Biodeterioration of funeral sculptures in La Recoleta Cemetery, Buenos Aires, Argentina: Pre- and post-intervention studies

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ABSTRACT

Stone materials exposed to weathering are subject to biological colonization and consequently to biofilm formation, causing biodeterioration. The color changes on the stone substrates caused by biogenic pigments, mechanical stress on the mineral structure due to extracellular polymeric substances (EPS) and the accumulation of atmospheric pollutants by the biofilm are some of the deteriogenic effects, which modify esthetic and functional aspects of the work. The aim of this study was to determine biodeterioration and biofilm formation on marble tombstones from La Recoleta Cemetery, Buenos Aires, Argentina. The effect of the biocide benzalkonium chloride on biofilm formation was studied, and a chart produced of the treated tombstones. Pre- and post-intervention microbiological studies, scanning electron microscopy and X-ray dispersion analysis showed a significant decrease of the biofilm flora after treatment. However, algae of the genus Trentepohlia were difficult to eradicate. These studies are a valuable contribution to determine restoration criteria against biofilm formation, to characterize chromatic variations of biological origin on the stone and to formulate conservation and restoration policies.

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1. Introduction

Biodeterioration is caused by microbiological, biological and physicochemical processes [1]. Biodeterioration can be defined as a change in material properties due to the vital activities of the organisms [2] and biofouling is the accumulation of biological deposits on a surface [1]. A wide range of materials can be affected, suffering physical, chemical and esthetic damage caused by insects, algae, lichens, fungi and bacteria.

Memorial sculptures are continually exposed to weathering agents, such as wind, temperature, rain, relative humidity, and condensation. These agents induce physical and chemical meteorization. The former affects stone matrix stability while the second causes chemical corrosion, or erosion, of the stone minerals, by oxidation and dehydration reactions, carbonate dissolution and dissolution of some mineral elements containing silicates [3]. In addition to this, the stone is subject to biological colonization. Stone surfaces and other inorganic materials are used as substrates by a wide variety of microorganisms, chemoorganotrophs, chemolithotrophs and phototrophs, actinomycetes, fungi and lichens [4].

Complex microbial communities immersed in extracellular polymeric substances (EPS) that constitute biofilms and biofouling on the surfaces of structural materials lead to biodeterioration. The major compounds of the EPS are polysaccharides [5]. Among the effects are (a) chromatic changes on the stone substrate produced by biogenic pigments, which, depending on the size of area they occupy, can alter the esthetic appearance and thus the interpretation of the work of art; (b) the mechanical stresses on the mineral structure caused by EPS, which increase with successive hydration and drying cycles; and (c) accumulation of atmospheric pollutants adsorbed by the biofilm [3].

The intensity of biological attack and biodeterioration processes is strongly influenced by water availability. Biodeterioration is

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determined by specific material parameters (porosity, permeability), their conservation status (low ductility, erosion, micro-cracks), as well as by environmental conditions, the morphology of the structure, its orientation and its conservation status, which tells us about the interactions between the support and the environment over time [6,7]. The natural biomass accumulated by the photosynthetic microorganisms and the anthropogenic pollutants (nitrogen compounds, hydrocarbons) of agricultural or industrial origin may be the only source of nutrients for the microorganisms colonizing the stone. Growth and metabolic activity of these organisms are controlled by natural parameters such as light and humidity. La Recoleta Cemetery, situated in a protected historic area of the Autonomous City of Buenos Aires (S.P.U. G.C.B.A. 1998) Argentina, contains many well established monuments, which date from the period 1900 to 1930 [8]. The tombstones chosen for this study were those of José C. Paz (1915), Rufina Cambaceres (1908), Carlos Federico Brandsen (1905), and Carlos Pellegrini (1910 approx.); all are constructed of marble [9], a monumental stone associated with the qualities of durability and continuing beauty.

The aim of this work was to study the biodeterioration of these marble tombstones, as well as the effect of benzalkonium chloride treatment on biofilm formation. Also it will provide information of procedure interventions, assessment of intervention success, and planning future post-intervention steps.

2. Materials and methods

2.1. Microbiological sampling and isolation of microorganisms

Pre- and post-intervention samplings were carried out in autumn. Pre-intervention samplings were as follows: Rufina Cambaceres and José C. Paz: May 2007, Carlos Brandsen: May 2002 and Carlos Pellegrini: May 2006. Post-intervention samplings were: Rufina Cambaceres and José C. Paz: May 2009, Carlos Brandsen: May 2009 and Carlos Pellegrini: May 2010.

Intervention procedures took about 2–3 months.

The following samples were taken: two at the José C. Paz memorial (1 and 2), three at that of Rufina Cambaceres (3–5) two at that of Carlos Brandsen (6 and 7) and two at that of Carlos Pellegrini (8 and 9) (Fig. 1a, c, e and g). Samples 1, 7–9 showed discolouration, sample 2 microkarst, samples 1–9 biological colonization, samples 4 and 8 differential erosion, sample 5 peeling and sample 6 soiling [10].

Microbiological sampling was performed "in situ" using Envirocheck® Contact YM (R) (Merck) culture slides, which give a quantitative assessment of the surface microorganisms. Samples were also collected by surface scraping, removing small flakes of marble and by adhesive tape [11]. They were homogenized in sterile saline solution and inoculated in suitable culture media. For total aerobic bacteria, dilutions were inoculated onto nutrient agar and incubated at 32 °C for 72 h. Acid-producing bacteria (APB) were enumerated in broth for total acidifying bacteria [12]. To determine the absence/presence of sulfate reducing bacteria (SRB) 1 mL aliquots of each sample were inoculated into Postgate B medium and incubated at 32 °C for 15 days [13]. Similar procedures were used for sulfite reducing bacteria (Clostridium sp.), using differential-reinforced Clostridium broth (DRCM) at 32 °C for 15 days. For fungi, dilutions of each sample were inoculated onto YGC agar (yeast extract, glucose and chloramphenicol) and incubated at 28 °C for 5–7 days prior to counting colonies [14]. Coliform bacteria were cultivated in chromogenic culture media [15]. All tests were performed in quadruplicate.

2.2. Microbiological sampling of air

Microbiological sampling was carried out by the sedimentation method as described by Omeliansky [16,17]. Petri dishes containing YGC agar for fungi and nutrient agar for bacteria were exposed for 30 min at approximately 30 cm above the ground. Because of the small area involved, only two different points were sampled, each in triplicate. YGC plates were incubated for 7 days at 28 °C and those containing nutrient agar at 32 °C for 72 h.

The number of colony-forming units per cubic meter of air (CFU m^{-3}) was estimated according to Omeliansky's formula:

$$N = 5a \times 10^4 (bt)^{-1}$$

where N is the microbial CFU m^{-3} of indoor air; a the number of colonies per Petri dish; b the exposed agar area in square centimetres, and t is the exposure time in minutes.

Relative microbial distribution was determined according to Smith [18] as (number of colonies of the genera or species/total number of colonies of all genera or species) × 100.

2.3. Identification of isolated organisms

Culture and morphological characteristics of fungal colonies were observed and the identification was performed according to Barnett and Hunter [19], as well as information published on the web [20,21]. Bacteria were grouped on the basis of Gram staining and biochemical tests described in Bergey's Manual of Systematic Bacteriology [22,23].

2.4. Characteristics of the environment

The average temperature of the area is 17 °C, RH 72%, and the average rainfall 1234.92 mm/year (data from the last 8 years recorded at the Buenos Aires Palermo station, close to the La Recoleta Cemetery).

Environmental pollution surveys in the area indicate that gases released by burning of fossil fuels, nitrogen oxides and suspended particulate matter (PM10 fraction), are critical pollutants [24–26].

2.5. Intervention

The whole surface of each monument was brushed with benzalconium chloride (3% dilution in distilled water), and on the areas with presence of biofilm, cotton compresses soaked in the same solution were applied, covered with plastic film to maintain the humidity over a treatment time of approximately 3 h. Finally removal of biofilm was done with a hair brush.

Benzalkonium chloride is a quaternary ammonium salt with microbicidal activity due to its cationic character; it leads to changes in structure and permeability of biological membranes.

2.6. Monitoring of biofilm formation by microscopy

Samples were placed on clean slides sprayed with sterile water. An Olympus BX-51 inverted microscope was used for observation.

Unfixed microsamples were coated with Au/Pd and processed according to Videla et al. [27]. Samples were observed with a Jeol 6360LV SEM.

3. Results and discussion

3.1. Biofilms before intervention

Numbers of total heterotrophic bacteria and molds are shown in Fig. 2. High concentrations of viable bacteria and molds were detected. Highest counts of microorganisms in air were 10^4 CFU m^{-3} in Jose C. Paz tombstone, which is surrounded by trees. In Rufina Cambaceres and Carlos Pellegrini tombstones oscillated

around 10^3 CFU m^{-3} and the lowest counts were in Carlos Brandsen tombstone (10^2 CFU m^{-3}), which has no trees around and a high exposure to the sun.

Acid producing bacteria (APB) was found in samples 1 and 2 from José C. Paz tombstone and samples 3 and 4 from Rufina Cambaceres Sulfate reducing bacteria (SRB) were not detected in any sample. Although it is unlikely to found these microorganisms on stones, in previous studies of archeological sites their presence has been demonstrated [28].

Sulfite reducing bacteria (*Clostridium* spp.) were detected in samples 1 and 2 from the José C. Paz tombstone. Total coliform bacteria were also detected at 20 UFC cm^{-2} on all tombstones.

The bacterial genera most frequently isolated were *Bacillus* spp. and *Pseudomonas* spp. The genus *Bacillus* was most commonly isolated in air samples. The metabolic activity of all heterotrophic

bacteria is subject to the availability of organic material. Given the location of the monuments, this organic material could come from the ground, dust or even from dead organisms (phototrophs and heterotrophs) [29].

Fungal genera commonly found were *Aspergillus*, *Alternaria* and *Penicillium* for the Rufina Cambaceres and José C. Paz tombstones. The yeast *Rhodotorula* sp. was detected in all tombstones.

There was a great diversity of microorganisms, especially fungi, in air samples (Table 1). Only some of them are able to colonize rocks and produce damage.

The greatest biomass was found on the Carlos Brandsen and Carlos Pellegrini tombstones, studied in autumn of 2002 and 2006 respectively. Algae of the *Trentepohlia* genus were identified in both tombstones. Similar pigmentation was also detected in a few other monuments. The detail in Fig. 1g (right side) shows the coverage of
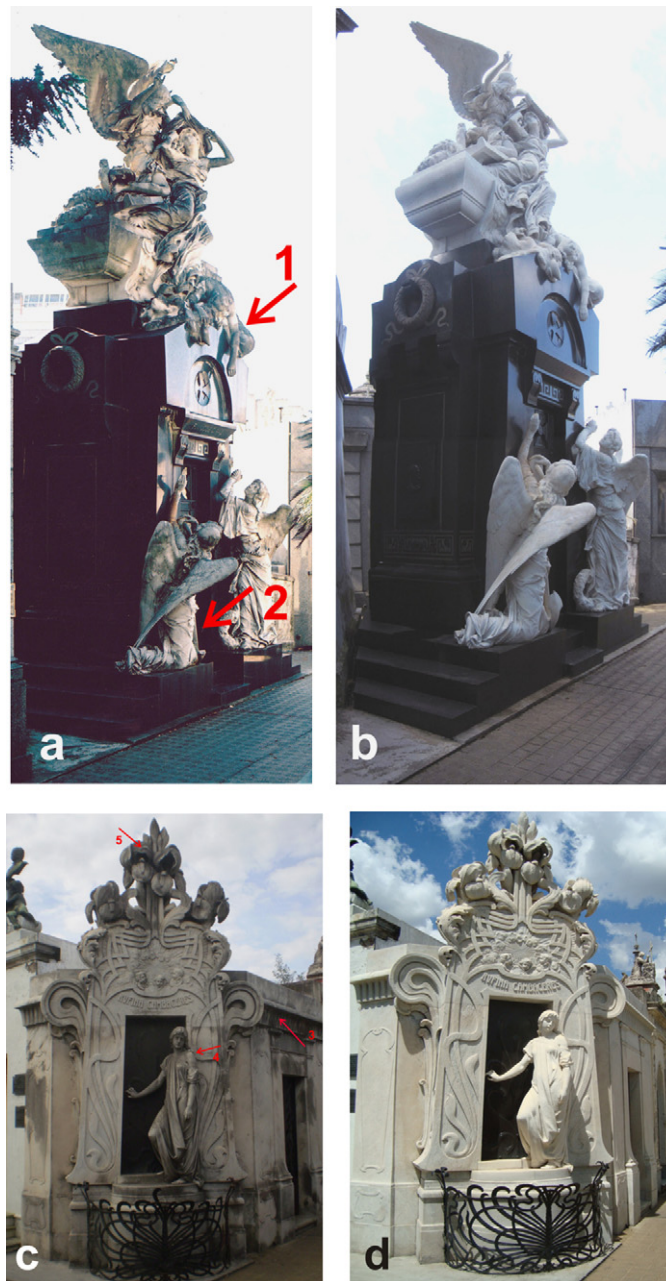

Fig. 1. Pre- and post-intervention of tombstones: José C. Paz (a and b), Rufina Cambaceres (c and d), Carlos Brandsen (e and f) and Carlos Pellegrini (g and h). Details of 1g right side: zone with red stained area; left side: optical microscope image with red carotenoid pigmentation due to algae *Trentepohlia* sp. (10×). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Fig. 1. (*Continued*)

Table 1
Fungal and bacterial diversity in air samples.

| Rufina Cambaceres	José C. Paz	Carlos Brandsen	Carlos Pellegrini
Fungi			
Alternaria sp.	*Alternaria alternata*	*Alternaria* sp.	*Alternaria* sp.
Aspergillus sp. 1	*Alternaria* sp.	*Aspergillus ochraceus*	*Aspergillus* sp.
Aspergillus sp. 2	*Aspergillus niger*	*Aspergillus* sp.	*Curvularia* sp.
Candida sp.	*Aspergillus* sp. 1	Mycelia Sterilia	*Fusarium* sp.
Cladosporium sp.	*Aspergillus* sp. 2	*Penicillium* sp.	*Geotrichum* sp.
Fusarium sp. 1	*Aspergillus* sp. 3	*Rhodotorula* sp.	Mycelia Sterilia
Fusarium sp. 2	*Cladosporium* sp.		*Penicillium* sp.
Penicillium sp. 1	*Fusarium* sp. 1		*Rhodotorula* sp.
Penicillium sp. 2	*Fusarium* sp. 2		
Rhodotorula sp.	*Mucor* sp.		
Trichoderma sp.	*Penicillium* sp. 1		
Ullocladium sp.	*Penicillium* sp. 2		
	Penicillium sp. 3		
	Rhizopus sp.		
	Rhodotorula sp.		
Bacteria			
Bacillus sp. 1	*Pseudomonas* sp. 1	*Bacillus* sp.	*Bacillus* sp. 1
Bacillus sp. 2	*Staphyloccoccus* sp.	*Pseudomonas* sp.	*Bacillus* sp. 2
Bacillus sp. 3	*Bacillus* sp. 1		*Bacillus* sp. 3
Micrococcus sp.	*Pseudomonas* sp. 1		
Pseudomonas sp. 1			
Pseudomonas sp. 2			
Staphylococcus sp.			

Fungi

Bacteria
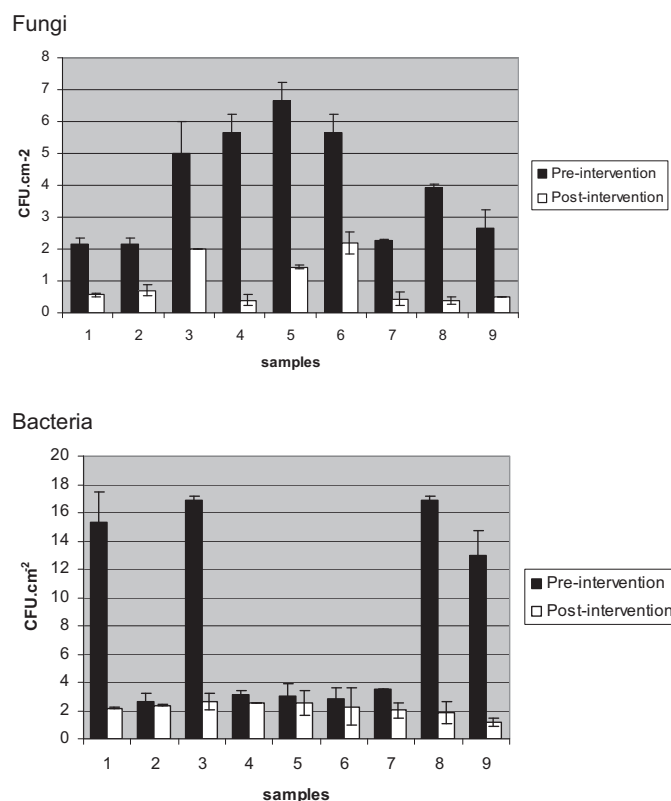
Fig. 2. Counts of CFU cm^{-2} by the slide culture technique error bars indicate standard deviations.

surface by *Trentepohlia*. This algal genus has long been recognized as biodeteriogenic on buildings and is well known for its production of pink/brown discoloration [30] caused by biogenic pigments. It has also been associated with mechanical degradation of monuments in Spain [31] and Mexico [28]. Pigment production in these biofilms is an adaptation that increases resistance against environmental stress [32].

The stone surface showed surface erosion in the areas of greater sun exposure (horizontal planes, convex shapes), as well as in the zones of rainwater drainage. Aggressive environmental agents such as rain and atmospheric gases, together with microbial growths, act preferentially in these areas. The erosion caused loss of surface brightness and increase in porosity can been observed in Fig. 1b, d, f and h. In parallel, in concave zones corresponding to crowning adornments, allegorical figures and portraits, a more humid microclimate is generated, protected from wind currents from the nearby river as well as from rain. This is favorable for the retention of suspended particulate matter of carbonaceous origin, registered as "critical pollutants" during measurements previously performed in this metropolitan area [24–26]. It was observed at the edges of these black zones from accumulation of particulate matter, a process of disintegration of the parental material up to 10 mm deep.

Stone disintegration can be linked to fungal growth. These microorganisms are particularly concentrated on the stone coatings. They are able to penetrate into the material with the grown of hyphae and to cause bioerosion, biosolubilization by organic and inorganic acid excretion (oxalic and fumaric acids) and cationic oxidation of the minerals [3,4,7,33,34] as well as by organic agents that chelate the metallic cations of the stone [35,36]. The abundance of biological material, the EPS and the adherence of a complex microbial community (molds and bacteria) (Fig. 3), may alter the structure and integrity of the stone causing cohesiveness

Fig. 3. SEM photomicrograph. Marble of the pre-intervention Rufina Cambaceres tombstone. Fungal microcolonies are observed.

loss in the marble grains and chemically attacking the substrate [37,38].

At the same time, when retaining water, the mycelium contributes to mechanical degradation of the stone, causing loss of cohesiveness in the stone grains by means of successive cycles of hydration and dehydration, which was observed in some areas of the monuments. Bird guano and remains of various arthropods may provide nutrients to favor later microbial colonization and subsequent biofilm development [33,17].

3.2. Biofilms after intervention

In the case of the Rufina Cambaceres tombstone, after intervention (Fig. 1b), fungal hyphae have obviously persisted in the substrate (or have recolonized the marble); it is of great importance to perform such biocide treatments periodically. In the four tombstones that formed this study, José C. Paz, Rufina Cambaceres, Carlos Brandsen and Carlos Pellegrini, the interventions allowed complete interpretations of the monuments to be recovered [39] (Fig. 1b, d, f and h). In the case of Carlos Brandsen and Carlos Pellegrini tombstones, it was of particular interest to detect the origin of small chromatic zones, which remain unchanged in area with the passing of time without changing the image of the monument. Results revealed that algae of the *Trentepohlia* genus were responsible for this discoloration, visible both pre- and post-intervention (Fig. 1g and h).

There were no APB or SRB detected at any of the places sampled post-intervention. *Clostridium* spp. was found in samples 2 from José C. Paz and 4 and 5 from Rufina Cambaceres. Counts of total heterotrophic bacteria and molds are shown in Fig. 2. The treatment was effective since it reduced the attached microbial load, even though there was no change in biodiversity.

Biocide treatment led to a decrease in the microbial flora colonizing the monuments and the removal of acidifying microorganisms that could alter the marble. This compound is a quaternary ammonium salt, with microbicidal activity due to its cationic character, allowing it to interact with cell membranes [40]. It had little effect on lichens, which are a resistant form of symbiotic microbial association (Fig. 2).

Detail of Fig. 1g (left side) shows marble discolored by carotenoid pigment released from *Trentepohlia*. This kind of discoloration was detected in samples from Carlos Brandsen and Carlos Pellegrini tombstones.
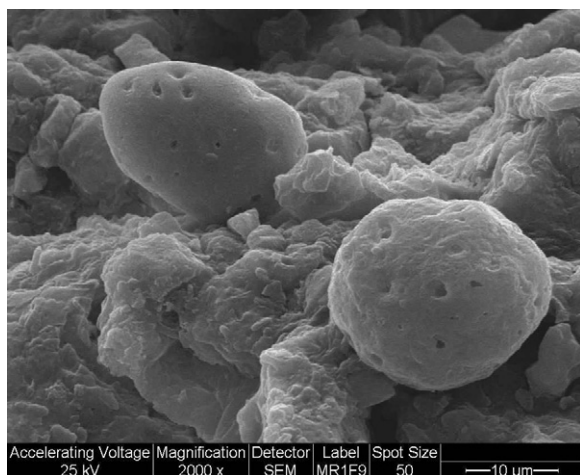
Fig. 4. SEM image of anthropogenic deposits on the marble from Rufina Cambaceres tombstone (2000×).

3.3. Biofilms by SEM

Pre-intervention images showed the presence of particles of anthropogenic origin (Fig. 4) deposited on the marble. Their morphology of spheres with holes is considered typical of sulfur and carbon compounds [41]. These particles are suitable for the growth of biological communities on the marble.

4. Conclusions

These studies are a valuable contribution to determine restoration criteria against biofilm formation and to characterize chromatic variations of biological origin on the stone.

The information obtained from this and future studies will allow the elaboration of schedules and maintenance procedures, treatment and conservation of the tombstones within the Conservation and Restoration Program of the Recoleta Cemetery.

These studies contribute to the understanding of material transformations under environmental influences and associated causes, allowing determination of restoration criteria to inhibit biofilm formation, as well as to characterize chromatic areas in the stone without altering the esthetic image. The removal of such areas would imply abrasive and irreversible treatments of the material, and so it is important that they be understood and accepted by conservators as a consequence of the passing of time.

The pre- and post-intervention analysis allowed us to evaluate the efficacy of benzalkonium chloride, which proved efficiency on a wide spectrum of biofilm microorganisms. It would have to be applied not only during restoration but also in the within post-intervention schedules.

The red chromatic zones were caused by the alga *Trentepohlia*; they did not increase in size during the post-intervention periods. However, their presence is connected with deterioration and so other biocides or alternatives should be tested.

Acknowledgments

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