Biofouling of crypts of historical and architectural interest at La Plata Cemetery (Argentina)

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A B S T R A C T

Cemeteries are part of the cultural heritage of urban communities, containing funerary crypts and monuments of historical and architectural interest. Efforts aimed at the conservation of these structures must target not only the abiotic stresses that cause their destruction, such as light and humidity, but also biofouling by biotic agents. The purpose of this study was to assess the development of biofouling of several historically and architecturally valuable crypts at La Plata Cemetery (Argentina). Samples obtained from the biofilms, lichens, and fungal colonies that had developed on the marble surfaces and cement mortar of these crypts were analyzed by conventional microbiological techniques and by scanning electron microscopy. The lichens were identified as *Caloplaca austrocitrina*, *Lecanora albescens*, *Xanthoparmelia farinosa* and *Xanthoria candelaria*, the fungi as *Aspergillus* sp., *Penicillium* sp., *Fusarium* sp., *Candida* sp. and *Rhodotorula* sp., and the bacteria as *Bacillus* sp. and *Pseudomonas* sp. The mechanisms by which these microorganisms cause the aesthetic and biochemical deterioration of the crypts are discussed.

1. Research aims

As the first city in South America to install electric light bulbs, La Plata represented the modernization of Argentina at the end of the 19th century and as such even aspired to become the capital of the Republic. Its progress was also recognized at the 1889 Paris EXPO, which awarded the city two gold medals, as the "City of the Future" and the "Best Built Project". The city of La Plata had been designed by Pedro Benoit, who was also the architect of its Cathedral and, in 1886, its public cemetery. Among the latter's notable architectonic structures are its portico and many of the family crypts, with their Neoclassic, Neogothic, Art Nouveau, Art Deco, and Egyptian Revival styles. These monuments were primarily built with marble and cement mortar [1]. Now, after 120 years, they show signs of advanced biodeterioration, including the appearance of biofilms and fungal colonies, both of which are often quite large and continue to spread. Biofouling has not only resulted in the aesthetic deterioration of the stones but also in the loss of historical information since in many cases it has become impossible to read the names, dates and inscriptions engraved in the headstones. An additional problem, pointed out by the Commonwealth War Graves Commission, is the disfigurement of soldiers' graves by colonies of lichens [2].

Efforts to rescue the crypts from further disfigurement and destruction must begin with a detailed analysis of the invading microorganisms. The appropriate measures can then be taken to preserve and restore these beautiful and culturally important funerary monuments.

2. Material and methods

2.1. Site, visual inspection, sampling and biofilm analysis

This work was carried out at La Plata Cemetery in Argentina. La Plata has an average annual temperature of around 16.3 °C and the average annual rainfall is 1023 mm. As the city is close to the La Plata River, it tends to be rather humid, with an average annual relative humidity of about 77%. The air temperature during sampling was around 20 °C and the relative humidity 50–55%.

Fig. 1. Portico of the crypt of Francisco Arrechea (crypt F87), which is of architectural interest because of its Art Nouveau style. The yellowish coloration is indicative of the presence of *Caloplaca austrocitrina*.

Visual inspection of crypts of historical and architectural interest showed clear evidence of significant biofouling (lichens, fungi, and dark biofilm patinas). Five sites from four crypts were selected for study:

- crypt C95 (marble column);
- crypt C77 (inner side of the marble);
- crypt F87 (front, cement mortar – Fig. 1);
- crypt A9 (front, cement mortar);
- crypt A9 (side wall and front).

Biofilms, fungi, and crustose lichens were sampled (1 cm^2) by aseptically scraping the external surfaces of selected monuments using a sterile scalpel. Foliose lichens were carefully detached with the help of a penknife. Biofilm samples to be further analyzed by scanning electron microscopy (SEM) were fixed overnight with a 2.5% glutaraldehyde solution in phosphate buffer, washed with distilled water, dehydrated in an acetone-water series, critical-point dried, and sputter-coated with gold. They were then examined with a Jeol scanning electron microscope (JSM-T100) at an accelerating voltage of 20 kV.

2.2. Lichens

Lichen samples were observed by stereoscopic and optical microscopy and identified using a specialized classification system [3,4], referring to the diagnostic characteristics of the different species; growth form, color, thallus, size, surface structures (isidia, soredia, etc.), fruiting bodies, spore form and number, and specific substances present in the thallus.

2.3. Fungi

Fungal samples were dispersed in physiological saline solution and a dilution series prepared. A volume of 100 μL from each dilution was used to inoculate YGC (yeast extract-glucose-chloramphenicol) agar plates, which were then incubated at 22 °C for two weeks. Moulds were identified based on their morphology [5], and yeasts using the API 20 C AUX system (bioMérieux).

2.4. Bacteria

Total aerobic heterotrophic mesophilic bacteria were determined by first dispersing the samples in physiological saline solution, which was then used to prepare a dilution series. Volumes of 100 μL from each dilution were plated on nutrient agar, plate count agar, and CPS (casein-peptone-starch) agar and then incubated at 28 °C for one week. Bacteria were subjected to Gram staining followed by biochemical testing using the API 20NE and API 50CH systems (bioMérieux) to obtain a preliminary identification prior to sequencing. Genomic DNA of the selected bacteria was extracted by three freeze-thaw cycles (−75 °C, +55 °C). 16 s rDNA fragments corresponding to nucleotides 5–531 in the *Escherichia coli* sequence were PCR-amplified using the primers 5F (5'-TGGAGAGTTTGATCCTGGCTCAG-3') and 531R (5'-TACCGCGGCTGCTGGCAC-3'). PCR was performed in a GeneAmp PCR System 2400 (Perkin Elmer) with the following thermocycling program: 5 min denaturation at 94 °C, followed by 30 cycles of 1 min denaturation at 94 °C, 1 min annealing at 55 °C, and 1 min extension at 72 °C, and a final extension step of 7 min at 72 °C. The reactions consisted of Ready Mix™ Taq PCR Ready Mix with $MgCl_2$ (Sigma) containing 5 μL of DNA and 25 pmol of each primer and were brought to a final volume of 25 μL with sterile water. PCR products were analyzed by electrophoresis in 1% (wt/vol) agarose gels in 1 × TBE buffer containing ethidium bromide (0.4 $\mu g \cdot mL^{-1}$) and then purified by filtration through Microcon-100 (Millipore) columns. Bacteria were identified by sequencing of the respective 500-bp rDNA fragment using the BigDye™ Terminator v1.1 Cycle Sequencing kit (L-7012, PE Applied Biosystems). Sequences were resolved in an ABI PRISM™ 310 Genetic Analyzer following the manufacturer's instructions and then compared directly to all known sequences deposited in the NCBI (National Center of Biotechnology Information) databases using the basic local alignment search tool Megablast.

In addition, acid-forming bacteria were quantified using the extinction technique, in which serially diluted cultures were maintained in glucose broth containing a pH indicator.

3. Results and discussion

3.1. Visual inspection and biofilm analysis

The studied crypts were those of Vicente Isnardi (crypt C77), Emilio B. Coutaret (crypt A9), Domingo Lastra (crypt C95), and Francisco Arrechea (crypt F87). Vicente Isnardi was one of the founders of La Plata University. Emilio B. Coutaret was a well-known architect who assisted Pedro Benoit with his plans for La Plata Cathedral, including the design for the Virgin's column, which is located in the garden surrounding the Cathedral. He also built the clubhouse of the La Plata Jockey Club. According to the La Plata Cemetery's records, Domingo Lastra was simply an employee worker. However, his funerary monument is interesting not only because of its method of construction and the quality of the materials used, but also because of its incomplete marble column, meant to symbolize

Fig. 2. Biofilm on the marble column of crypt C95.

that Lastra was unable to complete his task in this world. Francisco Arrechea was probably a prosperous merchant or landowner. His crypt is noteworthy because of its Art Nouveau style.

Lichens and dark biofilms were readily observed on crypts of historical and architectural interest at La Plata Cemetery. Biofilms were well established on crypts with E-SE exposures, as they receive light for only a short time during the morning and are exposed to the rain and humid winds coming from the La Plata River. In some cases, biofilms occurred under very specific conditions. For instance, on the column of crypt C95 (Fig. 2), a biofilm had grown remarkably well on the south-facing aspect. A dark biofilm growing on crypt C77 was particularly prominent on the inner side of the marble flowerpot (Fig. 3). Crypt A9 was found to be in very poor condition, obviously due to a lack of maintenance. Moreover, its location favored microbial colonization. Although this crypt is situated in front of a small open square, the door faces south and the building is tucked between two other crypts, under the shade of a tree. The façade of crypt F87, which is covered in shade in the afternoons, was colonized by lichens. The location of monuments in shady places facilitates the growth of lichens and other microorganisms by reducing exposure to the sun's harmful UV rays and by allowing the increased retention of water in the micropores of the edifice's substrate. This was seen even in south-facing crypts and

Fig. 3. Dark biofilm on a marble flowerpot across from crypt C77.

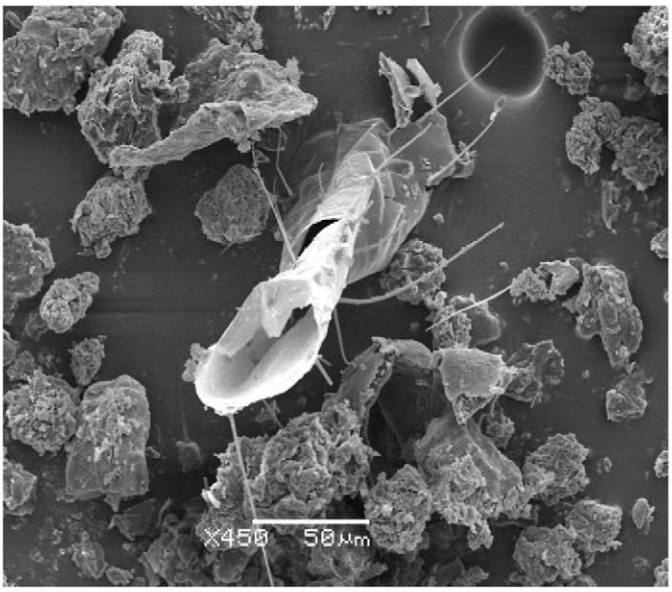

Fig. 4. Scanning electron micrograph of a sample from crypt F87, showing the presence of inorganic and organic materials (fragment of an insect).

in other monuments in La Plata, such as the Cathedral. However, other similarly constructed crypts exposed to the same conditions did not show signs of biodeterioration, most likely due to regular cleaning and maintenance.

Analysis of the biofilms by scanning electron microscopy revealed both fungal hyphae and bacteria embedded in a matrix of extracellular polymeric substances. Moreover, bdelloid rotifers were also found in the crypts (Fig. 4). Gorbushina and Petersen [6] observed that the remains of arthropod exoskeletons usually occurred in association with growing fungi. The authors concluded that the ingestion of fungal mycelium by arthropods, as a source of nutrition, is accompanied by the transportation of fungal spores in their intestinal tracts. Subsequent excretion of the spore mass results in further contamination at sites where the excreted material is deposited. Moreover, arthropods can directly cause substrate deterioration, through bioabrasion.

3.2. Lichens

Lichens identified as *Caloplaca austrocitrina* Vondrák, Riha, Arup and Søchting [7], *Xanthoria candelaria* (L.) Th. Fries, *Lecanora albescens* (Hoffm) Branth and Rostr., and *Xanthoparmelia farinosa* were found on cement mortar, especially on crypt F87. Approximately 95% of the affected surface was covered by *Caloplaca austrocitrina* and less than 5% by other lichens. Lichens belonging to the *Caloplaca* genus are known to colonize natural stone. Nimis et al. [8], in Latium and Tretiach et al. [9] in Sardinia, identified about 300 different species of lichens on archaeological remains. In that study, almost all *Caloplaca* sp. were found on calcareous rock with a SW, S, E, or SE exposure. The exception was *Caloplaca ochracea*, which occurred on northern exposures, specifically, in shaded sites near the soil. *Caloplaca* was also found to have colonized the carbonate rock (limestone) of the Jeronimos Monastery (Lisbon), where lichen thalli of *Thyrea*, *Aspicilia*, and *Verrucaria* were detected as well [10]. Colonies of *Caloplaca* sp., among others, were also identified on the pavement mosaics of the Romanesque town of Italica (Seville). Direct colonization of the tesserae was shown to have been preceded by colonization of the mortar [11]. Deruelle et al. [12] identified the nitrophiles *Caloplaca citrina*, *Xanthoria candelaria*, and *Lecanora albescens* on the Basilica of Notre-Dame de

Table 1
Microorganisms found in various crypts at La Plata Cemetery (Argentina).

Sample	Material	Lichens Absence/Presence	Fungi CFU/cm^2	Total aerobic bacteria CFU/cm^2	Acid-forming bacteria CFU/cm^2
Crypt C95 (marble column)	Marble	Presence	40×10^2	40×10^2	10×10^2
Crypt C77 (marble flower pot, internal side)	Marble	Presence	50×10^2	14×10^3	No growth
Crypt F87 (door)	Cement mortar	Presence	10×10^3	70×10^6	10×10^5
Crypt A9 (front)	Cement mortar	Presence	60×10^2	31×10^3	35×10^4
Crypt A9 (side wall)	Cement mortar	Presence	50×10^3	32×10^3	35×10^4

L'Epine (a calcareous monument in Marne, France). The presence of these species indicated that nutrient enrichment of the stone substrate had favored the lichens' growth. Overall, the recent increase in lichen colonization may be related to the widespread use of fertilizers and above all to the newly implemented method of spraying by pulverization. This was reflected by the broader extent of lichen populations, augmenting the disfigurement on the west-facing surfaces, which are more exposed to the dominant winds.

Bech-Andersen and Christensen [13] observed that in cement-based materials the cement paste was preferentially attacked by lichens, leaving the aggregates unaffected. More recent studies showed that *Caloplaca austrocitrina* can penetrate both cement and concrete. This species also produces oxalic acid, whose chemical action causes a loss of calcium from mortar substrates [14]. In a study of stone monuments in Rome, Seward [15] described the differential actions of lichen species in biodeterioration. Furthermore, not all species are harmful. While the sporadic growth of *Lecanora muralis* has been related to the mechanical decay of the substrate, species such as *Lecanora dispersa*, which also account for significant coverage, do not cause evident modifications. Monte [16] observed that *Lecanora campestris* and *Lecanora rupicola* preferentially grow on W to SW exposures whereas *Xanthoria calcicola* grows mainly on south-facing surfaces. These preferences are consistent with the fact that some species are stenoic, thriving only within a very specific range of pH, humidity, luminosity, and nitrogen supply, while others are euroic and thus tolerant of a wider range of conditions [17]. Although their role as primary colonizer seems doubtful, lichens nonetheless cause reduced substrate cohesion and therefore biocorrosion. In the crypts sampled in the present study, the surfaces were rough, eroded, and uneven due to the chemically targeted destruction of the cement matrix but not the aggregates, which were unaffected. Mechanical damage, by contrast, results from penetration of the substrate by lichen hyphae. The penetration depth of the thallus depends on the lichen species and the nature of the substrate [18]. Lichens may also cause substrate damage by the production of acidic substances, such as carbon dioxide, lichenic acids, and oxalic acid. A full review of lichens as agents of damage can be found in Lisci et al. [17].

While chemolithotrophic microorganisms have often been described in association with damaged inorganic materials, more recent studies have emphasized the significance of chemoorganotrophic bacteria and fungi, together with photoautotrophs, as the primary colonizers of building stones. The activities of these primary colonizers precondition the building for attack by chemolithoautotrophs and thus initiate the process of biological succession [19].

3.3. Fungi

Aspergillus sp. and *Penicillium* sp. were the predominant groups probably because they are ubiquitous air-borne fungi and easily cultivable. *Fusarium* sp., *Candida* sp., and *Rhodotorula* sp. were also detected among the fungi that colonized La Plata Cemetery's marble and cement mortar crypts. The fungal-mediated deterioration of stone was previously reviewed by May et al. [20]. Fungi were also identified by Wollenzein et al. [21] as the causative agents of the deterioration of marble and other calcareous materials used in the building of culturally important monuments in many localities in the Mediterranean area. Gaylarde and Gaylarde [22], in a comparative study of the microbial biomass of biofilms occurring on the exteriors of buildings in Europe and Latin America, found that fungi, although not an important contributor to the biomass invading stone, seemed to preferentially colonize painted surfaces rather than other substrates (cement, mortar, concrete). Perfettini et al. [23] isolated a strain of *Aspergillus* that produced gluconic and oxalic acids during the degradation of cement. After 8 months of contact with the substrate, these acids had induced the dissolution of portlandite (without the leaching of calcium) and had increased the porosity of the cement while reducing its bending strength.

In more recent laboratory studies, ordinary Portland cement pastes were severely attacked during bioleaching using *Aspergillus niger* cultures, especially by fungal biogenic organic (acetic, butyric, lactic, and oxalic) acids and to a lesser extent by respiration-induced carbonic acid [24]. However, this study was conducted under atypical conditions, in which large amounts of water were present, and cannot be related to the conditions to which most buildings are exposed.

The turgor pressure exerted by the hyphae of plant pathogens such as the rice blast fungus *Magnaporthe grisea* has been calculated on synthetic surfaces, including the plastic poly(vinyl chloride) [25]. Indirect measurement of turgor pressure indicated that the hyphae of this fungus can generate pressures in excess of 8.0 MPa, which is sufficient to allow fungal penetration of marble (tension resistance of 3.9 MPa), thereby causing mechanical damage at the ultrastructural level.

3.4. Bacteria

Total aerobic bacteria and acid-forming bacteria counts are shown in Table 1. Based on these counts, cement mortar was more highly colonized than marble. From the isolated bacteria, three isolates were selected for sequencing based on the Gram staining and API results. These bacteria were subsequently identified as *Bacillus* sp. and *Pseudomonas* sp. Their sequences were deposited in GenBank under the accession numbers JN837477 (97% similarity with JN208185), JN837478 (98% similarity with HM352366), and JN837479 (96% similarity with AY269246). *Bacillus* sp. (spore-forming bacteria) and *Pseudomonas* sp. (desiccation-tolerant bacteria) are able to survive in non-favorable conditions and can be easily found in environmental samples.

Heterotrophic bacteria have frequently been isolated on stone monuments and are known to cause biodeterioration [26]. Flores et al. [27] found several species of the genera *Bacillus* on weathered sandstones of a church. Vuorinen et al. [28] observed that Finnish granite is slowly degraded by cultures of *Pseudomonas aeruginosa*, resulting in morphological alteration of the stone surface and the elution of minerals from the stone.

In the crypts examined in this study, acid-forming bacteria were isolated from marble and from cement mortar. These bacteria

are likely to play a role in the biodeterioration of these historical monuments through the chemical and physical processes that are integral to biofilm formation [22].

3.5. Bioreceptivity of the building materials

According to Guillitte [29], bioreceptivity describes a material that can be colonized by living organisms but without necessarily undergoing biodeterioration. The bioreceptivity of stone is determined by its structure and chemical composition, and the intensity of microbial contamination by climatic conditions and anthropogenic eutrophication of the atmosphere [30]. Accordingly, given the same climate, the influence of the substrate on microbial colonization can be examined. Interestingly, our data (Table 1) show that in spite of similarities in microclimate, microbial growth on marble and cement mortar differed. While the cement mortar was almost completely covered by lichens, the marble was less colonized. In addition, bacterial and fungal growth on cement mortar was greater than on marble. A possible explanation for this is that cement mortar has a higher porosity than marble. In laboratory tests performed at the LEMIT ("Laboratorio de Entrenamiento Multidisciplinario para la Investigación Tecnológica", La Plata, Argentina), the water absorption rate of samples of Carrara marble was 0.924% (indicating a low porosity), whereas that of cement mortar was 6–9% (indicating a high porosity). Thus, regardless of the heterogeneity of cement mortars in terms of their composition, cement/water relationship, and age, they are much more porous than marble. This ability to absorb and retain relatively large amounts of water provides favorable growth conditions for microorganisms.

3.6. Conservation and restoration of crypts

Some authors are of the opinion that if biofilms do not cause serious damage or hinder conservation efforts then their presence may actually increase the cultural value of a building or monument [17]. However, this is not the case at La Plata Cemetery, where biodeterioration of the crypts is serious enough to warrant intervention.

The removal of lichens and other organisms from a surface is a delicate task that must be considered and planned with the greatest care. It differs from a simple cleaning, which allows regrowth. In Buenos Aires Province, for example, regrowth of the lichen *Caloplaca austrocitrina* on the region's stone monuments and cement mortar-covered buildings was observed 6 months after these structures had been cleaned using a hydrojet washing method (our unpublished results). Lichen reproduction occurs by the germination of spores or by multiplication of the cells of the soredia and isidia. These reproductive structures are dispersed in the atmosphere by wind or transported by birds and insects. If they settle into cracks, pores, or cavities that retain water, new growth may arise. On stone surfaces, the microorganisms comprising the lichen thallus are frequently accompanied by bacteria, cyanobacteria, free-living algae, or fungi, and their additional effects cannot be ignored in any removal strategy [30].

Before a decision on the removal method is reached and a biocide is chosen, the environmental conditions (for example, light and humidity) must be considered and, to the degree possible, changed; otherwise, the effects of the treatment will be short lasting. Unfortunately, this is not possible in the case of the La Plata funerary monuments, since they are regularly exposed to inclement weather. The nature of the material, the degree of its alteration, and the presence and density of the organisms causing the biodeterioration are critical factors in deciding whether cleaning and removal are required and whether a biocide will be needed [31]. The authors of the latter study emphasized that biocides must not alter the material (discoloration, salt efflorescences, etc.) and should be specific for the biodeteriorating organisms but not harmful to people, other animals, or plants.

4. Conclusions

Mature biofilms cover crypts of historical and architectural interest at La Plata Cemetery (Argentina). These biofilms have caused the biodeterioration of monuments of different periods, styles, and materials.

Lichens (*Caloplaca austrocitrina*, *Lecanora albescens*, *Xanthoparmelia farinosa*, *Xanthoria fallax*), fungi (*Aspergillus* sp., *Fusarium* sp., *Penicillium* sp., *Candida* sp., *Rhodotorula* sp.), and bacteria (*Bacillus* sp., *Pseudomonas* sp.) were identified based on morphological and biochemical characteristics and in the case of bacteria by rDNA sequencing as well. These organisms are not a complete list of those present on the crypts. Phototrophs (algae and cyanobacteria) were not sought, but there is no doubt that they are present as primary colonizers of building stones. The use of PCR-based molecular tools on fresh biofilms, rather than culture-dependent techniques, would have allowed many more microorganisms to be detected.

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