two representative FT-IR spectra (SMF2 and SMF9 from NW and SE area, respectively) are shown.

Marbles observed in ultrathin sections under a petrographic microscope (Figure 3) display the typical polysynthetic twinning of the calcite crystals which do not exhibit a preferred orientation; the grain-size is heterogeneous, mainly in the range $150\text{--}200\text{ }\mu\text{m}$, up to $500\text{ }\mu\text{m}$.

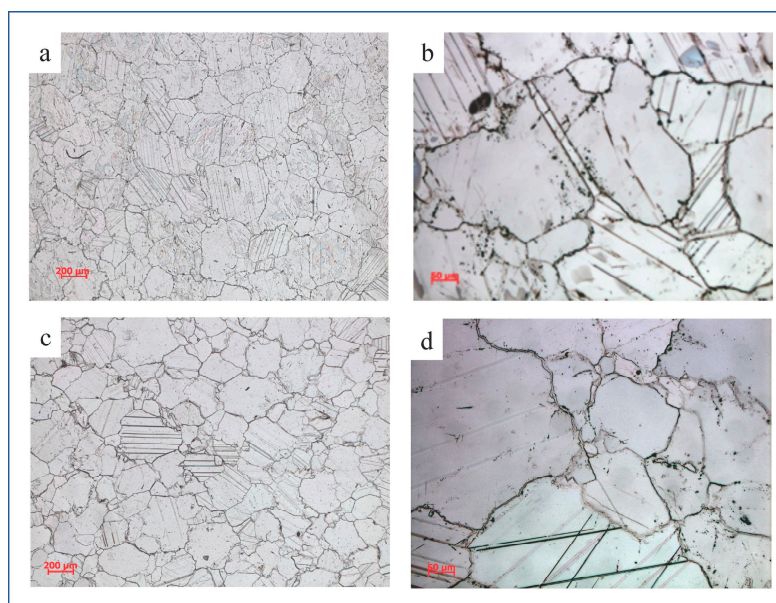


Figure 3. Photomicrographs at different scale of ultra-thin sections of marble in plane-polarized light. (a,b) NW sample (SMF1); (c,d) SE sample (SMF6).

The samples collected from the two areas display overall similar characteristics. They have a prevalent heteroblastic or, in some cases, homeoblastic mosaic texture and the grain boundaries show straight to lobate-curved and sutured shapes (Figure 3). All the samples show low macro-porosity; however, where the crystal boundaries are prevalently straight, a slight grain detachment is observed. In particular, the detachment and the presence of fine-grained recrystallized calcite along the grain boundaries are more evident in the samples collected in the SE area (Figure 3d).

4.3. Ex Situ Microscopic Observations

Observation of samples under the stereomicroscope confirmed the biocolonization patterns observed in situ and presumably attributable to cyanobacteria, algae, and fungi (Figure 2e,f). Suspensions of marble particulate used to cultivate fungi were also observed under the light microscope that showed dispersed aggregates of phototrophic microorganisms such as algae, cyanobacteria, and black fungi (Supplementary Figure S3). Observation of cells of black spots picked up with a sterile needle from marble samples showed very similar images of such aggregates (not shown). A few black spots sampled from SMF8 were plated on MEA, in one case leading to the growth of a meristematic colony (strain M1; Section 4.4.3).

RLM observations of cross-sections showed a variable degree of biodeterioration in different samples. The presence of an epilithic biofilm on marble surface was always

detected, thicker on the NW samples (Figure 4a,b) whose internal grains, however, showed a better state of conservation (Figure 4a,b,f). An endolithic biofilm developed in the SE samples. It penetrates as up to 3–4 mm in depth, surrounding the internal grains and causing their physical distancing (Figure 4c,d,g,h). In the superficial layer, the biofilm, growing around the grains, causes their detachment (Figure 4e,f). At the magnification used, we could not detect the kind of microbial cells participating to the epilithic or endolithic biofilm detected by PAS, except for the presence of endolithic algae, suggested by green spots successively stained by PAS (Figure 4g,h). Moreover, the presence of black fungi was clearly visible inside some samples (Supplementary Figure S4).

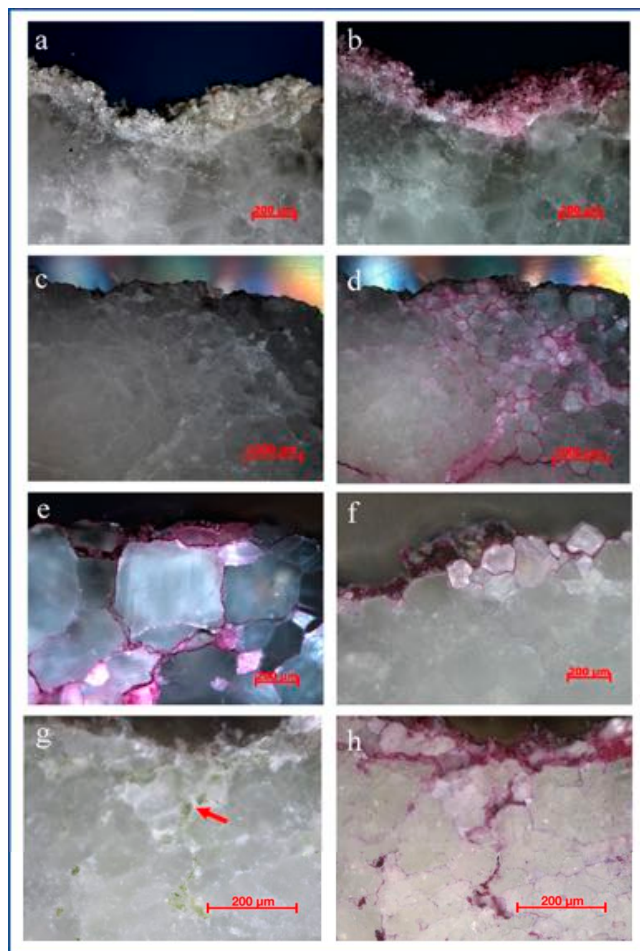


Figure 4. Biological colonization of SMFC marble observed by marble polished cross sections. SMF1 (NW) cross section before (a) and after (b) PAS staining; SMF6 (SE) cross section before (c) and after (d) PAS staining; (e) a detail of d; (f) PAS-stained cross section of SMF2 (NW); SMF9 (SE) cross section before (g) and after (h) PAS staining, endolithic algae (arrow) are visible in (g).

SEM observations confirmed the differences already noted on ultra-thin sections; the SE samples display a more evident grain detachment than NW ones (Figure 5a,b). Moreover, they also revealed the presence of a composite microbial community showing intimate relations with the marble grains. In a few samples, individual colonies are recognizable, as in the case of the cyanobacteria growing among marble grains (Figure 5b,c). In other samples, a well-developed biofilm is visible with differently shaped (filamentous and globular) microorganisms embedded into the slime of EPS; it completely covers the marble (Figure 5d,f) and contributes to grains detachment (Figure 5e).

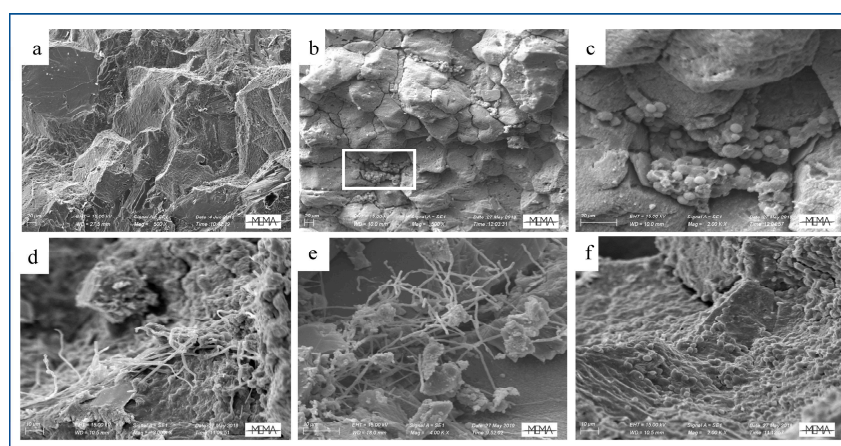


Figure 5. Scanning electron microscope images of SMFC marble. (a) NW sample (SMF1); (b) SE sample (SMF6), the grain detachment is clearly visible, colonies of unicellular cyanobacteria are scattered around the marble grains. (c) A detail of (b) (white rectangle), a colony with cells growing in the fissures among grains; (d–f) NW sample (SMF2), filamentous microorganisms connected through an EPS matrix to a multispecies biofilm adhering to marble (d), and detaching marble particulate (e). (f) Microbial biofilm completely covering marble surface with cells embedded within the EPS matrix.

4.4. Characterization of the Lithobiontic Microbial Community

4.4.1. Lichens

The examined areas were colonized mainly by crustose lichens, just one foliose species was present. Lichen thalli were better developed on the NW facing areas, nevertheless the identified species were the same. Although lichen species richness was extremely low, the abundance of some species was quite high.

Many thalli of *Myriolecis albescens* (Hoffm.) Sliwa, Zhao Xin, and Lumbsch and *Myriolecis dispersa* (Pers.) Sliwa, Zhao Xin, and Lumbsch were present (Figure 2d). They grew preferentially on biofilm black spots. Other species present on the examined areas were *Candelariella aurella* (Hoffm.) Zahlbr., *Flavoplaca citrina* (Hoffm.) Arup, Frödén, and Søchting and primordia of thalli of a foliose lichen belonging to *Physcia* genus.

4.4.2. Cyanobacteria and Algae

The most abundant phototrophic microorganisms were coccoid cyanobacteria belonging to the *Chroococcales* group *Gleocapsa* sp. and the unicellular green alga *Chlorococcum* sp. (*Chlorophyta*), being most spread on both the NW and SE facing areas, followed by filamentous cyanobacteria, belonging to the *Oscillatoriales* group. Other few genera were observed in the SE area, such as the cyanobacterium *Aphanocapsa* sp. (*Synechococcales*) and the filamentous green alga *Ulothrix* sp. The microscopic observations of the phototrophic cultures also showed the presence of black fungi associated with the phototrophic cells (Supplementary Figure S3).

4.4.3. Fungi

The fungal viable titer was $1.0 \times 10^4 \pm 4.1 \times 10^3$ CFU/g at the NW and $5.5 \times 10^3 \pm 4.7 \times 10^3$ CFU/g at the SE site, confirming our previous results [3]. Fungi from two different samplings (spring and fall 2019) were investigated for their biodiversity. The morphological analysis of all the colonies identified 28 morphotypes at the NW site and 20 morphotypes at the SE site (Supplementary Figure S5). DNA was extracted and rDNA ITS amplified from all the morphotypes. Results of sequence analysis are reported in Table 1. Although the ITS region is the universal barcode marker currently used for fungi, it has some limitations regarding the identification at species level, depending on the fungal group [51]. Although most of the isolated strains were identified as species, we found ambiguity in species assignment for strains of *Alternaria*, *Cladosporium*, and *Epicoccum*

(Table 1). For this reason, we summarize and discuss the data relative to the genera found. Overall, a total of 21 different genera were identified at the two study sites, with eight genera specific of the NW area and 10 specific of the SE area. Three genera (*Alternaria*, *Cladosporium* and *Epicoccum*) were common to both the areas. *Alternaria* and *Cladosporium* had apparently the highest number of species, all the other genera were represented by only one species, but *Coprinellus* by two.

Table 1. Identification of fungi isolated at the NW and SE study sites of SMFC by rDNA analysis. (a) Morphotypes isolated at the NW site. (b) Morphotypes isolated at the SE site. In the Morphotype column, bold indicates dematiaceous filamentous fungi; italics indicate dematiaceous meristematic fungi and black yeasts.

Morphotype	Organisms with the Most Similar ITS Sequences	Similarity (%)
(a)		
Nord A	<i>Parengyodontium album</i>	99.81
NB	<i>Dimorphoma saxea</i>	100
NC	<i>Alternaria alternata</i> /A. <i>tenuissima</i>	99.81
ND	<i>Alternaria tenuissima</i> /A. <i>alternata</i>	100
NE	<i>Hyphodermella rosae</i>	100
NF	<i>Cladosporium asperulatum</i> /C. <i>xylophilum</i>	99.80
NG	<i>Cladosporium halotolerans</i>	99.61
NH	<i>Alternaria alternata</i> /A. <i>tenuissima</i>	99.81
NT-N-1	<i>Diplodia seriata</i>	99.82
NT-N-2	<i>Neoscochyta exitialis</i>	97.25
NT-N-3	<i>Alternaria tenuissima</i>	99.81
NT-N-6	<i>Pithomyces chartarum</i> ¹ / <i>Leptosphaerulina chartarum</i> ¹	99.48
NT-N-7	<i>Alternaria ethzedia</i> /A. <i>infectoria</i>	99.64
NT-N-8	<i>Aspergillus niger</i> /A. <i>welwitschiae</i>	98.21
NT-N-9	<i>Alternaria ethzedia</i>	99.46
NT-N-10	<i>Cladosporium cladosporioides</i> /C. <i>perangustum</i>	98.85
NT-N-11	<i>Epicoccum nigrum</i> /E. <i>layuense</i>	99.23
NT-N-12	<i>Alternaria tenuissima</i> /A. <i>alternata</i>	99.81
NT-N-13	<i>Leptosphaerulina chartarum</i>	99.48
NT-N-14	<i>Aspergillus niger</i>	99.44
NT-N-15	<i>Cladosporium asperulatum</i> /C. <i>uredinicola</i>	99.61
NT-N-17	<i>Alternaria alternata</i>	100
NT-N-18	<i>Alternaria cumini</i>	99.39
NT-N-19	<i>Alternaria tenuissima</i> /A. <i>alternata</i>	100
NT-N-20	<i>Leptosphaerulina chartarum</i>	99.49
NT-N-21	<i>Alternaria alternata</i> /A. <i>malvae</i>	99.62
NT-N-22	<i>Pithomyces chartarum</i> ¹ / <i>Leptosphaerulina chartarum</i> ¹	99.65
M1	<i>Lithophila guttulata</i>	100
(b)		
SA	<i>Alternaria alternata</i>	100
SB	<i>Epicoccum nigrum</i> /E. <i>sorghinum</i>	99.80
SC	<i>Phoma conidiogena</i> ² / <i>Didymella glomerata</i> ² / <i>Coniothyrium aleuritis</i> ²	100
SD	<i>Bipolaris coffeana</i> ³ /B. <i>austrospiae</i> ³ / <i>Cochliobolus cynodontis</i> ³	100
SE	<i>Coprinellus xanthothrix</i>	99.70
SF	<i>Arthrimum arundinis</i>	98.95
SH	<i>Alternaria ethzedia</i>	99.63
SI	<i>Phoma conidiogena</i> ² / <i>Didymella glomerata</i> ² / <i>Coniothyrium aleuritis</i> ²	99.61
SL	<i>Phoma conidiogena</i> ² / <i>Didymella glomerata</i> ² / <i>Coniothyrium aleuritis</i> ²	99.61
SM	<i>Exophiala capensis</i>	97.90

Table 1. Cont.

Morphotype	Organisms with the Most Similar ITS Sequences	Similarity (%)
SN	<i>Elaphocordyceps</i> sp. ⁴ / <i>Tolypocladium</i> sp. ⁴	98.85
SO	<i>Aureobasidium pullulans</i>	99.64
Sud P	<i>Aureobasidium pullulans</i>	99.82
NT-S-1	<i>Phaeosphaeriopsis pseudoagavacearum</i>	99.81
NT-S-5	<i>Dothideomycetes</i> sp.	99.45
NT-S-6	<i>Cladosporium ramotenellum</i> /C. <i>puyae</i>	98.82
NT-S-7	<i>Alternaria citri</i>	99.81
NT-S-8	<i>Cladosporium sinuosum</i> /C. <i>tenellum</i> /C. <i>herbarum</i>	99.80
NT-S-9	<i>Paraconiothyrium hawaiiense</i>	100
NT-S-10	<i>Coprinellus micaceus</i>	99.69

¹ *L. chartarum* is the teleomorphic form, *P. chartarum* is the anamorphic form. They were considered as a single species. ² Some *Phoma* species are the anamorphs and their teleomorphic forms are described in genera *Didymella* and *Coniothyrium*. They were considered as a single species. ³ *Cochliobolus* is the teleomorphic form, *Bipolaris* is the anamorphic form. They were considered as a single species.

⁴ *Tolypocladium* genus includes some asexual morphs of *Elaphocordyceps* species. They were considered as a single species.

Most of the identified genera (8 out of 11, 72.7% of the genera at NW; 9 out of 13, 69.2% at SE) and species are known as dematiaceous (Table 1). Among these, one strain at NW (M1) and two strains at SE (SM and NT-S-5, the latter based on morphology) were meristematic fungi, while two other strains at SE (SO and SP, both identified as *Aureobasidium pullulans*) showed a yeast-like growth (Supplementary Figure S5).

4.5. CaCO₃ Solubilization Test

Twelve fungal strains isolated from SMFC marble and belonging to genera and species frequently detected on marble and limestone were selected to better understand their role in marble deterioration and tested for their ability to solubilize CaCO₃. Five strains resulted positive (Table S1, Figure S5): NT-N-8, NT-N-14 (both *Aspergillus niger*), NT-N-10 (*Cladosporium cladosporioides*), SO, and SudP (both *Aureobasidium pullulans*).

5. Discussion

The studied marbles consist of almost pure calcium carbonate; however, trace amounts of silicates and gypsum were revealed, in almost all samples, by the FT-IR analyses. The presence of gypsum is generally imputed to the sulphation process. This consists of the reaction of sulfuric acid, coming from the air pollutant SO₂, with the insoluble calcium carbonate of marble, which is consequently transformed into the sulfate dihydrate or gypsum (CaSO₄ * 2H₂O). The process is considered one of the main causes of carbonatic stone deterioration in urban environments and is known to be associated to the blackening of outdoor marble artworks (e.g., [52]). Since gypsum is soluble in water, it is usually washed away in rain-exposed areas, whereas in sheltered areas, its crystals form networks that entrap particles of dirt and airborne pollutants, such as carbonaceous particles, to form black crusts [24]. The Cathedral of Santa Maria del Fiore is exposed to the urban polluted air of Florence and, among pollutants, to SO₂. The annual average values of SO₂ emissions detected in Florence from 2007 to 2012 were around 1–2 µg/m³. Since 2014, the annual average values showed a reduction from 3 µg/m³ to 1 µg/m³ detected in 2019 [53] possibly due to the pedestrianization of this area since October 2009. Due to the long-term SO₂ exposure, the external marbles of the Cathedral display black crusts in several areas [3]; however, they are not present in the studied areas, as demonstrated by microscopic, mineralogical, and chemical investigations. The NW and SE study sites are rain-exposed, thus, soluble salts including gypsum are washed away; this is in agreement with the low amounts of gypsum found by FT-IR. The blackening of marble can thus be imputed to microbial colonization. The dark spots and patches on marble contain in fact dark colonies often present as multispecies aggregates mainly consisting of dematiaceous fungi and dark cyanobacteria, as shown by in situ (Figure 2) and ex situ microscopic observation of samples (Supplementary Figure S3). The presence of black fungi in these

aggregates was further confirmed by the growth of a meristematic colony, strain M1, from a black spot. On the other hand, cultivation confirmed the presence of dematiaceous fungi and dark cyanobacteria. Even if dark patinas show different levels of development at the SE and NW sampling sites, they can be attributed to the same biological cause.

Indeed, a complex multi-kingdom microbial community inhabits the SMFC marble. Cyanobacteria, green algae, and lichens have been detected and characterized by morphological criteria. The fungal community was deeper investigated through isolation and identification by molecular methods. Bacteria were also detected [3], and their analysis is the subject of an ongoing work.

Concerning the photoautotrophic community, the biodiversity seems to be very similar in the investigated areas. Lichen species richness is extremely low as it is expected in an area exposed to pollutants as the Cathedral is. Nonetheless, some species (*Myriolectis albescens* and *M. dispersa*) show a high prevalence. This result can be likely due to the pollution resistance of these species as well as to microenvironmental conditions. They grow preferentially on biofilm black spots.

Cyanobacteria and algae are considered primary colonizers of the bare stones. Cyanobacteria, in particular, can easily develop in harsh conditions, and they widely grow on NW and SE surfaces of the SMFC, being important contributors to the darkening alterations. Dark pigments such as mycosporine-like amino acids and carotenoids (common in cyanobacteria and some green algae) and scytonemin (found in sheathed cyanobacteria) contribute to the blackening phenomena even on other monuments [25,26]. The coccoid-sheathed cyanobacteria are dominant on the SMFC marbles, but the green unicellular algae showed a broad presence as well. Both unicellular cyanobacteria and green algae may be part of the symbiotic process in the presence of mycobionts, therefore leading to the formation of lichens. On the other hand, fungi can be prevalent in urban conditions since they utilize the airborne anthropogenic compounds [7].

The isolated fungi showed a high biodiversity, with genera and species richness similar at both study sites. Although some differences in species composition at the NW and SE sites were detected, the strong presence of dematiaceous fungi (more than 70% of the total 21 isolated genera)—in particular, of *Alternaria* and *Cladosporium* (the most abundant genera)—are nevertheless the common denominator of the fungal community at both the study sites. Dematiaceous or black fungi are an artificial heterogeneous group of darkly pigmented fungi with different morphological characteristics (and often pleomorphic behavior) forming dark-brown, green-black, or black colonies due to the production of melanin and melanoid pigments [54]. Among them, meristematic fungi are well known as rock inhabitants able to deal with varying microclimatic conditions. They have cell walls strongly melanized and form small black colonies on and inside the stone, often occurring in close association with lichens [55]. Black fungi are considered as major agents of microbial deterioration of building stones. Their occurrence on marble produces well-documented effects such as aesthetical damage due to darkening (from black spots to black layers completely covering stone) and other color changes, as well as surface erosion and exfoliation [8,20,27,30,32,55–57]. Their activities are favored by the urban “air eutrophication” that increases the occurrence of discolorations and crust formations [30]. Among the isolated dematiaceous fungi, *Alternaria*, *Aspergillus*, *Aureobasidium*, *Cladosporium*, *Epicoccum*, and *Phoma* are the most frequent genera present as airborne ubiquitous spores growing on stone in urban environments [16], and well known to cause blackening on marble and limestone [30,58]. These genera were also found on darkened areas of the external marble of the Milan Cathedral, where their growth was enhanced by naturally aged acrylic resins used as stone protectives and consolidants [19]. Some of them, particularly *Phoma* and *Alternaria*, are considered as ones of the most damaging organisms that attack and even penetrate the surfaces of stone monuments [29,30,56].

While for the above-mentioned dematiaceous genera the role in stone deterioration is well documented, other isolated fungi would be present as airborne spores occasionally deposited or entrapped in biofilms on marble, with an unclear role in stone colonization.

This would be the case for the plant pathogens *Bipolaris* (strain SD), *Arthrrium* (strain SF), *Hyphodermella* (strain NE), and *Coprinellus* (strains SE and NT-S-10). Concerning *Parengyodontium album* (strain Nord A), it has been frequently isolated from deteriorated materials of cultural heritage, stone included. Although several authors have linked the development of *P. album* on cultural heritage monuments to the presence of insects, its role in biodeterioration is debated [59]. A fungus frequently detected at the NW site is *Pithomyces chartarum* (or its teleomorph *Leptosphaerulina chartarum*; strains NT-N-6, NT-N-13, NT-N-20 and NT-N-22). *Pithomyces* strains are commonly isolated from a wide range of plant material; it has been previously isolated from marble [60], but its possible role in deterioration is unknown. For this reason, *Parengyodontium* and *Pithomyces* (strain NT-N-13) were included among the strains selected for the calcium carbonate dissolution test (Section 4.5). They resulted negative (Table S1), confirming what was found for *P. album* by Trovão et al. [58], and indicating that these fungi do not carry out this kind of chemical attack on the stone. On the other hand, the ability to dissolve calcium carbonate by isolated strains belonging to *Aspergillus*, *Aureobasidium*, and *Cladosporium* shows that such degradative potential is present among the SMFC microbial community members already known as stone deteriogens. This chemical activity is attributed to the production and excretion of organic acids that dissolve the stone carbonates acting as chelators of calcium and other cations. Other members of the SMFC fungal community, such as *Phoma* and *Alternaria*, are known to firmly attach to and penetrate deeper into the marble by a physical attack [30]. The combined action of chemical and mechanical processes allows fungi to actively penetrate marble forming euendolithic communities in the bulk of the substrate [8,32].

Concerning the meristematic fungi, strain SM was identified as *Exophiala capensis*, a species never isolated from stone, and M1 was identified as *Lithophila guttulata*, a fungus previously detected as a new species from marble artworks of the Vatican City [57]. On the contrary, strain NT-S-5 seems to be a not yet identified fungus, since ITS rDNA sequence analysis allowed to classify it up the *Dothideomycetes* class.

Overall, the picture of the fungal community emerging from the current cultivation data shows a prevalence of dematiaceous hyphomycetes with respect to meristematic fungi, according to the climate conditions of Florence. In fact, hyphomycetes including species of *Alternaria*, *Cladosporium*, *Epicoccum*, *Aureobasidium*, and *Phoma* dominate the fungal communities on monuments in moderate and humid climates, while microcolonial black fungi dominate the fungal community in arid and semi-arid environments [55]. However, since we used cultivation conditions favoring the growth of fast-growing fungal strains (Section 3.4), the presence of meristematic fungi on SMFC marble will be better assessed by using more appropriate cultivation conditions for their growth.

Other than biodiversity, the colonization pattern seems to be different at the NW and SE sites. At the NW site, the community grows mainly as a thick epilithic biofilm completely covering the marble surface and strongly adhering to it by EPS (Figures 2, 4 and 5). The biofilm does not invade the inner parts of marble, given the compactness of marble grains (Figure 5). At the SE site, microorganisms grow mainly as epilithic colonies or biofilms along boundaries of marble grains, and as endolithic biofilms that penetrate through and surround the grains' boundaries up to 4 mm in depth (Figures 2, 4 and 5). This penetration pattern relates to the observed detachment of marble grains (Figure 5b). Green unicellular algae, cyanobacteria, and fungi participate in this inward marble colonization due to their small dimension and low nutritive requirements, and also to the white marble transparency that allows the photosynthetic activity. The different growth patterns can be explained by the different microclimatic conditions of the two study areas. Solar radiation and temperature are higher for most of the year on SE-facing surfaces than on NW-facing ones, so the microclimates of the two areas vary during the year. It is widely known that marble thermal weathering is responsible for micro-cracks' formation at the boundaries between grains [17,61,62]. This process is more effective on calcitic marbles than on dolomitic ones due to the anisotropic behavior of calcite crystals. Indeed, when exposed to thermal

variations, calcite expands along the crystallographic c-axis and contracts perpendicularly to the same axis causing the detachment of grains, the enlargement of micro-cracks, and the opening of new ones. Experimental studies demonstrated that even day-night thermal excursions can be responsible for the detachment of crystals from their borders and that this process can begin at 40 °C in calcitic marbles [61]. This is the case of the studied marble, consisting of an almost pure calcite, subjected during the summer to temperatures higher than 40 °C. Moreover, the dark microbial patinas on white rock surfaces causes, in addition to the aesthetic damage, a selective absorption of solar radiation that influences the surface thermal behavior and enhances the physical stress leading to crystals' decohesion [2,16]. Such a process would act particularly on the SE-exposed marble, where the irradiation is much stronger and prolonged, causing the distancing of the superficial grains and favoring the penetration of microorganisms. In this way, microorganisms are protected against the irradiation stress and, in turn, cause grains distancing in the inner of marble as a result of biophysical and biochemical processes. On the other hand, NW-exposed marbles have higher water contents and, contrary to SE-facing surfaces, drying occurs at a very slow rate so that NW biofilms may remain wetted for longer periods. This favors a greater epilithic growth, as also indicated by the viable titer of fungi higher at NW than at SE (about double), and the more developed darkening observed at the NW site. According to Diakumaku et al. [30], the major aesthetic damage by fungi may occur when they are spread over the rock surface under favorable conditions (air eutrophication and constant humidity for longer periods) and not forced to grow deep into the crevices.

6. Conclusions

According to the obtained data, the growth of dark cyanobacteria and black fungi is the main cause of the widespread darkening of Santa Maria del Fiore external white marble at both the study sites. The biodiversity of the lithobiontic community seems to be similar in the investigated areas, especially that of the photoautotrophic components. On the contrary, the pattern of microbial colonization on/inside marble seems to be different at the NW and SE sites depending on the distinct climatic conditions of the two study areas, in particular on solar radiation exposure, which influence marble's bioreceptivity.

The results are relevant for the knowledge of the state of conservation of marble building facades in urban environments because they focus on (i) decaying phenomena of ancient marbles exposed in a changing urban environment over centuries without receiving chemical restoration treatments (e.g., synthetic protectives or consolidants); (ii) the darkening in two differently exposed areas with different climatic conditions; and (iii) the broad description of the lithobiontic community (photoautotrophic components and fungi). The acquired knowledge will be used to plan in situ tests with innovative methods to control microbial colonization of NW and SE sides of the Florence Cathedral.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/app11136163/s1>. Supplementary Figure S1: Marble samples (source), and analyses carried out. Supplementary Figure S2: FT-IR. Supplementary Figure S3: Light microscope observation of marble powder suspensions in PBS (a,b) and cultures in BG-11 medium (c,d). Supplementary Figure S4: Polished cross-sections of SE marble samples. Supplementary Figure S5: Some fungi isolated in this study. Supplementary Table S1: Carbonate dissolution test.

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