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SMITHSONIAN CONTRIBUTIONS TO MUSEUM CONSERVATION • NUMBER 2



Biocolonization of Stone: Control and Preventive Methods

Proceedings from the MCI Workshop Series

Edited by
A. Elena Charola,
Christopher McNamara,
and Robert J. Koestler

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ABSTRACT

Charola, A. Elena, Christopher McNamara, and Robert J. Koestler, editors. *Biocolonization of Stone: Control and Preventative Methods, Proceedings from the MCI Workshop Series*. Smithsonian Contributions to Museum Conservation, number 2, 116 pages, 87 figures, 5 tables, 2011. — The Smithsonian Museum Conservation Institute Workshop on Biocolonization of Stone was the second workshop in a series and was dedicated to research on removal and control of biocolonization in stone objects. Twelve presentations were made, and the workshop ended with a roundtable discussion open to the 71 attendees. The goal was to provide a discussion forum for biologists, material scientists, and conservators interested in stone biodeterioration. Seven papers were presented, ranging from microbiological laboratory studies to combination of on-site testing and laboratory evaluation for World Heritage Sites such as Angkor Wat, to a literature overview. Five case studies were also presented, covering control of biodeterioration at Veterans Affairs cemeteries, experience gathered from the installation of zinc strips at the Stanford Mausoleum in San Francisco, the red staining found on the marble of the Memorial Amphitheater at Arlington National Cemetery, problems posed by deer stones in Mongolia, and the site test installed at San Ignacio Miní Jesuit mission in Misiones, Argentina. The roundtable and discussions drew attention to the importance of exploring new methods to prevent microbial colonization of stone. Finally, in a closed session, suggestions were offered for developing criteria to evaluate microbial growth and determine when treatment is necessary. It was recommended that a database be prepared on stone biocolonization and its control.

Cover images, from left to right: Details of Figure 5b by Warscheid and Leisen and Figure 1b by Delgado et al.; Figure 8 by DePriest and Beaubein.

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Contents

Preface

This volume brings together the papers that were presented at the Smithsonian Museum Conservation Institute (MCI) Workshop "Biocolonization of Stone: Control and Preventive Methods" that was held at the MCI on 20–22 April 2009. Seven papers and five case studies were presented. The papers dealt with such diverse topics as microbiological laboratory studies; combination of on-site testing and laboratory evaluation for World Heritage Sites, such as Angkor Wat; and a literature overview. The five case studies presented practical experiences from work with a diverse range of subjects: Veterans Affairs cemeteries, the Stanford Mausoleum in San Francisco, the Memorial Amphitheater at Arlington National Cemetery, the deer stones in Mongolia, and the San Ignacio Miní Jesuit mission in Misiones, Argentina.

An objective of the workshop was to obtain answers to the following three questions:

1. What should be the criteria for determining when microbial colonization and growth on stone heritage materials is problematic?
2. Are there new methods with minimum environmental impact that will prevent microbial deterioration and colonization and control recolonization of stone after a conservation intervention?
3. What future research directions can help achieve these goals?

The answers to these questions were discussed during the meeting and are summarized in the conclusions included in this volume.

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Microbiological Studies on Stone Deterioration and Development of Conservation Measures at Angkor Wat

Thomas Warscheid and Hans Leisen

ABSTRACT. Microbiological studies at Angkor Wat and surrounding temples in the Angkor site have shown that the natural microflora on the stones is composed of a complex and stable microbial community of algae, bacteria, fungi, and lichens. The microbial biofilms are mainly located in the uppermost layers of rocks, but certain microbes can penetrate deeper into the stone profile. The metabolic activity of the microflora, i.e., respiration and photosynthesis, is at a very high level, especially during the rainy season, and results in potentially biocorrosive and bio-oxidative activities. Nevertheless, in mature biofilms, such as those resulting from lichen colonization, the biodeteriorating activities reach a balanced state. This equilibrium should not be interfered with unless a thoroughly considered conservation program can be developed. This is fundamental since the microbial biofilm regulates the moisture and thermal absorption of the stones at the Angkor site. Cleaning and biocidal treatments should be applied only where needed for stone consolidation treatments or in order to achieve better visibility and readability of details in the historic structure. Organic- and chloride-containing biocides should be avoided because of their toxicity, short-term effectiveness, and possible nutritive value for microflora. Any conservation activity needs to be complemented by constructive water management. The long-term effect of conservation treatment has to be monitored during ongoing maintenance of the monuments and temples at the Angkor site.

INTRODUCTION: BIODETERIORATION PROCESSES

The importance of microbial impacts in the alteration and deterioration of porous inorganic materials used in cultural artifacts has been widely acknowledged in the course of many recent investigations (May et al., 1993; Warscheid and Braams, 2000). In the past, biodeterioration problems on cultural artifacts were not thoroughly analyzed before being subjected to biocidal treatments. However, a deeper interdisciplinary understanding of the environmental factors and material properties regulating the biogenic damage will permit a more-practical control strategy. The contamination, growth, and metabolic activity of individual or complex microorganism communities, such as algae, bacteria, cyanobacteria, fungi, and lichens, will influence the complex interaction between the various types of materials present leading to their physical as well as chemical damage (Koestler et al., 1994).

Biofouling can be defined as the presence of colloidal microbial biofilms on or inside materials. In the course of its development by the microflora, it leads to

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an unaesthetic discoloration by biogenic pigments, such as green chlorophyll, brownish melanin, or red carotenoids, and to the alteration of physicochemical characteristics of the materials with regard to their mechanical properties, superficial absorbency/hydrophobicity, diffusivity, and thermohygric behavior (Warscheid, 1996). Subsequently, the microbial consortia may cause a biocorrosive attack leading to the alteration of the structure and stability of materials by (1) the phototrophic enrichment of organic biomass, (2) the selective cellular enrichment and redox processes of cations and anions, such as iron, manganese, and nitrates, (3) the excretion of immediate corrosive metabolic products, such as organic and inorganic acids, and (4) the enzymatic mineralization of the respective organic materials (Warscheid and Krumbein, 1996).

It is important that during the survey and documentation process the environmental conditions that are favorable for allowing the development of microbial infection, contamination, and biodeterioration processes are recorded. This record allows the basic parameters for effective countermeasure strategies to be established. Microbial contamination on and in materials is basically determined by the availability of water (provided by rainwater, rising damp, and condensation) and depends on the sorption isotherms of the material. Two parameters are critical: water activity, a_w, the ratio of the vapor pressure of the air in equilibrium with a substance or solution to the vapor pressure at the same temperature of pure water, and time of wetness (TOW). Fungal growth is enabled at $a_w > 0.6$ and TOW > 0.5, e.g., more than 12 h during the day, and optimal conditions for fungal growth are $a_w \sim 0.75$ (Adan, 1994). Other microorganisms, such as algae or bacteria, usually need higher moisture levels ($a_w > 0.9$), but when a biofilm is present, the microorganisms may survive under less-favorable moisture conditions because of the ability of the biofilm to retain moisture (Flemming and Schaule, 1994).

Overall material characteristics, such as surface roughness, absorbency/hydrophobicity, porosity, and inner surface, determine the adhesion, colonization, and spreading of the microorganisms on and within the material (Warscheid et al., 1993). In addition, the chemical composition of the material may support the microbial succession by providing inorganic and organic nutrients. Further decomposable nutrient sources may be offered by exposure to light, leading to the enrichment of the photosynthetic biomass, as well as by the deposition of natural and anthropogenic aerosols, such as ammonia, nitrate, or combustion of biogenic hydrocarbons (Warscheid et al., 1991, 1993; Saiz-Jimenez, 1995; Mitchell and Ji-Dong, 2000). When evaluating the nutrition conditions for particular microbial consortia, it is important to consider that microorganisms settling on material surfaces are able to survive or even grow under oligotrophic conditions, i.e., with low concentrations of nutrients (May et al., 1993). The contamination process will even be extended when buffering capacities for biogenic metabolic compounds with acidic properties are in the material since the optimum pH for most of the microorganisms studied on cultural artifacts falls around the neutral point.

The optimal temperature for most of the microorganisms involved in the biodeterioration of cultural artifacts ranges between 16°C and 35°C. The rate of oxygen supply will not exclude microbial activity, but it will determine the type of the metabolic pathway, whether oxidative or fermentative. Finally, the possible pathways of contamination, e.g., airborne transmission, have to be considered as potential causes of biodeterioration processes on historical objects.

Control of biodeterioration processes on materials can be achieved basically by measures limiting the aforementioned growth conditions for microflora. Therefore, "good housekeeping" and climate control are the basic means of reducing the biocolonization problem. Furthermore, the application of appropriate, especially microbial-resistant, protective products is preferable to the application of ecotoxic, questionable, and unhealthy biocides (Warscheid, 1999, 2003).

THE ANGKOR SITE: THE GERMAN APSARA CONSERVATION PROJECT

The temple complex of Angkor, built between the ninth and fourteenth centuries, is located near the town of Siem Reap, close to the Tonle Sap Lake in the center of Cambodia. The region lies in a tropical climate with alternating dry and rainy seasons. The temple complex constitutes the largest religious monument in the world, with more than 100 temples over an area of some 400 km^2. The largest temple of the Angkor complex is Angkor Wat (Figure 1). The temples are decorated with bas-reliefs depicting mythological scenes and Apsaras, celestial nymphs; these bas-reliefs are badly deteriorating through flaking and scaling of the sandstone. Figure 2 shows a detail of one of the reliefs and the losses that have occurred over time.

In order to prevent total loss of this important heritage, it was necessary to develop an effective conservation strategy. This in turn required analysis of the causes for stone deterioration and an evaluation of the microbial impact within this process. In 1995, studies for the

FIGURE 1. View of the Angkor Wat temple (a) around 2004 and (b) in 2009. The color variation is mostly the result of different lighting and of the damp stone in the later photograph. Note that incipient biological recolonization is becoming evident.

FIGURE 2. Detail of one of the Apsara reliefs showing increased loss over time, induced by scaling and flaking of the sandstone: (a) 1959, (b) 1995, and (c) 1996.

conservation of the Apsara reliefs began at Angkor Wat. This initiative, the German Apsara Conservation Project (GACP), was undertaken by the Restoration and Conservation Department of the Cologne University of Applied Sciences (Leisen et al., 2000, 2008).

PRELIMINARY STUDIES

On the basis of the experiences and results of former and recent microbiological studies by French and Japanese scientists (Pochon et al., 1960; Hyvert, 1968; Fusey, 1991; Japanese Government Team for Safeguarding Angkor, 1995, 1996a, 1996b, 1998, 1999, 2000, 2002a, 2002b, 2003a, 2003b) the most typical sites for biodeterioration processes at Angkor Wat were selected for detailed and long-term microbiological studies. Additional microbiological investigations were performed at Preah Ko, Preah Khan, Bayon, and Banteay Srei in order to obtain a comprehensive view of the biodeterioration processes at the Angkor Site. The microbiological studies during 1997 and 2004 included the assessment of both quantity and quality of microbial infestation, i.e., algae, lichens, fungi, bacteria, and actinomycetes, within the stone profile; the monitoring of the microbial metabolic activity over time and climatic conditions; the analysis of the possible impact of microorganisms in the deterioration of stones; the evaluation of previous conservation treatments; and the development and testing of conservation and biocidal treatments in regard to possible biodeterioration control strategies.

FIELD STUDIES

Site Studies: Locations and Tests

The microbiological investigations were carried out at different sites in the Angkor Wat temple complex over a five-year period (1997–2001). Forty sites in seven different areas were established and monitored regularly for microflora metabolic activity. These ranged from areas that had practically no biocolonization to those with algae and lichens and others with scaling of enriched iron oxide crusts to areas that had been treated with an acrylic resin (Figure 3).

In addition, eight stone samples were analyzed in detail for composition and quantity of microorganisms. Also, different biocide test treatment areas were established in the third enclosure of the temple: two with a northern exposure on the North Library, in 1997 and 1998 (biocide test field [BT] I); two with a southern exposure in the both the North and the South Libraries; one additional northern exposure on the North Library in 2001 (BT II); and the roofs and facades of the central East Gopura, in 2004 and 2005 (BT III).

The application of biocides was performed by spraying (100 mL/m^2), once with and once without pretreatment of medicinal alcohol (i.e., 70% ethanol), without prior brushing or any other mechanical cleaning so as to avoid destroying the original stone surface. The biocides applied included:

- Thymol (1% by volume in isopropanol),
- Aseptin A (active ingredient: quaternary ammonium compounds; 3% by volume in *n*-butanol),
- Barrycidal (active ingredients: quaternary ammonium compounds [ready-made solution]),
- Borax (5% w/v in water),
- algal wash, a specifically developed copper-complex formulation, later nicknamed Melange d'Angkor,
- Mergal S 90 (active ingredients: carbendazim and triazin; 1.5% volume/volume (v/v) in water with 0.1% weight/volume (w/v) ethylenediaminetetraacetic acid (EDTA), Troy Inc., Hanover, Germany),
- Mergal S 88 (active ingredient: carbamate; 2.5% v/v in water with 0.1% w/v EDTA, Troy Inc., Hanover, Germany),
- Zinc Omadine (active ingredient: pyrithione 2% in water, Arch Chemicals, Atlanta, Georgia),
- Algophase (active ingredient: tetrachloro-methylsulfonyl pyridine; 3% v/v in isopropyl alcohol + 20% acetone, PHASE, Florence, Italy), and
- Parmetol DF 12 (active ingredient: isothiazolinone; 3% v/v in water, Schülke & Mayr, Norderstedt, Germany).

Nondestructive methods, such as adenosine triphosphate (ATP) measurements, respiration measurements, and contact agar plates, were used to assess the microbial infestation at the mentioned test sites as well as on other areas in the Angkor temple complex that had been treated previously with various biocidal formulations. For example, ammonium formaldehyde (also called formamide or formamine) was applied by French scientists in the late 1960s at Bayon; ammonium zinc fluosilicate, tributyltin oxide (TBTO), and pentachlorophenol (PCP) were used by Indian restorers at Angkor Wat around 1990; copper sulfate solutions and sodium hypochlorite applications were made by Frank Preusser (Getty Conservation Institute) for the World Monuments Fund Project around 1995 at the "Preusser Hall" in Preah Khan; and Metatin

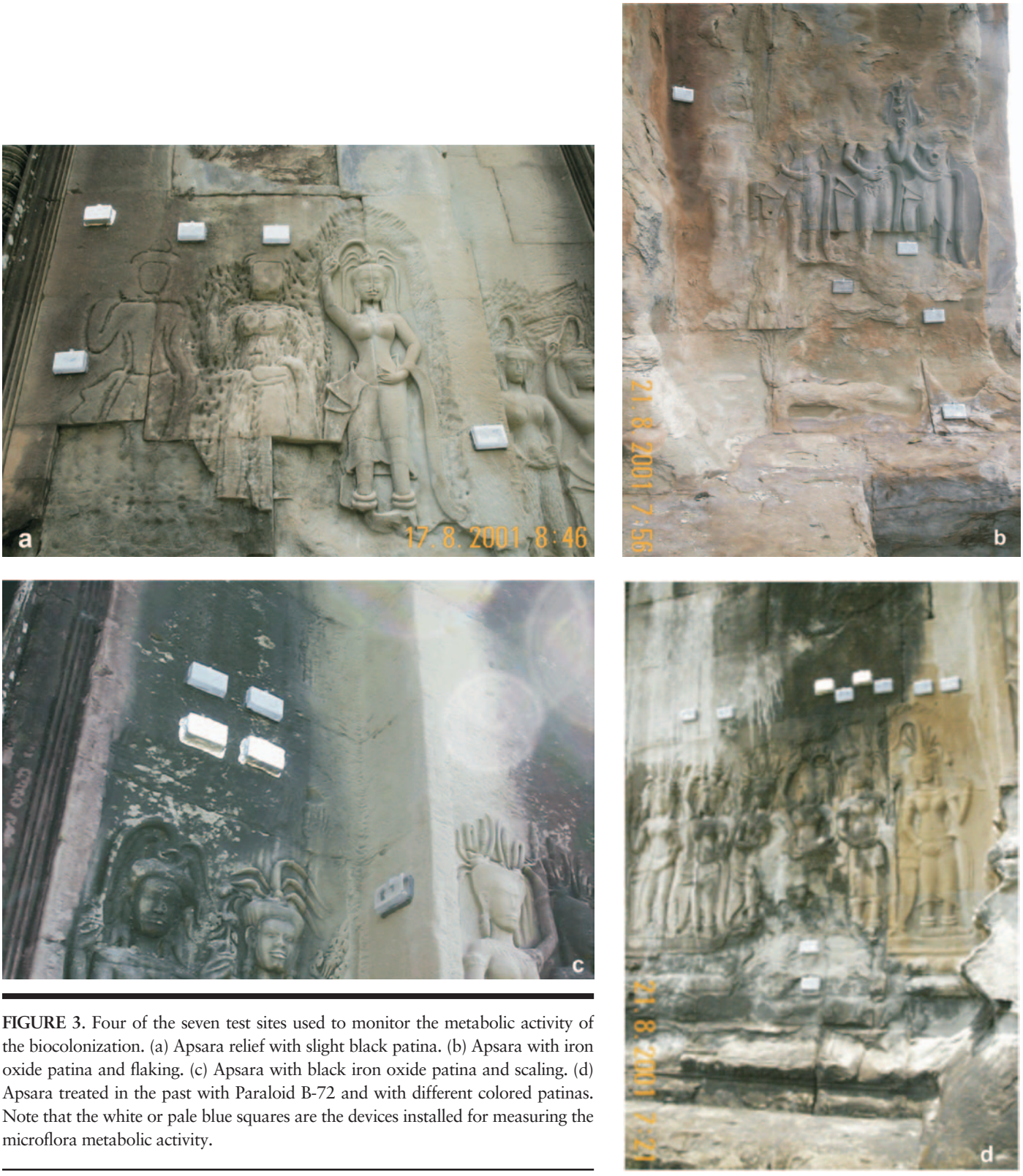

FIGURE 3. Four of the seven test sites used to monitor the metabolic activity of the biocolonization. (a) Apsara relief with slight black patina. (b) Apsara with iron oxide patina and flaking. (c) Apsara with black iron oxide patina and scaling. (d) Apsara treated in the past with Paraloid B-72 and with different colored patinas. Note that the white or pale blue squares are the devices installed for measuring the microflora metabolic activity.

58-10 was applied by Hungarian restorers to plaster at Preah Ko around 1996. Also, at Banteay Srei the biological infestation on the stone carvings was investigated in cooperation with the Swiss Banteay Srei Conservation Project in 2005.

Finally, laser cleaning to remove the black patina from the stone was carried out in cooperation with the Los Angeles Laser Conservation Research Center. Different wavelength laser beams were used, such as ultraviolet (355 nm, energy of 60 mJ), green (532 nm, energy of 100–190 mJ), and near infrared (1064 nm, energy of 300 mJ), with the following characteristics: beam diameter of 5 mm, pulse length of 3–6 ns, and frequency of 3 Hz.

Microbiological Studies

The stone-colonizing microflorae were examined by (1) microscopical analyses of the biofilm formation and distribution, (2) enumeration of the different microorganism groups, (3) quantification of their actual metabolic activity, (4) culturing and identification of typical microbial strains, and (5) physiological investigations on physicochemical deterioration mechanisms. The environmental parameters and characteristics of the sample sites were visually examined and documented by detailed photographs, registration of the relevant data concerning site exposure, and descriptions of the weathering pattern, its degree, and extent.

Microscopic Analysis

The microscopic analysis of the collected stone specimens was performed, after staining of the microbial biofilm with periodic acid Schiff reagent (PAS), with a stereomicroscope using magnifications up to 320× in combination with digital documentation equipment. The PAS staining method is suitable for staining carbohydrate-rich microbial biofilms since the stain reacts with organic substances such as glycogen, starch, cellulose, mucin, chitin, protein-carbohydrate complexes, and many glycolipids. The procedure was carried out in screw cap test tubes, and the amount of added reagent was approximately four or five times greater than the size of the sample under investigation. After each treatment step, the added solution was completely taken out with a Pasteur pipette.

On selected samples, the microscopic investigations were extended to fluorescence microscopy, after the addition of fluorescent stains such as acridine orange (AO) or fluorescein diacetate (FDA). Scanning electron microscopy (SEM) was also used, and selected stone samples were fixed in 4% glutaraldehyde for 24 h and then dehydrated in an ethyl alcohol series (25%, 50%, 75%, 90%, and 100%, each concentration applied thrice for l h). After critical-point treatment the specimens were gold coated.

Determination of Metabolic Activity

In order to quantify the metabolic activity of the microflora on the stone in situ, the respiration rate of the microflora was analyzed using respiration boxes, following the method of Isermeyer that measures the release of carbon dioxide from a particular stone area. The respiration rate box method is a nondestructive technique for the rapid and semiquantitative estimation of potentially damaging microbial activity on materials of historical buildings and monuments (Becker et al., 1994).

Metabolic activity was also assessed using a sterile cotton swab to nondestructively collect adherent particles from the material surface. These particles were then analyzed for the concentration of ATP using a luminometer.

Culture Enrichment of Microorganisms

After a careful selection of the sites that were most characteristic of typical weathering patterns at Angkor Wat, small cubes of approximately 20 × 20 × 4 mm were cut from the stone surface using a diamond drill. The specimens were removed from the stone using a chisel and were transferred into sterile petri dishes and transported to the laboratory in Germany.

In the laboratory, the collected specimens were powdered in a ceramic mortar. Then, 1 g of the powder was transferred into 10 mL of a 0.001% Tween 80 solution and shaken for 1 h. The resulting solutions were plated out on the selected enrichment media, applying 37 µL on the plate using a spiral plater device in order to quantify the concentration of microorganisms in the sample material under study. The inoculated enrichment media were incubated at 22°C for 14 days. Then the cultivated sample was counted, photographed, and selected for further microbiological studies (i.e., pure culturing, simulation tests). The counts were computed as colony forming units (CFU) per gram of stone (Becker et al., 1994). Subsequently, nondestructive enrichments were performed using Rodac impression agar plates, simply attached to the stone surface at the site and incubated in the laboratory under the same conditions as mentioned above.

The following solid nutrient media were used for the enrichment and quantification of the microflora present on the stone specimens:

- Czapek-Dox medium for copiotrophic fungi (Merck-Diagnostica No. 5460),
- Bunt-Rovira medium for oligotrophic bacteria (Bunt and Rovira, 1955),
- Glycerine-nitrate-casein medium for actinomycetes (Küster and Williams, 1964),
- VM-T (synthetic full medium, Merck-Diagnostica No. 5450 with tyrosine) for melanin-producing microorganisms,
- BF-Mn (Bromfield agar with $MnSO_4$) for manganese-oxidizing microorganisms (Bromfield, 1955), and
- BF-Fe (Bromfield agar with $FeSO_4$) for iron-oxidizing microorganisms (Bromfield, 1955).

The enrichment of nitrifying, sulfur-oxidizing, and photosynthetic microorganisms was performed in liquid solutions and analyzed quantitatively by the most probable number (MPN) method using the following enrichment media:

- BNM or BNB media for nitrifying bacteria (Krümmel, 1978; Bock and Engel, 1966),
- S5 and S6 medium after Hutchinson for sulfur-oxidizing bacteria (Hutchinson et al., 1965), and
- BG11 for photosynthetic algae (Ripka et al., 1979).

The counts of nitrifying bacteria, sulfur-oxidizing bacteria, and algae were examined as prescribed by the MPN method. Nitrifying and sulfur-oxidizing bacteria were incubated for six weeks in the dark at 28°C, and the counts were computed as MPN/g stone. The algae and cyanobacteria were incubated for six weeks in the light at 16°C, and the counts were also computed as MPN/g stone.

Biochemical Investigations

The biochemical investigations on the stone specimens taken from the selected sites were based on determination of the protein content as the parameter for the concentration of microbial biomass, dehydrogenase activity with tritetrazoliumchloride (TTC) as the biochemical indicator for the extent of actual microbial metabolic activity, presence of chlorophyll as the biochemical marker for photosynthetic microorganisms, and assessment of microbial acid excretion as the marker for biocorrosion processes. To measure protein content, the analytical protocol of Lowry was used (Lowry et al., 1951). The dehydrogenase activity provides a rapid method to confirm and evaluate microbial activity on decaying stones (Warscheid et al., 1990). To test acid production, the isolated microorganisms (i.e., fungi, bacteria, and algae) were cultivated in a glucose-containing mineral solution, referred to as ANG (Warscheid et al, 1990), for 14 days at 28°C. After this, the pH of the culture filtrate was measured with a pH meter (Warscheid, 1990). Selected stone samples, characterized by algal and lichen infestation, were crushed, and 200 mg of the crushed sample were introduced into 2 mL of methanol and centrifuged for 10 minutes; the supernatant was then removed and analyzed in a UV-visible spectrophotometer for its absorption profile.

Substrate Characterization

Four in situ methods were used to characterize the substrate: (1) capillary water uptake with the RILEM or Karsten tube, (2) rebound hardness with a recently developed impact hammer, (3) surface temperature measurements, and (4) drilling resistance. The RILEM or Karsten tube allows determination of the time-dependent water uptake of the stone at the monument. The plot of the water uptake (kg/m^2) as a function of the square root of time is linear, except for the initial water uptake period. The water uptake coefficient w ($kg/m^2\ h^{0.5}$) can be calculated by the slope of the initial straight line. The measurement takes, in general, about 1 h but has to be stopped if wet stains appear on the sample surface, as this indicates a faulty seal of the tube to the substrate. If the water penetration coefficient B ($cm/s^{0.5}$) of the substrate is known from laboratory tests, the depth of penetration of water as a function of time can be calculated (Wendler and Snethlage, 1988).

Surface temperature measurements were carried out on different sunlit and shaded stone surfaces with various microbial infestation characteristics. Fixed measuring points were monitored by using either a surface thermal sensor or a thermohygrometer.

The rebound hardness, measured with an impact hammer, correlates to the compressive strength of a material (Bögner, 1999; Grassegger and Leisen, 2005). A calibration diagram is required to translate the reading obtained by the impact hammer into a compressive strength value.

The drill resistance equipment provides a plot of the depth of drilling as a function of time. From this plot, the drilling resistance is obtained from the slope dt/dx (s/mm) at each specific depth (Leonard and Kiessl, 1990). Calibrating in the laboratory with materials of known strength, a high-resolution (±1 mm) strength profile can be obtained. The method is very useful in detecting subsurface flaking or detachment. In addition, the drill powder obtained can be used for chemical analysis of soluble salts.

RESULTS

The evaluation of the macroflora showed that the stones have a significant cover of lichens, such as the *Parmelia*, *Caloplaca*, *Pyxine*, *Dirinaria*, and *Lepraria* species. Only in very humid areas can green algae, such as *Pleurococcus* sp., be found. There are also some distinct red and black areas that could be related to green algae, such as the *Trentepholia* sp., which appear red-brown under normal conditions, and cyanobacteria, such as *Gloeocapsa* and *Scytonema* sp., which can appear black (Figure 4).

The natural microflora found on stone and stuccos, although very complex, is well balanced, so that overgrowth and domination from one specific group of microorganisms is avoided. The trees that grow in the Angkor enclosures shelter its monuments from rapid moisture and temperature changes, thus preserving the original stone surface and the readability of their historic testimony, despite the detrimental mechanical effects of the roots to the structural integrity of the temples.

In the early 1990s, a biocide-based cleaning was carried out under an Indian conservation project that removed the surface biocolonization. Subsequent years showed an intensive blackening of the treated stone surfaces due to uncontrolled overgrowth of black-colored cyanobacteria. The resulting thermal effect of the blackened surfaces, especially under tropical conditions, added to the normal thermohygric stress suffered by an already weakened and sensitive clay-bearing stone, leading to increased delamination, scaling, and flaking of the surface. Thus, the removal of the biopatina of lichens can be considered a major threat to the endangered stone material (Figure 5).

The natural microbial biofilm is located mainly on the uppermost layers of the stone surfaces (Figure 6), but metabolically active cells can also penetrate into deeper parts of the rocks (Figure 7). SEM analyses support the finding that photosynthetic microorganisms, i.e., lichens, algae, and cyanobacteria, are found mainly on the surface of the rock, whereas chemoorganotrophic fungi and bacteria can also be found in the interior (Figure 8).

Analysis and measurement of the enriched microorganisms from selected stone samples from the Angkor Wat temple indicate that they consist mainly of phototrophic algae and cyanobacteria. This was proven by the increased photosynthetic activity when exposed to light through ATP measurements and by the presence of chlorophyll in a methanol extract obtained from black surface samples as determined by UV-visible spectrophotometry. Culture enrichment methods showed that both actinomycetes and chemoorganotrophic bacteria dominate compared to fungi, which have been found only in small quantities, although their presence clearly correlates with the occurrence of black patina, as a result of the melanin-producing strains (Figure 9).

The isolated fungi mainly consisted of the so-called "black" fungi, such as *Alternaria alternata, Cladosporium cladosporoides*, *Cladosporium sphaerospermum,* and *Ulocladium chartarum*. They also included "fungi imperfecti," such as *Aspergillus niger*; ubiquitous fungi, such as *Curvularia geniculata* and *Trichoderma viride*; "dirt fungi," such as *Fusarium oxysporum*; some biocorrosive penicillia, e.g., *Penicillium glabrum* and *P. griseofulvum*; and mycotoxin-producing fungal strains, such as *Paecilomyces variotii*, *Stachbotrys chartarum,* and *Acremonium strictum*.

The presence of nitrifying bacteria is scarce, and so far, the presence of sulfur-oxidizing bacteria cannot be established, although their presence is always referred to in the literature dealing with microbiological studies at the Angkor sites (Pochon et al., 1960). To complement this information, it should be mentioned that sulfates have often been found in relation to the habitats of bats around the towers and pillars of the galleries.

The protein content of the stone samples investigated was quite high (up to 4.48 mg/g), as was the metabolic activity (up to 0.391 µg CO_2/g). The latter was highest during the rainy season, but it could easily be increased by the addition of moisture during the dry season, as proven by higher respiration and photosynthesis rates.

The isolated microbial strains showed a high potential for biocorrosivity due to the significant excretion of organic acids that could lead to stone surface sanding. Finally, the presence of iron-oxidizing microorganisms could be established, particularly at locations that show a significant reddish-orange coloration resulting from the migration and oxidation of iron minerals in the stone (see Figure 3b).

From the studies carried out it is evident that the microflora on the stones of Angkor Wat and other temples has biocorrosive and bio-oxidative properties, affecting the mineral structures of stones. However, results from the comparative analysis of the water transport data suggest that the natural biofilm of a stable, long-term developed macroflora, i.e., lichens, has an important moisture-controlling function for the environmentally stressed stones. The measurement of the capillary water uptake at different places on the temple (Figure 10) shows that lichens protect the stone from rapid water uptake, whereas certain algal and blackened cyanobacterial biofilms significantly increase it. This phenomenon is especially disadvantageous during rainy seasons, when the heated stone

FIGURE 4. Colored biofilms of different photosynthetic organisms, e.g., lichens, algae, and cyanobacteria, found on the stones at the Angkor monuments, depending on exposure and microclimate.

is rapidly cooled by rain, thus increasing the thermohygric expansion-contraction processes that lead to delamination, scale formation, and flaking (see Figure 2). It should be noted that the "dead" biofilm remaining on the stone after a biocidal treatment also slows down water uptake, at least initially, though presumably the stone's surface will shift to that of an unpatinated stone over time. The surface temperature differences measured on stones covered with the dark black patina as compared to those with the grayish-colored lichen infestations, or even the uninfested stones, can be as high as 15°C.

Rebound hardness and drill resistance measurements taken on mature microbial biofilms, i.e., lichens, have not shown any evidence that they significantly affect the mechanical properties of the stones. Consequently, it can be stated that the mature microbial biofilms (i.e., lichen patina) regulate the humidity, thermal transmission, and water vapor diffusion, reducing thermohygric stresses to the stone at Angkor Wat. Any ongoing conservation activities

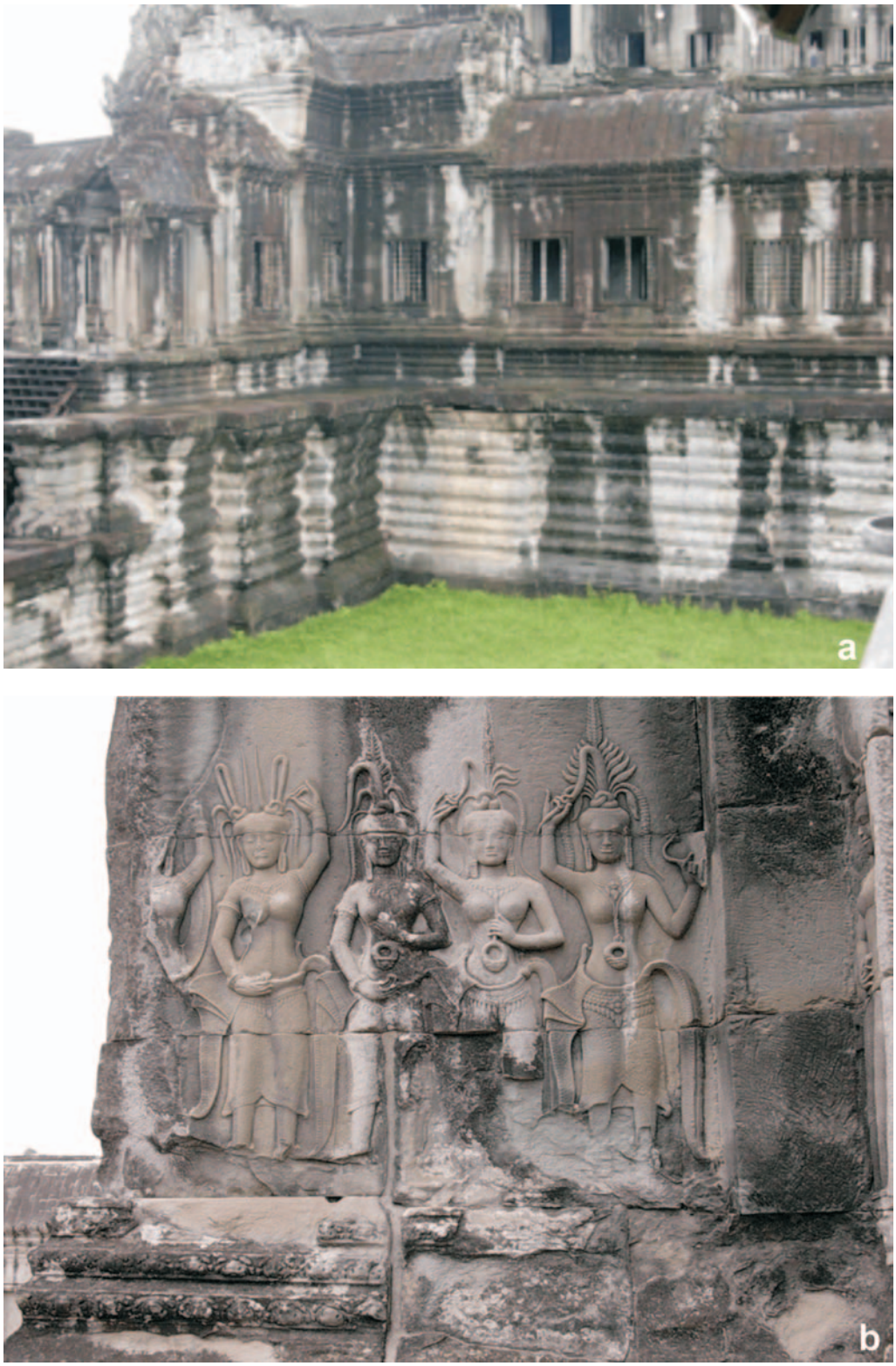

FIGURE 5. (a) The removal of the natural lichen biopatina at Angkor Wat resulted in an intense colonization by blackening microorganisms. (b) The increased thermohygric stresses to the clay-bearing sandstone resulted in severe flaking and delamination on the exposed carved surfaces.

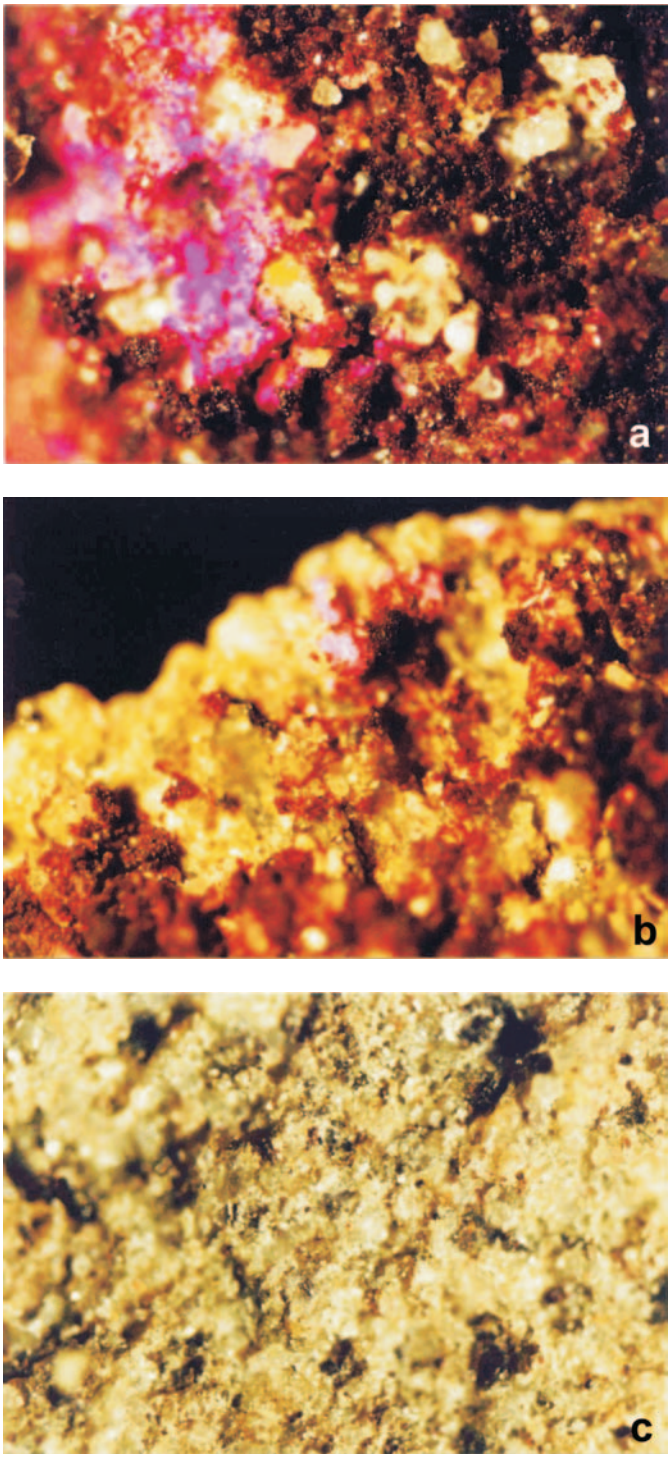

FIGURE 6. Microbial biofilm on a sample of Angkor Wat stone surface visualized by PAS staining. (a) Surface is completely covered with a microbial film. (b) The visible biofilm is reduced in the subsurface. (c) Almost no biological biofilm is visible in the interior of the stone.

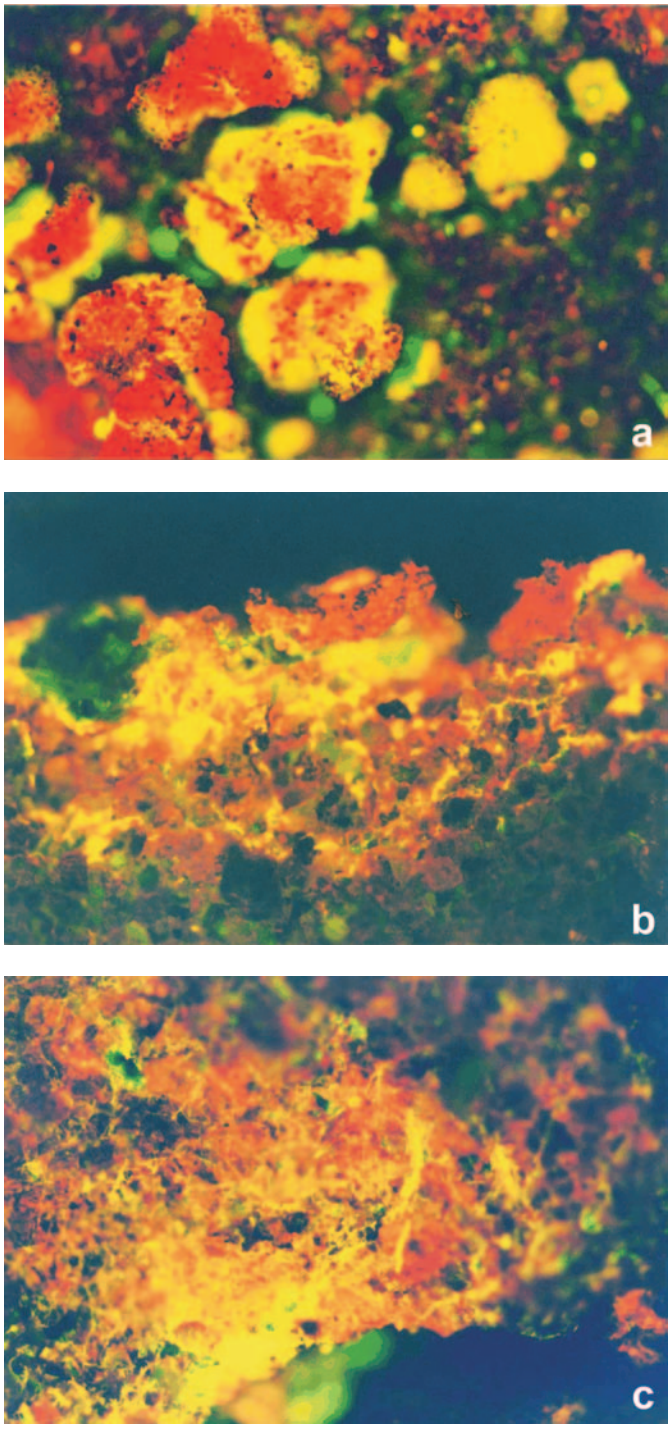

FIGURE 7. Microbial biofilm, mainly due to lichen infestation, on a rock flake at Angkor Wat visualized by fluorescent acridine orange staining. Fluorescent staining indicates metabolically active cells. (a) The surface shows round-shaped lichen thalli. (b) The biofilm penetrates only slightly into the sandstone. (c) A fungal infestation is present in the interior of the stone flake.

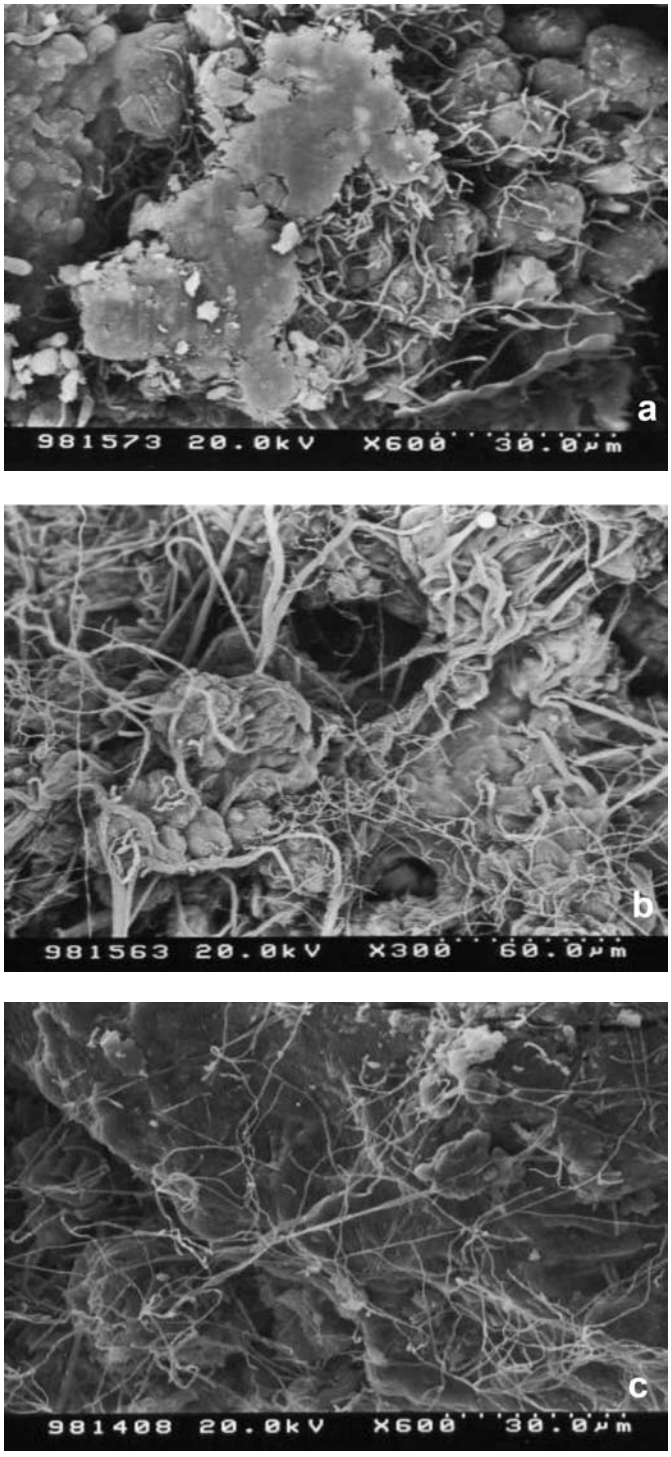

FIGURE 8. SEM examination of a microbial biofilm on a stone sample from Angkor Wat. (a) Lichen thallus grows on the surface of the stone. (b) At the subsurface, the concentration of photosynthetic microorganisms diminishes while fungal and bacterial cells prevail. (c) Fungal contamination dominates in the interior of the sample.

should take into account the preservation of these acceptable microbial infestations while removing those microbial biofilms that enhance deterioration or where the visibility and readability of historic artifacts is essential.

The results of the biocide test sites evaluated during the seven-year period (1997–2004) have shown that the sequenced application of disinfecting and biocidal solutions, provide an effective strategy for the long-term control of local microbial infestation. As disinfectants, medical ethanol was used to destabilize the microbial biofilms of either lichens, algae, or fungi, and this was followed by the application of effective but fairly ecologically sound biocidal compounds, such as algal wash (based on a low concentration copper complex solution), or the Parmetol DF 12 (based on an aqueous solution of a mixture of various isothiazolinones, biodegradable by bacteria).

Also investigated was the effectiveness of previous biocide treatments applied at different sites in the temple complex. The evaluation revealed that mostly weak effects were obtained by the application of "soft" biocides, whereas the use of highly toxic and environmentally critical biocides gave only moderate and temporary relief from microbial infestation and often resulted in recolonization by blackening cyanobacteria, as seen at Angkor Wat (see Figure 5).

The long-term growth-controlling effect of the algal wash, the copper complex formulation, could be observed even after seven years of exposure at the first biocide test field (BT I) treated in 1997. Figures 11 and 12 show two of the sections of the second test site (BT II) after four years of the application of algal wash in 2001. It should be noted that no mechanical cleaning had been performed at or after the application of the biocide.

Laser cleaning also gave positive results on test cleaning of stone fragments and shows potential for cleaning stone surfaces at carefully selected areas, such as very friable stone that needs to be consolidated. The portable laser with 532 nm (green) wavelength and an energy of 190 mJ gave the most promising cleaning. Nonetheless, the thermal impact on the stone needs to be further analyzed in order to avoid microstructural damage of the mineral lattice.

Finally, it has to be considered that conservation treatments, such as cleaning, consolidation, or hydrophobization, need to be evaluated with regard to their possible contribution toward the recurrence of microbial colonization. For example, the use of local well water for soaking bricks prior to their use resulted in an algal infestation of the finished wall constructed with them (Figure 13a), whereas the application of a water repellent at the temple of Ta Nei resulted in the selective reinfestation by *Trentepholia* sp., the red-colored green algae (Figure 13b).

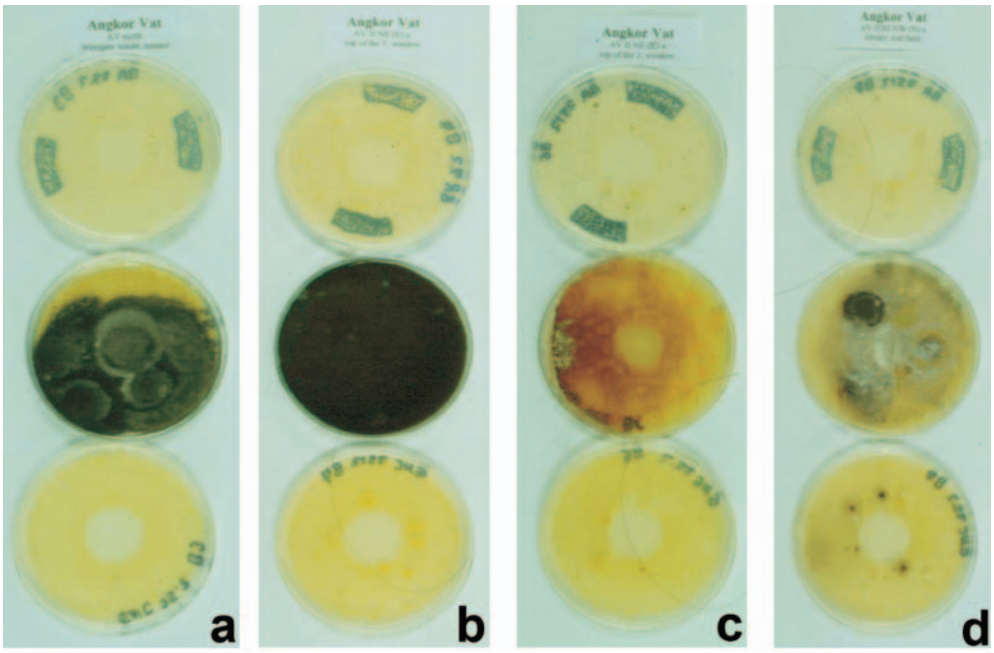

FIGURE 9. Cultural enrichment of bacteria, fungi, and actinomycetes, respectively, from top to bottom, on different stone samples characterized by (a) a black patina, (b) a slight black patina, (c) no patina, and (d) a lichen patina.

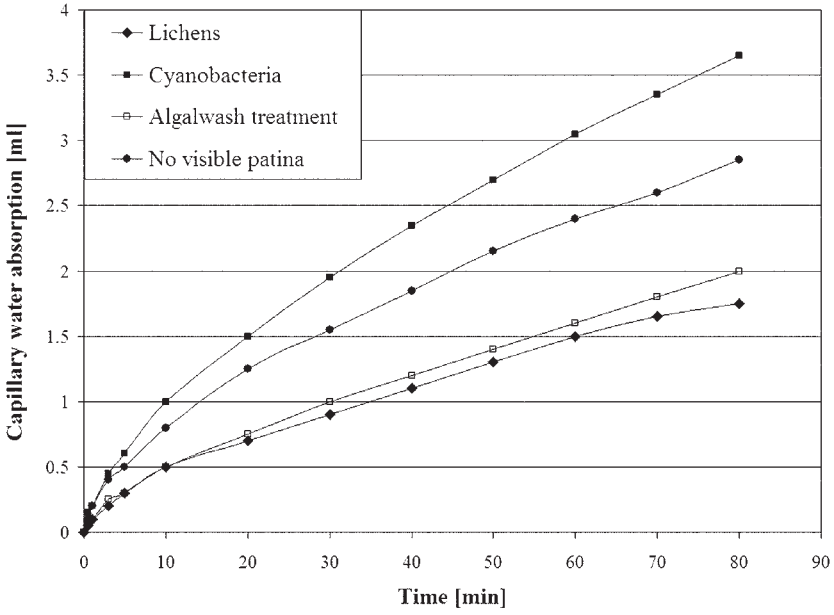

FIGURE 10. In situ capillary water absorption measurements at stone areas with and without visible biofilms and after three years of application of algal wash (copper-complex-based product) to a cyanobacterial biofilm. Note that the lichen biofilm shows the lowest water absorption and the applied treatment nearly lowers the absorption to this level.

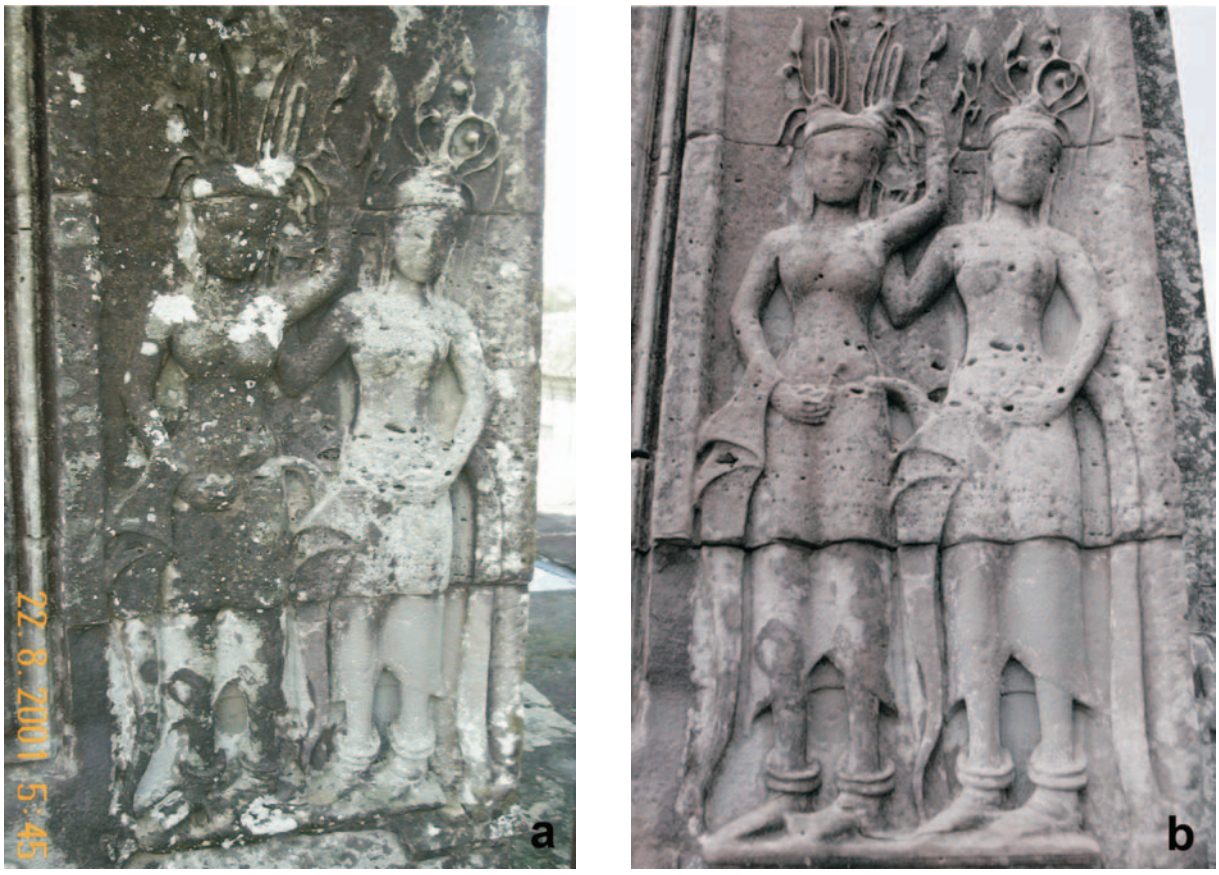

FIGURE 11. Comparison of an area on the northern library, northern exposure, (a) before treatment and (b) four years after the application of Parmetol DF 12.

FIGURE 12. Successful application of algal wash at the southern library, southern exposure, four years after treatment.

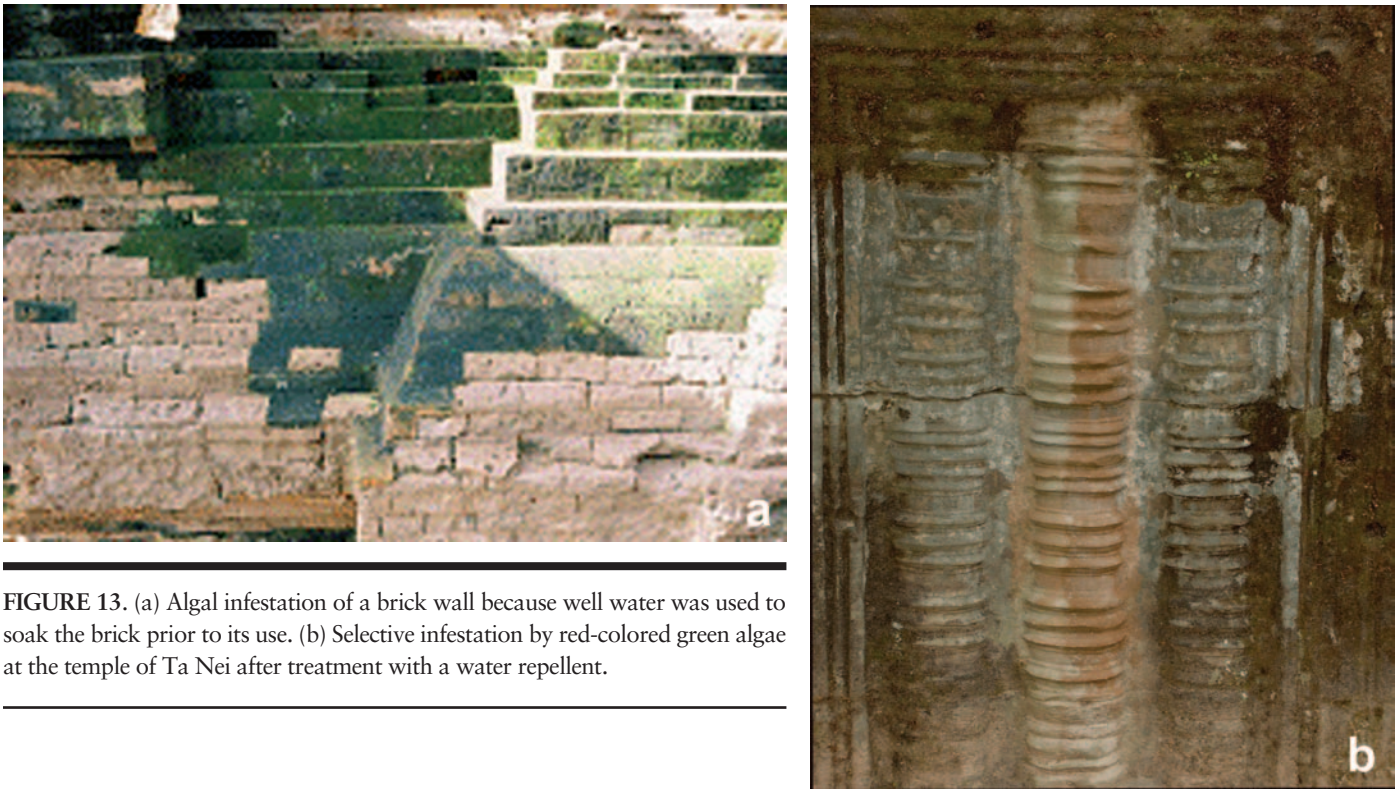

FIGURE 13. (a) Algal infestation of a brick wall because well water was used to soak the brick prior to its use. (b) Selective infestation by red-colored green algae at the temple of Ta Nei after treatment with a water repellent.

Within the GACP Project, applied products were regularly screened for contaminants and microbial resistance during the conservation work.

CONCLUSIONS

The studies carried out have shown that although the microflorae present on Angkor Wat and other temples have biocorrosive and bio-oxidative properties, there are significant differences between different biofilms. Stable, long-term developed algal biofilms possess an important moisture-controlling function for the stone substrate, whereas certain algal and black-colored cyanobacterial biofilms significantly increase moisture as well as surface temperature. This is a particularly negative feature for the clay-bearing sandstone of the monuments, given its thermohygric expansion-contraction properties, which lead to delamination, scale formation, and flaking.

Consequently, any conservation activities should take into account the preservation of these acceptable microbial infestations while removing those microbial biofilms that enhance deterioration. For this purpose, the application of effective, long-term biocides, tested on site, should be used. Furthermore, it is recommended that any other conservation treatment, be it cleaning, consolidation, or the application of a water repellent, needs to be evaluated with an eye to the potential of applied products for enhancing or changing the existing biocolonization.

Acknowledgments

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Microbial Community Diversity and the Complexity of Preserving Cultural Heritage

M. Carmen Portillo and Juan M. Gonzalez

ABSTRACT. In addition to physical and chemical factors, microorganisms also affect the conservation of stone monuments. The development of novel methods of detection has significantly advanced the study of microorganisms. Whereas classical microbiological techniques are based on culturing bacteria, the use of molecular methods based on nucleic acid technology permits the detection of numerous bacteria that previously remained unnoticed. Besides the detection of bacteria from DNA, using RNA for bacterial identification permits us to determine the bacteria showing active metabolism within the community. The metabolism of most bacteria is unknown, and the effect of bacteria on stone monuments can hardly be predicted. This drastic change in our understanding of bacterial communities and their effect on cultural heritage is leading to different perspectives in preventing, controlling, and monitoring biocolonization of stone monuments. The incredibly high bacterial diversity detected in monuments suggests a great complexity, thus complicating any attempt to prevent microbial colonization of cultural heritage. This study focuses on the bacterial diversity of caves and natural shelters with prehistoric paintings and the consequences of this diversity for the conservation of this cultural heritage in these specific environments.

INTRODUCTION

Stone materials, including natural structures, buildings, monuments, and other cultural heritage objects, are exposed to the action of physical, chemical, and biological factors. Among the latter, the role of microorganisms has been proposed to have a high deteriorating potential to stone because it is acting constantly over time. The small size of these living creatures (at the micrometer scale) makes their study difficult. Thus, different methodological strategies are being applied to determine which microorganisms are present and what they are doing. This research aims at elucidating the role and potential impact of microorganisms on stone biodeterioration.

Most of the pioneering studies on microorganisms were performed by growing these cells on culture media. This strategy allows the cells to multiply so that colonies of macroscopic size can be visualized. Also, culturing microorganisms permits the investigation of their physiological characteristics. When using these culturing methods, natural microbial communities appear to be composed of just a few microbial types. The microorganisms detected by culturing techniques are those that grow optimally under the conditions provided. However, recent

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studies are changing this simplistic perspective, and the introduction of advanced detection methods for microorganisms is drastically expanding our knowledge of the real scale of the problem.

By the end of twentieth century, the introduction of molecular methods based on the detection of specific DNA sequences contributed to the identification of a large number of novel microorganisms previously undetected. Presently, it is widely accepted that standard culturing methods in natural environments only permit the detection of about 1% of the total number of microorganisms present in the samples (Ward et al., 1990). Therefore, nearly the complete microbial community remained undetected. This scenario was drastically changed when molecular methods were introduced because they greatly contribute to our understanding of the actual composition of microbial communities and their potential as a deterioration factor.

In spite of being able to detect a large number of the microorganisms in nature, recent studies have demonstrated an even higher diversity of microorganisms in natural environments than were ever expected. In fact, microbial diversity appears to be so high that a true estimation is not possible with currently available methods (Curtis et al., 2002). Because of this huge diversity, how can we discriminate which microbes are important in the biogeochemical processes carried out by the community and what role is performed by specific microorganisms? Understanding which microorganisms are present and their role in a natural community is not a trivial subject.

In order to understand which microbes play important roles in biogeochemical transformations it is necessary to identify and study the physiology of those microorganisms actually carrying out significant metabolic activity within a microbial community (Mills et al., 2004; Portillo et al., 2008, 2009; Portillo and Gonzalez, 2009a). This has been achieved by detecting those microorganisms through their RNA because the amount of RNA per cell is proportional to the cell growth and activity (Molin and Givskov, 1999). RNA is highly unstable, so its handling greatly increases experimental complexity. Furthermore, even if we know which microbes are active within a community, this does not tell us the function of those microorganisms in a given environment.

Because the metabolic capabilities and physiology of most microorganisms detected in samples through molecular techniques are unknown, their influence on stone deterioration cannot be inferred. For this purpose, it is necessary to rely on culturing techniques that allow evaluation of the metabolic capabilities and physiology of microorganisms.

This study presents some examples of the microbial diversity, activity, and metabolism detected in two culturally important prehistoric paintings locations in Spain: one in a cave and the second in a natural stone shelter. From these examples, the consequences, problems, and future perspectives of microbial deterioration are discussed.

EXPERIMENTAL METHODS

Two locations were studied. One site was the Altamira Cave (Cantabria, northern Spain), containing unique polychromatic Paleolithic paintings, and the other was a natural shelter, Muriecho L (Aragón, northeastern Spain), with Neolithic paintings. Microbial colonies that had developed in these two environments were analyzed in order to detect the microorganisms involved in these communities. Molecular surveys of microorganisms were based on both DNA and RNA. Microbial detection through DNA was directed at identifying those microorganisms present in the studied areas, whereas the microorganisms showing significant metabolic activity in these communities were detected through RNA-based molecular methods.

DNA and RNA were extracted separately using the NucleoSpin Food (Macherey-Nagel, Düren, Germany) and RNAqueos-4PCR (Ambion, Inc., Austin, Texas, United States) extraction kits, respectively (Portillo et al., 2008). The complementary DNA to the extracted RNA was obtained by a reverse transcription reaction using the primer 518R (5′-ATT ACC GCG GCT GCT GG; Muyzer et al., 1993).

In order to obtain a visual image of the complexity of the microbial communities and to carry out comparisons among these communities, a molecular profiling technique was utilized. This allowed visualization of the major bacterial constituents of the communities. The protocol was optimized to obtain relative quantification of the contribution of major bacterial types to total DNA and RNA from the microbial community. The method used was the denaturing gradient gel electrophoresis (DGGE) as described by Gonzalez and Saiz-Jimenez (2004) with the modifications for quantitative analyses and the statistical procedure for profile comparison recently described by Portillo and Gonzalez (2008a).

For cloning purposes, amplification of the 16S rRNA genes was performed by PCR with primers 27bF (5′-AGA GTT TGA TYM TGG CTC AG; Zimmermann et al., 2005) and 907R (5′-CCC CGT CAA TTC ATT TGA GTT T; Gonzalez et al., 2003) and 27bF and 518R for DNA- and RNA-based analyses, respectively. The amplification products were used to identify the bacteria represented in the DNA and RNA extracted from the samples under study. In order to identify these bacteria, 16S rRNA gene libraries

were constructed using the TOPO TA cloning kit (Invitrogen, Carlsbad, California, United States). The screening procedure for selecting the clones in each library was previously described in detail by Gonzalez et al. (2003). Selected clones were sequenced and analyzed as previously described (Gonzalez et al., 2006; Portillo et al., 2008). This procedure represents a rapid and efficient method to detect specific sequences within complex gene libraries.

The obtained sequences were differentiated, and accumulation curves of the number of processed clones versus the number of newly found different operational taxonomic units (OTUs) or bacterial phyla were constructed. These curves were constructed as described by Hughes and Hellmann (2005) and were used to approach the grade of coverage of the detected sequences with respect to the total bacterial community. Thus, when the curve reaches a horizontal asymptote, most groups in the analyzed community have been detected.

MICROBIAL DIVERSITY IN ALTAMIRA CAVE

Altamira Cave is located in Santillana del Mar (Cantabria, Spain) near the northern coast of Spain. It contains some of the most famous paintings from the Paleolithic period. Most of these representations were painted in two colors, red (mostly hematite) and black (carbon), and were designed to take advantage of natural three-dimensional structural features of the rock (Lasheras, 2002). Three major types of bacterial colonies have been detected in the cave, and they have been named according to their coloration as white, yellow, and grey (Figure 1). At present, the effect of microorganisms on the substrate or rock forming the cavity is unknown, although the phenomenon of carbonate deposition and dissolution has been suggested (Sanchez-Moral et al., 1999) as a potential long-term effect caused by the development of these communities. Nevertheless, the aesthetic effect produced by their presence on cave walls represents per se a serious biodeteriorating phenomenon. The risk of a potential expansion of bacterial colonies is driving the investigation into understanding their composition and ecology, with the aim of being able to control or reduce their presence in the cave. The present study focuses on the biocolonization found on the substrate.

Using the dual strategy, DNA and RNA, for molecular detection of microorganisms offers the possibility of differentiating those metabolically active from those just present (Figure 2). Microorganisms showing no detectable metabolic activity represent a large fraction of the communities, which are waiting for more favorable conditions to appear. They can become active by changes in the environmental conditions or nutrient availability as well as by the elimination of a fraction of the community, which could allow the development of those previously inactive microorganisms. The use of biocides is an example that leads to

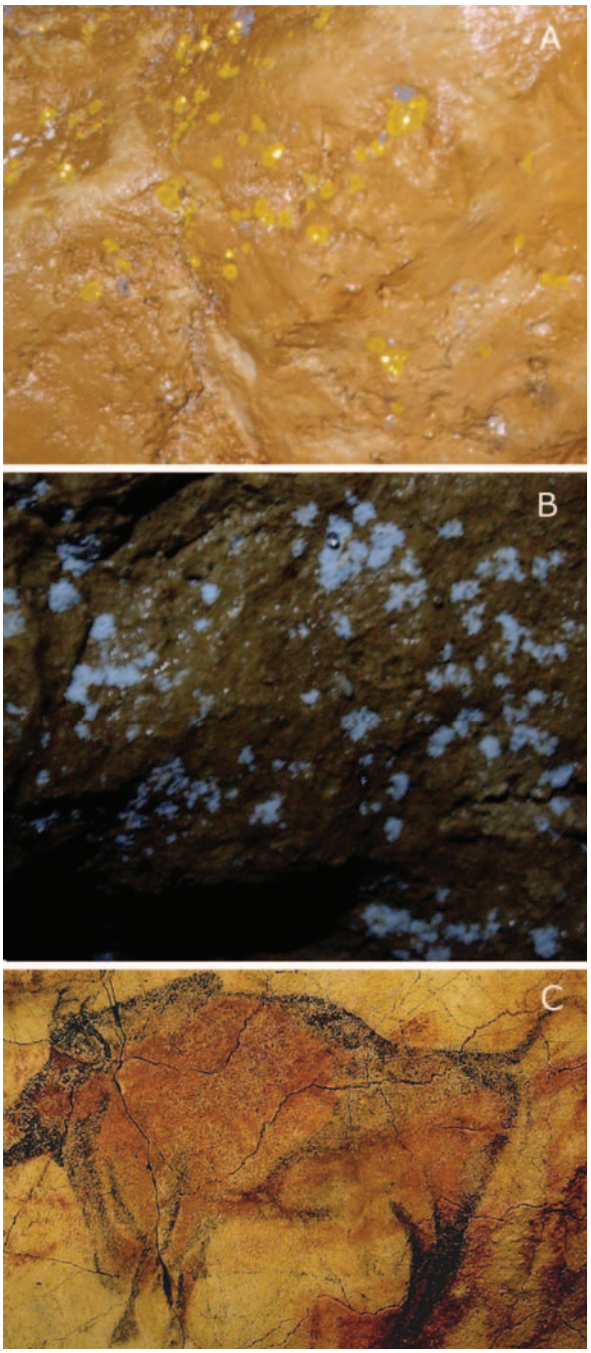

FIGURE 1. Photographs of the three different microbial colonies observed in Altamira Cave. (A) Yellow and grey colonies and (B) white colonies are shown. (C) One of the Altamira's polychromatic paintings representing a bison is also presented.

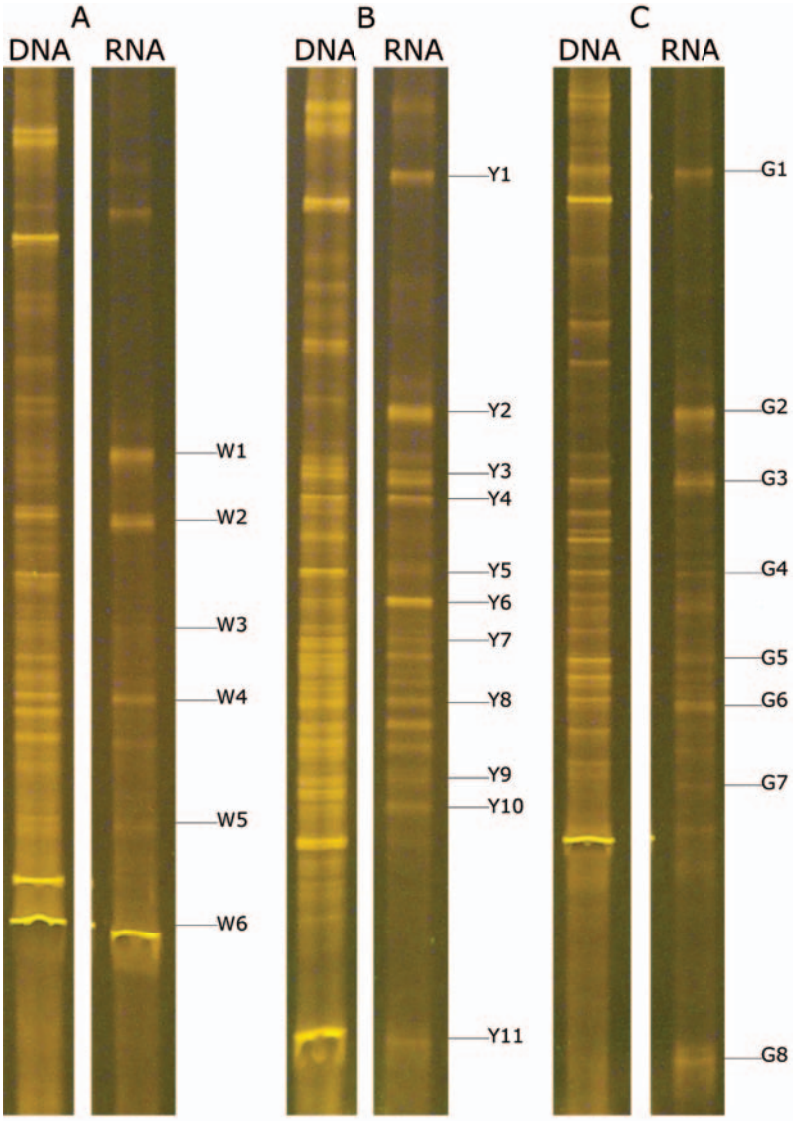

FIGURE 2. Microbial community fingerprints by DGGE for (A) white, (B) yellow, and (C) grey colonies sampled in Altamira Cave analyzed by molecular techniques based on DNA and RNA. The microorganisms corresponding to the major bands on RNA-based community profiles were identified. Identification codes correspond to those in Table 1.

a modification of microbial communities and their equilibria. The consequences of these changes in the communities are normally unpredictable. Generally, any change of the microenvironment or its conditions results in altering the long-term equilibrium in that ecosystem, and so it could imply the development of different microorganisms into a series of uncontrolled unstable associations until a new equilibrium is reached in the system. A typical example of this scenario is the excessive growth of specific types of microorganisms, which usually results in undesired effects for the conservation of the object under study.

White colonies in Altamira Cave are formed by a large number of different microorganisms as detected through DNA-based molecular surveys (Portillo et al., 2009). To identify the microorganisms that are responsible for the major proportion of metabolic activity within the microbial community in white colonies, the use of RNA-based molecular techniques has been proposed. For instance,

results showed that heterotrophic bacteria such as Alphaproteobacteria (e.g., *Sphingomonas* sp.), Gammaproteobacteria (e.g., *Pseudomonas* sp.), Acidobacteria, and Deltaproteobacteria are the major metabolically active bacterial groups in these white colonies (Table 1). In addition, many other microbial groups representing minor fractions of the total number of microorganisms present in these colonies were detected through RNA- and DNA-based techniques.

Another critical aspect was whether the white colonies located in different areas in the cave had identical or similar composition. Portillo and Gonzalez (2008a) proposed a statistical method for comparing molecular profiles and corroborated that the white colonies from different points of the cave were composed of the same microbial community, which had been able to spread and colonize specific locations along the cave (Portillo et al., 2009).

Yellow bacterial colonies have also been observed, although mainly at the entrance to Altamira Cave. The major bacterial groups constituting the metabolically active fraction of the community in these colonies were Gammaproteobacteria, Deltaproteobacteria (e.g., *Desulfovibrio* sp.), Acidobacteria, and Betaproteobacteria, (Portillo et al., 2008). The metabolism and physiology of this last group in the cave is practically unknown. Acidobacteria have been reported to represent a huge diversity in Altamira Cave, almost equivalent to that reported in the whole world (Zimmermann et al., 2005). The presence of active members of the Gammaproteobacteria (i.e., Chromatiales and Xanthomonadales) has been reported to be responsible for the yellow coloration of these colonies (Portillo et al., 2008).

The major components of the grey colonies in the cave were Gammaproteobacteria (e.g., *Pseudomonas*, enterobacteria), Acidobacteria, and Betaproteobacteria (e.g., *Thauera*).

In addition to finding the major bacterial groups in each type of colonization, it was realized that Altamira Cave has a huge microbial diversity. Many of these microbes had not been previously identified, but they could pose threats to the preservation of the cave. An example is the presence of a sulfate-reducing bacterium (SRB) that has been recently reported (Portillo and Gonzalez, 2009a). Sulfate-reducing bacteria, mainly belonging to the Deltaproteobacteria, have been detected as metabolically active bacteria in the cave. The most typical examples detected in this group are members of the genera *Desulfovibrio* and *Desulfomicrobium*. The SRB are anaerobic bacteria that reduce sulfates to sulfides, thus helping to maintain localized anaerobic conditions, darkening the rock surfaces by reacting with iron minerals present in the rock, increasing pH, and enhancing calcium carbonate precipitation. The SRB serve as an example of a microbial group that could be proposed as a target for monitoring efforts directed to detect massive colonization development in the cave.

Another unexpected microbial group detected as metabolically active is represented by the Archaea classified as Crenarchaeota (Gonzalez et al., 2006). The diversity of this group is high, and they represent practically the totality of the Archaea ever detected in the cave (Gonzalez et al., 2006). The metabolism and physiological characteristics of these temperate Crenarchaeota are practically

TABLE 1. Identification of the major metabolically active bacteria detected in white, yellow, and grey colonies on the rock of Altamira Cave. Bands are named as in Figure 2. OTU, organizational taxonomic units.

Major bands	OTU accession number	Taxonomic affiliation
		White colonies
W1	EF188810	*Pseudomonas* (Gammaproteobacteria)
W2	EF188320	Alphaproteobacteria
W3	EF188427	Alphaproteobacteria
W4	EF188619	Actinobacteria
	EF188426	Alphaproteobacteria
W5	EF188321	Betaproteobacteria
W6	AY960228	Acidobacteria
		Yellow colonies
Y1	AY960221	Chromatiales (Gammaproteobacteria)
	AY960228	Acidobacteria
Y2	AY960248	Betaproteobacteria
	AY960249	Betaproteobacteria
	AY960253	Gammaproteobacteria
Y3	AY960231	*Desulfovibrio* (Deltaproteobacteria)
Y4	AY960236	Actinobacteria
Y5	AY960233	Acidobacteria
	AY960224	Gammaproteobacteria
Y6	AY960234	Deltaproteobacteria
Y7	AY960269	*Microbacterium* (Actinobacteria)
Y8	AY960254	Acidobacteria
Y9	AY960257	Desulfovibrionales (Deltaproteobacteria)
Y10	AY960257	Desulfovibrionales (Deltaproteobacteria)
Y11	AY960220	Acidobacteria
		Grey colonies
G1	AY960273	*Pseudomonas* (Gammaproteobacteria)
G2	AY960260	Enterobacteriaceae (Gammaproteobacteria)
	AY960268	Enterobacteriaceae (Gammaproteobacteria
G3	AY960261	Gammaproteobacteria
G4	AY960270	Betaproteobacteria
G5	AY960263	*Pseudonocardia* (Actinobacteria)
G6	AY960244	*Thauera* (Betaproteobacteria)
G7	AY960238	Acidobacteria
G8	AY960220	Acidobacteria

unknown. Recently, the first cultured member of the low-temperature Crenarchaeota was reported (Konneke et al., 2005). It has an ammonium-oxidizing metabolism, and if this is also their role in the cave, massive consumption of ammonium ions could cause a significant reduction of the pH, resulting in calcium carbonate dissolution.

Other microbial groups have been detected in Altamira Cave as present (on the basis of DNA analyses) and/or as metabolically active (from RNA-based analyses), although they represent low proportions of the total bacterial communities (Gonzalez et al., 2008). The accumulation curves shown in Figure 3 suggest that most large bacterial groups (at the phylum level; Figure 3A) present in Altamira Cave have been detected because the curve tends to an asymptote. A linear increase of the final portion of the curve in Figure 3B indicates that a great number of the bacterial OTUs remain undetected. So there is a huge diversity of different bacterial taxa (Figure 3), which introduces a large potential for response to environmental changes in the cave and for generating highly dynamic microbial communities.

Most of the bacteria found in Altamira Cave are understudied; little is known about their metabolism. Therefore, their potential importance for cave conservation is unknown. Filling this gap will be an important step for predicting the potential risk involved in the development of specific microorganisms or microbial communities, including bacteria and fungi, and their ability to respond to any change in the environment.

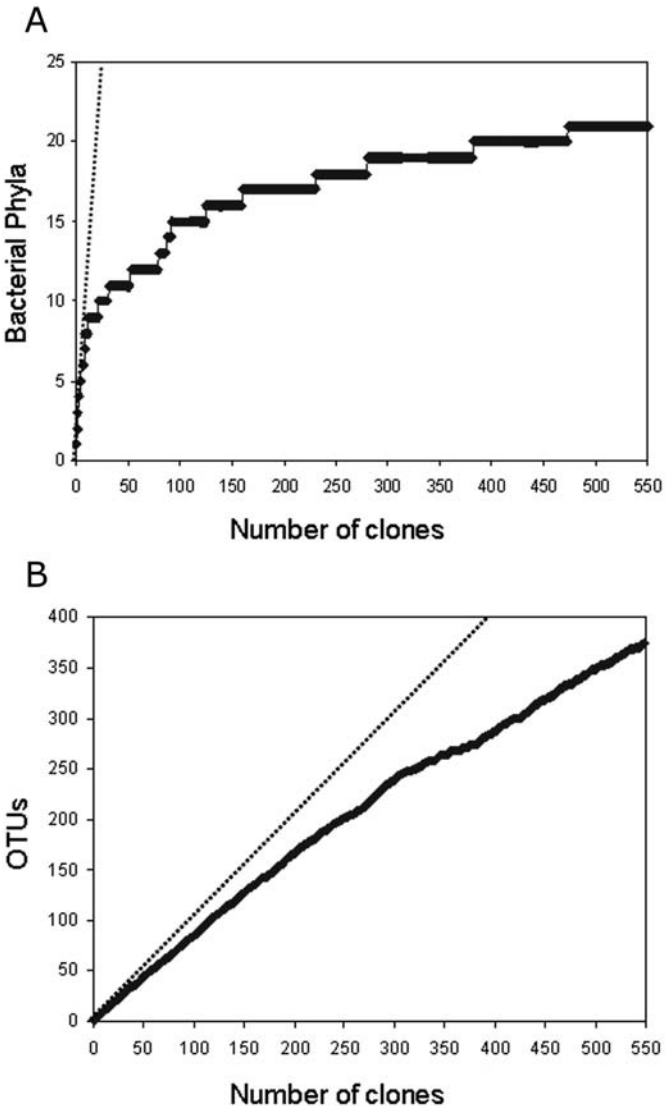

FIGURE 3. Accumulation curves for the identification of microorganisms detected in the microbial colonies sampled at Altamira Cave. Plots corresponding to the (A) identified bacterial phyla and (B) organizational taxonomic units (OTUs) are shown. The 1:1 lines (dotted lines) are presented for reference.

MICROBIAL DIVERSITY IN THE NATURAL MURIECHO L SHELTER

The region of Aragón harbors a large number of natural shelters containing prehistoric paintings dating from the Neolithic period. These represent numerous human and animal figures (Figure 4). Different areas covered by microorganisms have been detected in the Muriecho L shelter (Hameau and Painaud, 2004).

At this natural shelter three characteristic colonizations have been observed (Figure 4). Each has a specific microbial community. These colonizations occurred in areas where microbial development was induced by continuous runoff water, in a black crust that covers the upper portion of the shelter, and in a cryptoendolithic microbial community. These communities were studied using molecular methods based on DNA and RNA.

Results showed that the three microbial communities were clearly different ($P < 0.001$) in their microbial components even when their locations were only separated by about 1 m. This reflects the importance of the microenvironments for microbial development and the great capacity of these microbes to adapt to highly specific conditions. The phototrophic microorganisms detected in this study characterized these communities because they represented a major portion of their total DNA and RNA (Table 2). Also, they are likely to support the growth of other bacteria with a heterotrophic metabolism based on the consumption of photosynthates produced by cyanobacteria and other microscopic phototrophs (generally belonging to the Bryophyta).

FIGURE 4. Three characteristic types of microbial colonies observed in the natural Muriecho L shelter. (A) A black crust, (B) the development of a cryptoendolithic community, and (C) a zone affected by runoff water are shown. (D) Some of the paintings representing human and animal figures in this shelter are also presented.

The zone influenced by surface water paths showed the dominance of the cyanobacteria *Anabaena*, *Nostoc*, and *Microcoleus*. Bacteria associated with these phototrophs were mainly represented by Actinobacteria (e.g., *Pseudonocardia*). Chloroflexi represented a major group in DNA-based analyses, suggesting that this group might be dominant at some seasonal periods, depending on the environmental conditions. In addition, these chloroflexi were highly related to phototrophic bacteria, specifically to the genera *Cloroflexus* and *Roseiflexus*. These two genera are generally thermophilic photoheterotrophic bacteria, suggesting that they probably become active and develop mainly during the summer period when annual temperatures are highest (frequently over 40°C).

An area covered by a black crust was also dominated by cyanobacteria and was characterized by Actinobacteria (e.g., *Crossiella*) as the major bacterial component. Other bacteria in this area belong to the Gammaproteobacteria (e.g., Enterobacteria), Firmicutes (e.g., *Bacillus*), and Alphaproteobacteria (e.g., *Sphingomonas*), all presenting significant metabolic activity because they were detected through RNA analyses.

Cyanobacteria related to the genera *Chroococcidiopsis, Phormidium*, and *Cylindrospermum* represented most of the RNA from a cryptoendolithic microbial community. Bryophyta were also well represented in this community. Heterotrophic bacteria detected in this group included mainly Bacteroidetes (e.g., *Hymenobacter*, *Chitinophaga*), the candidate division WYO (which has no cultured representatives), and Actinobacteria (e.g., *Rubrobacter*). Table 2 summarizes the major microbial groups detected and quantified through DNA- and RNA-based molecular analyses at the three differentially colonized zones studied at the natural Muriecho L shelter. Some microorganisms were detected by RNA only. This might be a result of the low copy number of DNA sequences, which made their detection difficult. An abundance of RNA sequences from these microorganisms allowed an easier detection through RNA than DNA analysis. Results show that most of the major microbial components of these communities were detected, as suggested by the leveling off of the accumulation curve obtained for the studied shelter (Figure 5).

Clear differences can be observed between the environments of Altamira Cave and a natural shelter. The former is an almost-closed site, whereas the latter is in an outdoor environment. Thus, different factors might have different influences. Whereas a cave has no illumination (other than artificial lights) and generally has relatively constant

TABLE 2. Identification of the major bacterial groups detected from DNA and RNA analyses, comprising the communities of a zone affected by runoff water, a black crust, and a cryptoendolithic colonization, from the natural Muriecho L shelter. The frequency of encountered clones corresponding to a specific bacterial group in 16S rRNA gene libraries is shown as a percentage. A dash (-) indicates that data are not applicable.

	Runoff water affected area		Black crust		Cryptoendolithic community
Bacterial group	**DNA**	**RNA**	**DNA**	**RNA**	**RNA**
Alphaproteobacteria	20.0	-	-	7.1	-
Gammaproteobacteria	-	-	25.0	21.4	-
Betaproteobacteria	-	-	5.0	-	-
Deltaproteobacteria	-	-	5.0	-	-
Actinobacteria	-	22.2	-	57.1	6.7
Bacteroidetes	-	-	5.0	-	20.0
Firmicutes	-	-	-	14.3	-
Candidate division WYO	-	-	-	-	13.3
Chloroplasts (Bryophyta)	-	-	-	-	20.0
Chloroflexi	40.0	-	10.0	-	-
Cyanobacteria	40.0	77.8	50.0	-	40.0

humidity and temperature, the natural shelter is exposed to natural solar radiation and to a wide range of humidity and temperature conditions. In caves, phototrophs only have the opportunity to develop if artificial light is provided, usually for visitors entering the cave. This is not the case in Altamira Cave, where permanent illumination has been removed to avoid the development of phototrophic microorganisms. The natural shelter, however, suffers the consequence of large daily and seasonal environmental fluctuations in humidity and temperature as well as natural illumination. Phototrophs develop well on the natural shelter and represent the basis of the microbial trophic chain. Thus, environmental conditions are a critical factor that needs to be analyzed in order to evaluate the potential of specific microorganisms able to develop at different scales, both at the whole site under study and their microhabitats.

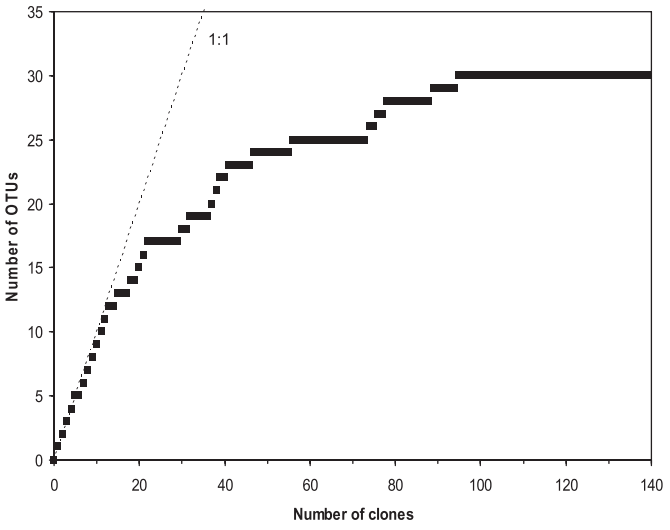

FIGURE 5. Accumulation curve of OTUs for the results obtained during the study of the natural Muriecho L shelter. The 1:1 line (dotted line) is shown for reference.

CONSEQUENCES OF MICROBIAL DIVERSITY ON BIODETERIORATION

The ecology and distribution of microorganisms and microbial communities are highly complex. Recent studies have shown a huge potential for microorganisms to disperse through different mechanisms, such as air currents and transportation by macroorganisms (Gorbushina et al., 2007; Portillo and Gonzalez, 2008b). Accordingly, microorganisms live almost everywhere, and stone materials are no exception. Combining this fact with the existing huge diversity of microorganisms on our planet, it can be easily inferred that microorganisms are the first colonizers of almost any material or site available or underexploited by other organisms (plants or animals). However, it is clear that even if microorganisms can reach any place on Earth, the environment, by providing a given set of conditions, will constrain the growth to those better adapted to develop under them (Portillo and Gonzalez, 2008b; Martiny et al., 2006).

Furthermore, microbial communities are dynamic and can show alterations with climatic or seasonal changes

(Tayler and May, 2000). Yet to be determined is the rate at which microbial colonization occurs. Results from Portillo and Gonzalez (2008b) suggest that microorganisms are continuously reaching uncolonized environments and that the arrival of a high number and variety of microorganisms can occur within the time frame of a few minutes.

Microorganisms cannot be totally eliminated, and even if that were possible, it is questionable whether elimination would be a wise decision; what is important to determine is the acceptable level of biocolonization, especially for the case of buildings and monuments. Biocolonization will be undesirable if the growth is excessive and, for example, conspicuous colonies or films are formed and if these lead to appreciable deterioration of the stone or discolorations. However, is there a possibility of controlling stone colonization by microorganisms? The use of biocides has been reported in some studies, although pre- and postapplication monitoring has rarely been carried out. Although biocides can be effective in eliminating, temporally or definitely, specific types of microorganisms, sooner or later, biocolonization will reoccur. If one fraction of the microbial community has been inhibited or removed by biocides, the rest of the community will certainly take over. If there are no survivors, new microorganisms will arrive. In both cases, the resulting microbial community will be unpredictable, as will be its effect on the colonized surface. Furthermore, most biocides can be subject to physical, chemical, or biological degradation, and so they can become a nutrient or result in the release of nutrients for microorganisms. An example is benzalkonium chloride, which can be degraded by a range of fairly common bacteria (Patrauchan and Oriel, 2003). The products of this degradation can also become carbon and energy sources for other bacteria, and the ammonium produced leads to changes in pH and fosters the growth of ammonium oxidizers or nitrogen-limited microbial species (Patrauchan and Oriel, 2003). Finally, dead biomass can become a nutrient source for microorganisms, fomenting novel microbes and leading to accelerated colonization events. The removal of microbial communities is generally a short-term event that may have serious consequences and, in many cases, adverse results.

The best strategy to control the development of microorganisms appears to be limiting their source of nutrients, or substrates for growth, as well as controlling the environmental conditions, such as light, temperature, pH, and humidity. By knowing which microorganisms are implicated in a specific colonization process, their physiology and metabolism could be investigated, allowing us to identify the specific compounds or elements needed for their growth so as to limit them. This is not an easy strategy but appears to be a promising alternative for the long-term success in controlling the biodeterioration of stone.

Natural or human-caused alteration in the natural equilibrium of microbial communities on stone may be the cause of undesirable microbial colonization and the resulting secondary effects. The interactions among microorganisms and the environment characterize a highly dynamic system representing a fascinating area to explore. Although some changes can occur rapidly, in some cases the consequences of these interactions may only be evident after some years. This can best be exemplified by anthropogenic air pollution and the damage it caused to stone materials. Climate change (Battisti and Naylor, 2009), including temperature alterations and changes in rain and drought events, may well represent a new factor to be monitored in the near future because climate change will certainly influence not only microorganisms but the world population as a whole.

FUTURE PERSPECTIVES

To ensure that future generations will have the opportunity to appreciate the monuments we enjoy today, a careful study of potential physical, chemical, and biological deteriorating factors is required to guarantee their conservation. From the microbiological perspective, the microbial colonization of stone may result in damage to monuments. Microorganisms are numerous and diverse. Their action on stone substrates may not be fast, but in the long-term their effect may be important. Because stone monuments cannot be sterile, at present, a promising strategy for stone conservation is to reduce or eliminate microbial unbalanced or massive growth. This objective can be approached in two major ways. The first and obvious line of research is to develop novel, rapid, and simple methods for the monitoring and evaluating microbial colonization on stone materials. This could be achieved by using rapid methods of detection and quantification of microorganisms and their activity. As an example, the use of fluorescent methods specific for biomolecules (e.g., Portillo and Gonzalez, 2009b) shows great potential. The second initiative involves the advancement of environmental microbiology toward closing the current gap of information existing between microbial phylogeny and physiology so that microorganisms can be identified and their metabolism easily deduced or quickly understood. A result of this major progress is that it will allow the development of strategies for limiting or eliminating the growth of specific microbial communities or microorganisms.

The analysis of the microbial communities found in the sites studied showed that although the level of diversity in these two environments may be different, the overall microbial diversity is too high to be easily understood by current methodological standards. Microorganisms can reach any environment on Earth, and removing one type of microorganisms will certainly bring others to replace it. Considering this scenario, the strategies of choice should focus on monitoring and controlling excessive microbial development. With this aim in mind, a variety of factors should be considered, and they range from physical and chemical aspects of the environment surrounding the stone materials to the microbiology of the site and specific microenvironments.

Acknowledgments

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Characterization of Bacterial Colonization of Stone at Global and Local Scales

Christopher McNamara, Nick R. Konkol, Brandon P. Ross, and Ralph Mitchell

ABSTRACT. A wide range of microorganisms, including bacteria, archaea, cyanobacteria, algae, fungi, and lichens, have been found on historic stone. Data are presented on bacterial colonization of stone at two different scales: global, comparing communities on stone monuments in Europe and southern Mexico, and local, comparing communities on different locations of the same monument. The purpose was to determine if differences in bacterial communities on stone at these two scales could be detected. At the local scale, samples of bacterial communities on a headstone in a Massachusetts cemetery were collected and compared to 16S rDNA from different locations. The bacterial community from the headstone was dominated by cyanobacteria and many species were found at both deteriorated and undeteriorated locations. Richness of the cyanobacterial community was greater in the undeteriorated location. At the global scale, a meta-analysis comparison of published 16S rDNA sequences from bacteria on historic stone was carried out. Globally, microbial communities on historic stone were dominated by similar bacterial groups, such as the Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria and the Actinobacteria. However, at a finer level of resolution (97% similarity of 16S rDNA sequences), communities from around the world appear to be quite different.

INTRODUCTION

Microbial colonization of historic stone may have two effects. First, there are aesthetic changes caused by the growth of pigmented microorganisms on or just beneath the stone surface. Cyanobacteria, algae, fungi, lichens, and some pigmented bacteria are responsible for these effects. Second, many microorganisms play an important role in the deterioration of stone in historic buildings, monuments, and archeological sites (e.g., Saiz-Jimenez, 1999), often through the action of organic and inorganic acids produced as metabolic by-products of biofilm growth.

Whereas aesthetic problems are, almost by definition, obvious, detection of microbial deterioration of culturally important stone objects is inherently difficult. In some cases, detection is based on macroscopic observations of pitting or etching of stone (Wakefield and Jones, 1998). However, attributing deterioration to chemical, physical, and/or biological agent in a specific case is extremely difficult (Koestler et al., 1994). There is no standard technique used to measure the potential of a community to cause biodeterioration. Laboratory experiments have been used to determine the potential for biodeterioration, and changes in

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stone have been analyzed by a number of methods. Some examples include measurement of changes in stone mass, measurement of calcium loss (from limestone or marble; Di Bonaventura et al., 1999; McNamara et al., 2005), scanning electron microscopy, nuclear magnetic resonance (Alesiani et al., 2000), acoustic wave velocity (Papida et al., 2000), and X-ray (Bentz et al., 1995; Jacobs et al., 1995) or microcomputed X-ray tomography (McNamara et al., 2002, 2003).

Laboratory experiments generally require that the microorganisms first be cultured. However, studies from many environments, including cultural heritage materials, have demonstrated that the majority of microorganisms cannot be cultured (Jannasch and Jones, 1959; McNamara et al., 2003). Furthermore, the metabolic (and thereby biodeteriorative) activity of microorganisms growing on laboratory media may differ significantly from the activity of the same organisms in situ. Recent application of tools from molecular biology has revealed a wide range of microorganisms on historic stone, including bacteria (Sand and Bock, 1991), archaea (Rölleke et al., 1998), cyanobacteria and algae (Tomaselli et al., 2000), fungi (Gorbushina et al., 1993), and lichens (Garcia-Rowe and Saiz-Jimenez, 1991). In particular, the application of molecular techniques has revealed the presence of an extremely diverse bacterial assemblage (Gurtner et al., 2000; Schabereiter-Gurtner et al., 2001a, 2001b, 2002a, 2002b; McNamara et al., 2006).

However, information is still lacking about the activity and function of these uncultured bacteria. For example, in one of the few studies to measure microbial activity on historic stone, Tayler and May (2000) found that roughly 20%–50% of bacteria on sandstone monuments were active. However, their methodology did not provide information about the activity of specific taxonomic groups. New studies assessing the presence of 16S rRNA have begun to address the issue of activity in uncultured bacteria (Gonzalez et al., 2008) but do not provide information about function (i.e., metabolism, biochemistry, or deteriorative activity).

A current assessment of the situation in the field of microbial deterioration of stone suggests that there is substantial knowledge of the taxonomy of microorganisms that colonize stone but little information about their in situ activity and function. The purpose of this study was to test the observation that the microbial community on culturally important stone has been well described. Data are presented on bacterial colonization of stone at two different scales: global, comparing communities on stone monuments in Europe and southern Mexico, and local, comparing communities in different locations of the same monument.

METHODS

Local Scale

Samples were collected from a headstone at the Woodlawn Cemetery located in Ayer, Massachusetts. Samples from locations on the same headstone were compared to minimize variation in the microbial community caused by other factors (e.g., age, type of stone, exposure, prior cleaning, or conservation treatments). Collection locations were chosen to represent undeteriorated and deteriorated areas. The condition of the stone was determined by visual examination. Stone from the undeteriorated locations was characterized by smooth surfaces and sharp edges and corners. In deteriorated locations, the stone surface appeared rough, and edges or corners appeared weathered and rounded. Microbial growth was not visible to the naked eye at either location.

Microorganisms were removed nondestructively from stone using swabs. Swabs were immersed in a dilute detergent solution to facilitate removal of microorganisms, and a 1.0 cm^2 area was swabbed. After sampling, swabs were placed on ice and returned to the lab. DNA was extracted using the UltraClean Soil DNA Kit (MoBio Labs, Carlsbad, California). The 16S rDNA was amplified using the polymerase chain reaction (PCR) as previously described (Perry et al., 2005) with primers 27f and 1492r (Lane, 1991). PCR products were cloned into the pCR 2.2-TOPO vector and transformed into competent *Escherichia coli*, as described in the manufacturer's instructions (TOPO TA Cloning Kit K4500-01, Invitrogen, Carlsbad, California).

Clone inserts were sequenced at the Dana Farber/Harvard Cancer Center High-Throughput DNA Sequencing Facility (Cambridge, Massachusetts) using a 3700 DNA Analyzer (Applied Biosystems, Foster City, California). Unaligned sequences were compared to the National Center for Biotechnology Information (NCBI) database using the BLAST search program to find closely related sequences (Altschul et al., 1997). Sequences were aligned using ClustalX (Thompson et al., 1997). Phylogenetic analysis was performed using PAUP 4.0 beta 10 (Swofford, 2003). Trees were constructed using neighbor-joining distances with 1,000 bootstrap replicates. Groupings that occurred in less than 50% of replicates were excluded. The number of operational taxonomic units (OTUs) and the Chao statistic were calculated using the DOTUR and SONS software packages (Schloss and Handelsman, 2005, 2006). Proportional Venn diagrams were calculated with VBVenn (Granato, 2002) and drawn using Microsoft PowerPoint.

Global Scale

The DNA sequences were collected from the NCBI database using accession numbers found in the publications listed in Table 1. Sequences were aligned using the myRDP auto aligner feature of the Ribosomal Database Project (RDP) (Cole et al., 2009). Multiple sequences of insufficient length (<700 base pairs) or homology were eliminated from the alignment. Neighbor-joining trees were assembled using the Jukes-Cantor distance correction model and bootstrapped using the Tree Builder feature of the RDP.

The number of OTUs was calculated as described above. Sequences were divided into three groups for comparison: sequences from tropical locations, sequences from temperate locations, and sequences from caves. Three-part proportional Venn diagrams were calculated using DrawVenn (http://theory.cs.uvic.ca/euler/DrawVenn/) and drawn using Microsoft PowerPoint.

RESULTS

Local Scale

A total of 62 clones were analyzed from deteriorated and undeteriorated locations. Rarefaction analysis indicated that most of the taxa present in the two communities were identified (Figure 1). At the 97% level of similarity, which is the agreed upon level for delineating different species, 15 different OTUs were found on the stone (Figure 2). Three OTUs (20%) were shared between the two libraries (27% of the undeteriorated OTUs and 43% of the deteriorated OTUs). Using the chao statistic, it was estimated that 7.5 OTUs were present in the deteriorated sample, and 11 OTUS were present in the undeteriorated sample (at the 97% similarity level).

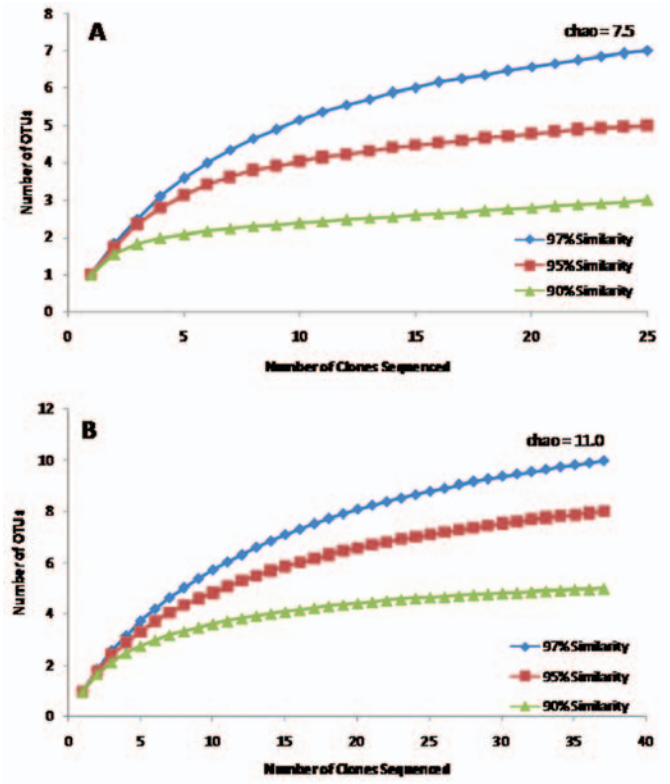

FIGURE 1. Rarefaction analysis of clones sequenced from (A) deteriorated and (B) undeteriorated locations. The number of operational taxonomic units (OTUs) at the 97% similarity level was calculated using the chao statistic.

TABLE 1. Sources used in the meta-analysis of bacterial 16S rDNA sequences.

Source	Number of sequences
Altenburger et al. (1996)	3
Eppard et al. (1996)	8
Gurtner et al. (2000)	82
Heyrman and Swings (2003)	7
McNamara et al. (2003)	10
McNamara et al. (2006)	84
Perry et al. (2003)	8
Piñar et al. (2001a)	3
Piñar et al. (2001b)	13
Rölleke et al. (1996)	10
Schabereiter-Gurtner et al. (2001a)	27
Schabereiter-Gurtner et al. (2001b)	23
Schabereiter-Gurtner et al. (2002a)	41
Schabereiter-Gurtner et al. (2002b)	22
Schabereiter-Gurtner et al. (2003)	30
Schabereiter-Gurtner et al. (2004)	58
Urzì et al. (2001)	6
Vlasceanu et al. (2000)	2

Neighbor-joining analysis demonstrated that sequences from the deteriorated and undeteriorated clone libraries were closely related to each other (Figure 3). In both locations, the microbial community was dominated by sequences related to Cyanobacteria, such as *Gleothece*, *Chroococcidiopsis*, and *Nostoc*.

Global Scale

Analysis of sequences obtained from the literature indicated that there were 247 OTUs at the 97% similarity level. However, there was minimal overlap between the

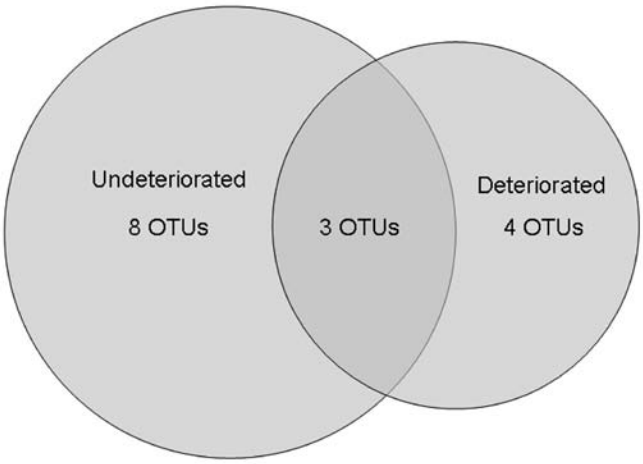

FIGURE 2. Venn diagram depicting the relationship between clones identified from deteriorated and undeteriorated locations of the same headstone. Total number of OTUs is 15, and distance level is 0.03.

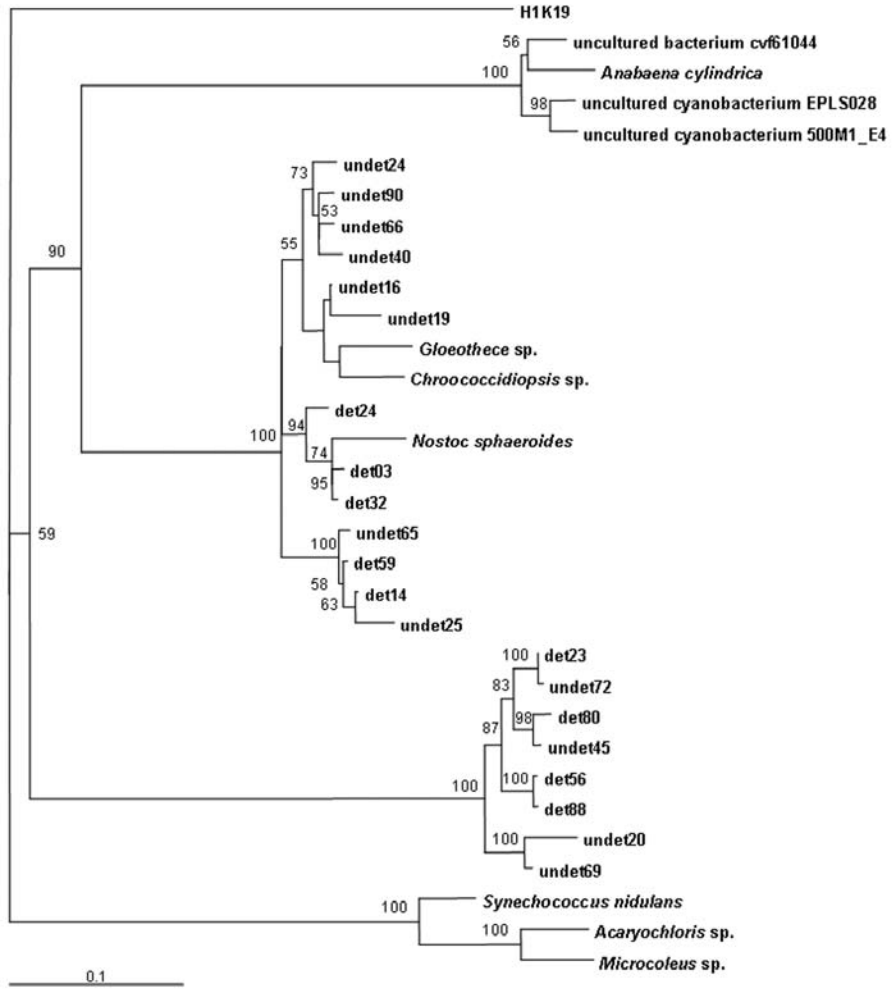

FIGURE 3. Phylogenetic relationships based on partial 16S rDNA sequences of clones from deteriorated and undeteriorated locations. For neighbor-joining trees, bootstrap values based on 1,000 replicates are indicated for branches supported by >50% of trees. Scale bar represents 0.1 nucleotide changes per position.

communities at this level (1 OTU in common between temperate and tropical locations and 1 OTU in common between cave and temperate locations). Overlap among the three groups of sequences was first observed at the 95% similarity level, but only 7 of the 221 OTUs were found in more than one group (Figure 4a). It was not until similarity decreased to 80% that overlap of the communities reached levels similar to those seen in the local samples. At 80% similarity there were 103 total OTUs, and 26 of these (25%) were found in more than one group (Figure 4b).

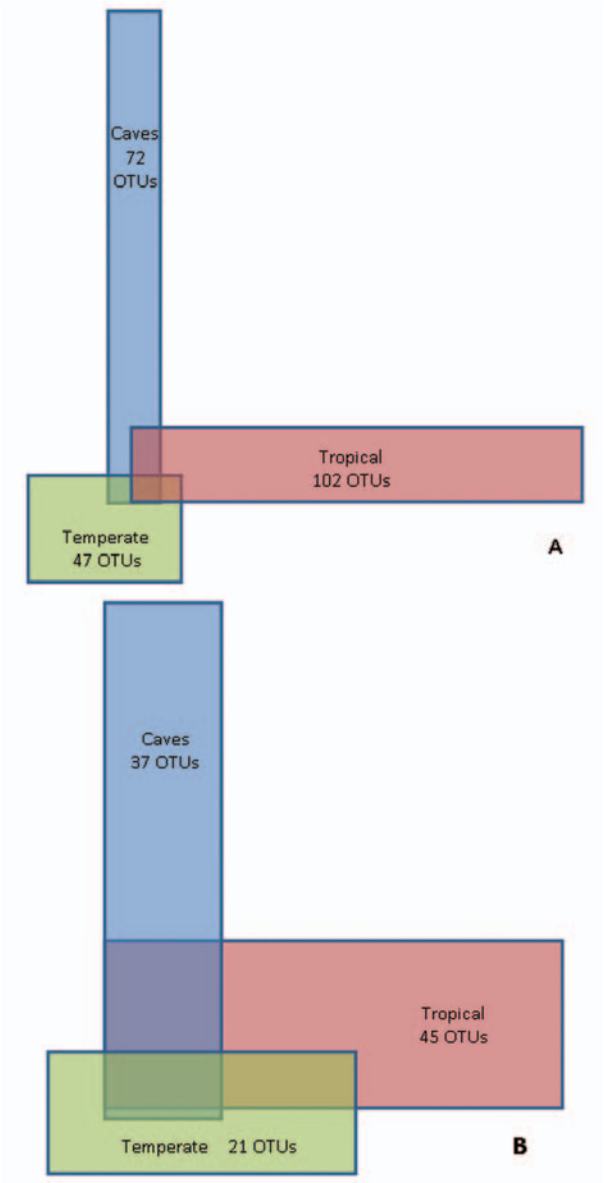

FIGURE 4. Overlap between OTUs in bacterial communities among cave, tropical, and temperate locations at the (A) 5% and (B) 20% similarity levels.

The neighbor-joining tree constructed with sequences from tropical, temperate, and cave locations presented a different picture of the relationship between the three communities. This analysis demonstrated that sequences from all three locations were interspersed among bacterial phyla (Figure 5). The presence of clusters exclusive to sequences from one location were absent.

DISCUSSION

Bacterial communities from deteriorated and undeteriorated locations of the same headstone were similar. A large percentage of OTUs was found in both locations. Furthermore, neighbor-joining analysis indicated that in many cases, sequences from deteriorated and undeteriorated locations were closely related. This is not surprising given that the sample locations were in close proximity. The low level of biological growth suggests that the deterioration observed in some locations was the result of nonbiological or, more likely, a combination of biological and nonbiological processes.

Differences in the richness of sequences (i.e., the number of different OTUs or taxa) found in deteriorated and undeteriorated locations were apparent. Higher microbial diversity was expected on the deteriorated stone because the rough surface provides for additional microniches that can be colonized. In fact, greater richness was observed on the undeteriorated stone. Multiple factors that influence richness or diversity have been reported for microbial communities, such as the supply of organic matter (Zhou et al., 2002; Waldrop et al., 2006) and interactions with other organisms (Taylor et al., 2004; Weinbauer and Rassoulzadegan, 2004) or the environment (Rickard et al., 2004). The importance of these factors on historic stone is unknown. If consistent differences in community richness or diversity could be demonstrated between deteriorated and undeteriorated stone, it is possible that a metric could be developed to indicate deterioration or biodeterioration.

Analysis of 16S rDNA sequences from bacterial communities found on heritage stone throughout the world revealed different results when compared by similarity of OTUs and by constructing a phylogenetic tree. The phylogenetic analysis (Figure 5) indicated that sequences obtained from locations around the globe were closely related. This is in agreement with previous work on photosynthetic microorganisms. Although there appear to be differences in the photosynthetic microorganisms that colonize various types of stone and building materials (Tomaselli et al.,

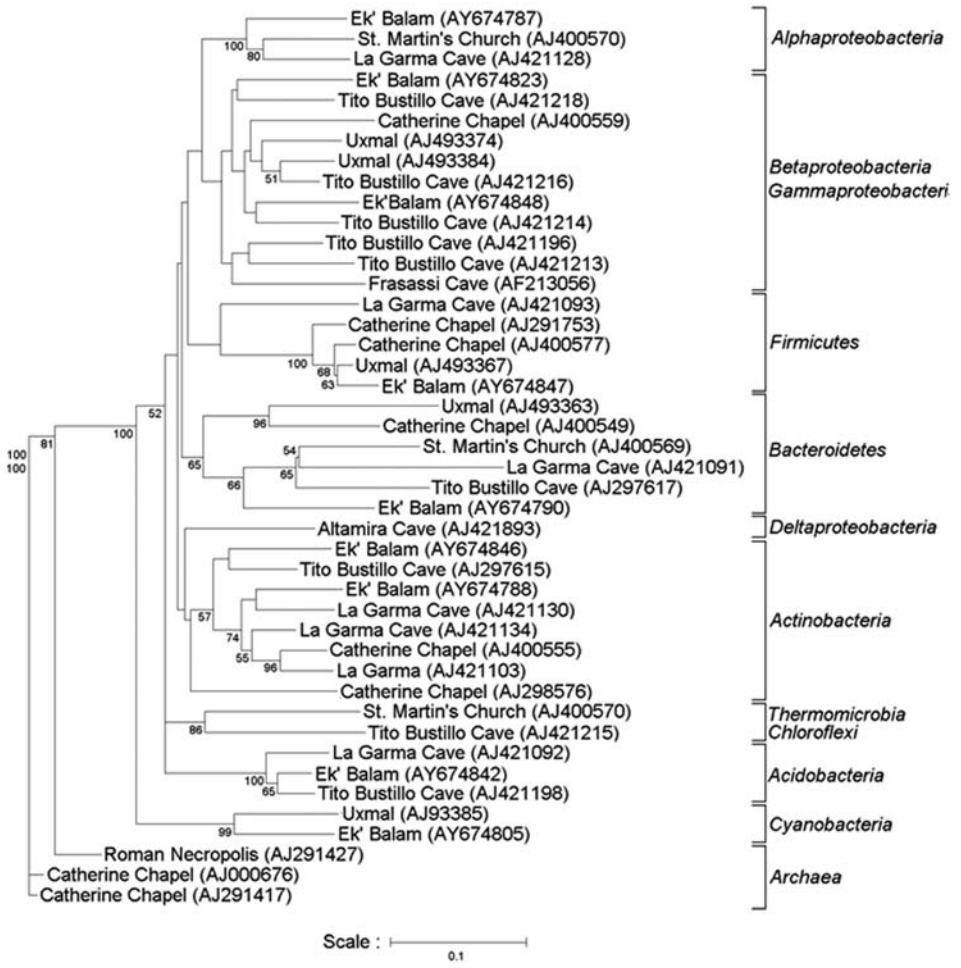

FIGURE 5. Phylogenetic relationships based on partial 16S rDNA sequences of selected clones from publications describing the microbial communities on historic stone (see Table 1). For neighbor joining trees, bootstrap values based on 1,000 replicates are indicated for branches supported by >50% of trees. Scale bar represents 0.1 nucleotide changes per position.

2000), the organisms found in tropical and temperate climates are quite similar (Crispim et al., 2003).

In contrast to the phylogenetic tree, comparison of OTUs from locations around the globe indicated that the communities were quite different from each other. This may represent true differences between the communities and could be the result of a biogeographic distribution of bacteria around the globe. Alternatively, the differences could be artifacts of the methodology employed.

Many factors may have contributed to the observed differences. For example, sequences used in the "global" portion of this study were obtained using different protocols and different primer sets. Also, it is not clear how well the communities were sampled or if all sequences were published. Furthermore, the sequences were grouped into broad categories on the basis of their general location (temperate, tropical, and cave). Grouping of the sequences in other categories could provide a different picture of the relationship between bacteria on stone in different locations.

Differences observed among OTUs could also reflect real differences among the microbial communities. Each of the sites studied has its own history of environmental exposure, materials, and conservation interventions. Analysis of the community at one point in time provides a snapshot of the community that, in some sense, reflects this history but that also may not well represent the community. Moving forward, it will be important to more thoroughly characterize microbial communities throughout the year and

to determine which historical and environmental factors are important in shaping the community.

CONCLUSIONS

Sequencing of bacterial communities on stone heritage materials has yielded a great deal of information about those communities. However, a better understanding of the interaction between microorganisms and the stone is needed.

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Methods to Prevent Biocolonization and Recolonization: An Overview of Current Research for Architectural and Archaeological Heritage

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ABSTRACT. The paper presents an overview of both the results of relevant case studies and the current trends in research that address the control and prevention of biocolonization. Recolonization of architectural and archaeological heritage depends on various factors, such as the biocide used and the application methodology, the nature of the stone and its state of conservation, the type and degree of colonization before the treatment, the application of other products, and, last but not least, on the micro- and macroenvironment surrounding the object. The application of preventive measures to discourage recolonization is especially valid for archaeological areas because of the weathered type of stone structures and their location. It is known that the effectiveness of biocidal treatments is limited in time if not associated with other control methods. Although the application of water repellents retards biological colonization as long as the surface retains its hydrophobicity, they may interfere with the biocidal application or result in the development of disfiguring patterns. The sequential application of these two products, regardless of the application order, may result in negative interactions. However, the application of mixtures of both products appears to provide a more-effective long-term protection that needs to be further researched. Recently developed experimental approaches to control microbiological growth involve the use of substances, such as pigment and polysaccharide inhibitors as well as permeabilizers, that make biofilms more vulnerable to biocides and the use of antifouling agents derived from plants or marine animals.

INTRODUCTION

This overview deals primarily with methods to prevent biocolonization and recolonization. It has focused on *visible* biocolonization. The presence of microorganisms and organisms on stone surfaces outdoors is generally made evident by the soiling, fouling, discoloration, patinas, etc., that they cause. Therefore, attention to biodeterioration problems, and consequently the study of them, is very often given only after the appearance of visually perceptible signs. It is well known that biological alterations may also be "atypical," difficult to recognize and interpret as of biotic nature without any appreciable evidence (i.e., the presence of unpigmented or endolithic microorganisms). The presence of endoliths

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on monuments has rarely been reported, mainly because of the sampling and identification techniques generally used (Salvadori, 2000).

It is common knowledge that biological growth on stone is highly dependent on climatic and microclimatic conditions, such as humidity, temperature, and light, and on chemical and biological pollution levels. In closed environments, climatic and microclimatic conditions can be managed up to a point through microlimate controls and what is referred to as "good housekeeping," whereas the possibility of controlling them outdoors is rather limited (Nugari and Salvadori, 2003a; Warscheid, 2003). Control of environmental factors becomes extremely difficult in archaeological sites, where the decision to install covers and roofs is a permanent dilemma. Since reducing the water absorption of a material is an important step in reducing the tendency for biocolonization, for the case of outdoor exposed stone or wood, the most common solution is the application of water-repellent products (Charola, 2003; Panov and Terziev, 2009).

The number of factors influencing biocolonization of stone is so large that it is practically impossible to make any generalizations. Nonetheless, the type of substrate, such as limestone, granite, or mortar, and its bioreceptivity, i.e., the potential of a material to be colonized by microorganisms, which is related to its mineralogical and petrographic properties, are probably the most important ones (Tiano et al., 1995; Shirakawa et al., 2003; Miller et al., 2006). Surface roughness, the presence of cavities, and porosity, i.e., pore sizes and pore size distribution, in a stone have critical importance for moisture retention and, consequently, colonization (Caneva et al., 2004). Next in importance is the location of the object, whether in a closed, semiclosed, or open environment, and its exposure. If outdoors, its geometry will define the areas that receive higher moisture input and that will be colonized first. When a comparison is made of objects with similar substrate, environment, and exposure, the more relevant factors are climatic conditions, previous history, and biogeographic characteristics of the site (Caneva et al., 2008).

Objects located outdoors are directly exposed to meteorological phenomena, such as rain, solar irradiation, thermal variations, and wind. These will depend upon the climate of the site, such as desert, Mediterranean, temperate, or tropical, and the geographic and topographic location of the site in question. A further distinction can be made between rural and urban environments, where microclimatic effects resulting from the presence of vegetation and pollution exert an important positive or negative influence over biocolonization. Weathering of stone exposed outdoors, added to the action of microorganisms and pollutants, results in an increase in the natural bioreceptivity of the material since a deteriorated surface presents a higher surface roughness and porosity, which facilitates the attachment of microorganisms to it (Guillitte, 1995). Furthermore, all conservation interventions, such as cleaning and the application of various products (e.g., biocides, consolidants, and water repellents), will modify the bioreceptivity of the substrate. In most cases, they will decrease it, but sometimes they may increase it, as synthetic polymers can represent potential substrates for the development of heterotrophic microorganisms (mainly fungi) that can partially degrade and modify them (Nugari and Salvadori, 2008a).

It is known that the deposition of particulate matter on surfaces is higher for sheltered areas than for those regularly washed by rain, on rougher than on smoother surfaces in a given environment, and, in general, proportional to the length of the exposure time. Particulate matter is composed of many different materials of inorganic, organic, or biological origin. The latter can be subdivided into those with a vital nature, such as cells, spores, conidia, and nonvital, such as vegetal and animal debris (e.g., leaves, feathers, and feces). Surfaces treated with water-repellent products may show a tendency to accumulate a higher amount of particulate matter with respect to untreated ones, with significant differences depending upon the nature of the applied products, e.g., silicone resins, acrylic resins, or fluoroelastomers (Bracci et al., 2002; Moreau et al., 2008b).

Few studies have dealt with the problem of biological recolonization, and most of these are recent. In cases when objects undergo conservation interventions, apart from the normal factors affecting biological colonization mentioned above, the products applied during the intervention and the methodology used will influence the recolonization rate. Thus, the number of factors that regulate biocolonization is even higher. Statues in parks and gardens present an excellent opportunity to study recolonization of stone objects (Charola et al., 2008; Nascimbene and Salvadori, 2008). In general, the presence of higher vegetation induces an increase in humidity and a decrease in temperature, both of which result in an increase in relative humidity. They also reduce the effects of the wind and chemical air pollution while providing nutrients for microorganisms, thus fostering their development. All these factors will influence the mode and timing of recolonization and, in general, increase biodeterioration.

Nascimbene and Salvadori (2008) have studied lichen recolonization of some limestone statues located in parks in three Venetian villas in Italy. A significant difference between recolonization times was observed and could be related to the consolidation and water-repellent products that had been applied, rather than the biocides used

because thorough rinsing had eliminated most residues of the latter, and to the environmental conditions. For example, recolonization started after a few weeks on statues treated with a fluorinated polymer, whereas it took several years for those treated with a silane and/or polysiloxane. A comparative study on the durability of different products applied to sandstone after exposure in a natural environment for five years evidenced different performances for them: acrylic resins and fluoroelastomers were degraded in a short time, whereas silicate-based products maintain their good performance over time (Bracci et al., 2002).

The time it takes for biological recolonization is fundamentally important when developing a maintenance plan or when planning major conservation interventions to prevent objects from attaining a complex biological community since, generally, the latter (recolonization) will induce the most significant deterioration (Nascimbene et al., 2009). Thus, the minimum intervention criteria should be implemented, reducing both costs and efforts (Charola et al., 2007).

ISSUES IN ARCHAEOLOGICAL AREAS

In situ conservation of archaeological structures requires both active and preventive conservation. Active conservation indicates those interventions required for the preservation of the site. These can be subdivided into emergency interventions, conservation, restoration, regular maintenance, and the installation of seasonal or permanent protection systems. In most cases, archaeological remains have lost their roofs and coverings, so that the structures and their surface finishes are exposed to climatic factors, e.g., rain, wind, and thermal changes. Thus, the installation of permanent coverings over archaeological sites reduces their deterioration rate by serving to control some of the deterioration factors. However, this reduction occurs only if the covering is appropriately designed and well constructed since otherwise the deterioration rate will be increased (Laurenti, 2006). The effectiveness of these systems depends on several factors, among which shape and material are critical. For example, transparent roofs increase the temperature of a protected object and allow light to reach its surface, resulting in enhanced biocolonization. The greater the number of transparent elements present near the surface of the structure, the higher the colonization by photosynthetic microorganisms and plants will be, as shown in Figure 1.

In tropical climates, characterized by alternating dry seasons and wet seasons with torrential rains, the only way to reduce biological recolonization is to change the environmental conditions and, in particular, the moisture

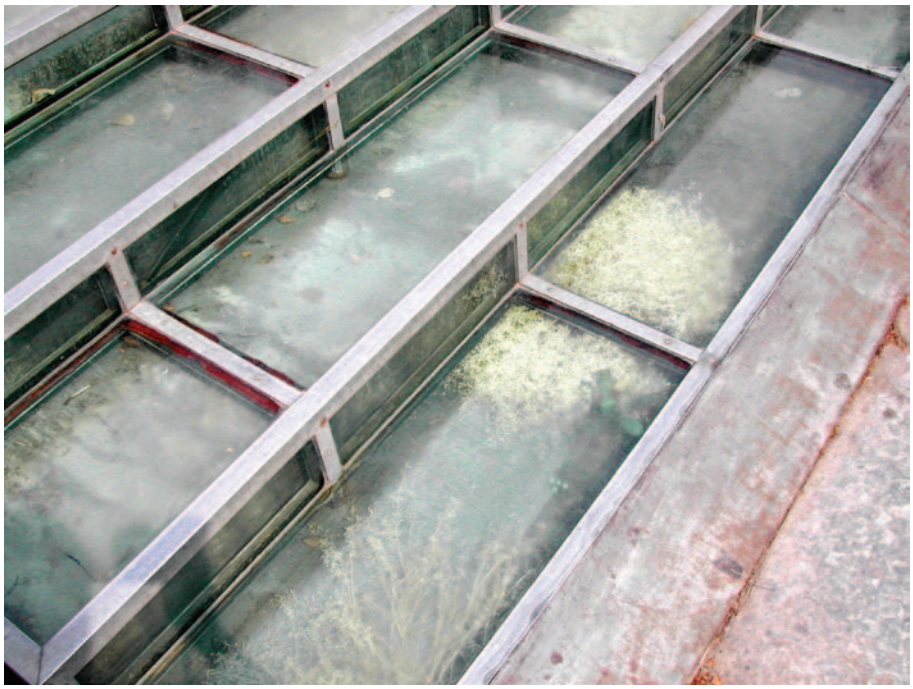

FIGURE 1. A glass cover over a marble stairway on the archaeological site of Ding Ling, China. Note the moisture condensation and the thick growth of plants underneath the glass, completely obscuring the sculpted marbles.

FIGURE 2. (a) Protective shelter installed over the Hieroglyphic Stairway in Copán (Honduras). Nonprotected stones to the left of the stairway show a thick blackish biocolonization. (b) View of the stairway under the tarpaulin. Only a slight covering of cyanobacteria and algae remain, and all lichens have disappeared. Note that the stone has a natural greenish hue.

content in the substrate. Some positive examples can be cited, such as that of the Mayan Hieroglyphic Stairway in the Copán (Honduras) archaeological site. This site had been highly colonized by microorganisms and lichens, and many biocidal treatments were carried out since the late 1970s. Starting in 1985, the monument was covered with a tarpaulin protective shelter, initially only during the rainy season, but after 1987 the shelter was modified to permanently cover the structure; this improved its protective function and the visibility and presentation of the stairway (Getty Conservation Institute, 2006) (Figure 2a). In 2000, a study was begun to determine the result of this measure and showed a drastic reduction of biological colonization. This reduction was reflected in the complete disappearance of the lichens with only a relatively small amount of cyanobacteria and green algae remaining (Caneva et al., 2005), as shown in Figure 2b. Treatment with biocides produced a clean surface (Figure 3a), which remained clean if the surface was protected (Figure 2b) but was recolonized if the surface was unprotected (Figure 3b).

The implementation of the use of metallic strips of copper or zinc to prevent recolonization on buildings was carried out by Wessel (2003). The toxic effect of heavy metals on microorganisms is well known and was the basis of many biocides in the past, but because of their negative impact on the environment, their use has been discontinued. The effectiveness of the metallic strips relies on the slow and limited dissolution of metal ions when rain water

FIGURE 3. (a) Altar 41, Copán (Honduras). Appearance of a nonprotected carved stone immediately after cleaning and the application of a biocide. (b) Detail of the same area, 15 years later, showing intense recolonization by lichens.

washes over them. They prove effective for preventing recolonization of clean surfaces but are extremely slow in reducing existing biocolonization. The main problems are the installation on the structure, its design, and environmental conditions, such as rain frequency, exposure, and shading (Guillitte, 1993). Among the problems that this system can induce is that some of the ions released can stain the substrate, especially if it is a white, porous stone. Copper oxides and hydroxides from bronze or copper elements will stain it blue, and zinc compounds from zinc alloy objects may induce a grayish color, as commonly seen on the stone bases of metal statues. Eventual deterioration from the presence of these ions has also been reported (Gaylarde et al., 2008). However, the application of the system is fairly common in areas with high precipitation to protect wooden or masonry buildings.

The application of this system in archaeological sites presents a challenge. A pilot project installed metal strips on one of the walls of the former houses in the Jesuit-Guaraní mission of San Ignacio Miní, Misiones, Argentina. The wall was partly cleaned, and three areas on the top were covered by a zinc mesh, a bronze mesh, and lead strips, respectively (Magadán et al., 2007).

Finally, soft-wall capping of grasses and other plants, as an alternative to hard-top capping, has been shown to be viable (Viles et al., 2002), but further study of this method is needed.

BIOFILMS AND BIOCIDES

Biocolonization results from the colonization of a surface by a single species of microorganism or, more frequently, from a community of them. If a community forms, it usually produces a biofilm. A biofilm results from the secretion of extracellular polysaccharide substances (EPS) by the microorganisms, which will enclose and shield the community from desiccation and other factors, such as biocides. The different microorganisms may be in different physiological states. Elimination of a biofilm is usually more difficult than treating a solitary species because biocides have to penetrate through the biofilm to reach the microorganisms.

There are three approaches for the elimination and prevention of biofilms: (1) regular disinfection of the surface to prevent the attachment of microorganisms and the formation of biofilms (Meyer, 2003); (2) elimination or destruction of the biofilm using biocides, oxidizing or non-oxidizing substances (Meyer, 2003); and (3) reduction and/or retardation of the biofilm formation by modifying the bioreceptivity of the surface. In this last case, both the type of surface and the accumulation of organic matter, which would enhance the formation of a biofilm, especially under oligotrophic (i.e., low-nutrient) conditions, are crucial.

It is known that biocide effectiveness can be limited by the biocide's penetration capability into a biofilm and its reaction with the EPS material. Each biofilm, although having common characteristics, is unique and is characterized by its microbial composition, amount and type(s) of EPS, and nutrient availability, among other factors. Even for similar biofilms, the effectiveness of a biocide will depend on its chemical composition, concentration, solvent type, and application method, as well as on the substrate of the biofilm, the presence of organic matter on its surface, and meteorological conditions (Nugari and Salvadori, 2003b). With regard to the substrate, both its mineralogical composition and its porosity are fundamental. Thus, to have similar biocidal effects on biocolonization, different concentrations should be used depending on the stone type and taking into consideration that some biocidal compounds, such as the quaternary ammonium compounds, are strongly adsorbed by clay minerals, particularly the expansive ones like smectites (also called the montmorillonite group of clays), which can retain them in variable amounts (Young et al., 1995). Therefore, sandstones may require higher concentrations of these biocides as compared to limestones, but the biocidal effect will last far longer for sandstones because of their mineralogical composition. It should be noted that the concentration at which these products are applied is not usually adapted to the type of substrate requiring the treatment.

The application of biocides to biocolonized stone can be made with two different objectives and methods: (1) application of the biocide and, after its action has been completed, proceeding with the complete removal of any residue so as to avoid any negative effects on the substrate and/or products to be applied after it and (2) application of the biocide without residue removal, so as to retard as much as possible the recolonization of the surface. In general, the first approach is predominantly used, given the uncertainty of the recolonization rate and the usual insufficient information provided for the commercial products (Nugari, 1999).

The second approach is most often used for products that have low or no solubility in water and therefore will not be easily washed off by rain. One of the active ingredients that has been tested using the second approach is a derivative of pyridine (2,3,5,6-tetrachloro-4(methylsulfonyl) pyridine; Gomez-Bolea et al., 1999; Pietrini et al., 1999). This has been shown to be effective in removing photosynthetic microorganisms, fungi, and lichens and in keeping the surface clean for five or six years after application. Eight years after the application of this product, the

presence of a biological patina made up of bacteria, algae, and fungi was apparent but was limited to the lower part of the base of the object, up to a height of half a meter because of rising damp (Urzì et al., 2000).

It is important to mention that even after half a century of studies, testing of biocides is mostly limited to evaluation with regard to their effectiveness in killing target microbes. In the past, the assessment of biocide treatments was empirically performed in situ with the naked eye, often providing misleading results; today, this evaluation is carried out with laboratory and/or in situ tests with portable instruments (Nugari and Salvadori, 2008b; Tretiach et al., 2008). Only rarely are any substrate interactions with these compounds considered (Tudor et al., 1990; Nugari and Salvadori, 2003b). Some studies in the past 10 years have clearly shown the effects that some biocides may induce on the stone, for example, changes in color, capillary water absorption, and mobilization of the calcium ion (Altieri et al., 1997; Tretiach et al., 2007). Recurrent biocide treatments can enhance negative effects on stone, cause resistance in treated microorganisms, and change biofilm composition to favor the development of more harmful microorganisms.

Comparing the capillary absorption curves before and after the application of a biocide (active ingredient: alkylaminotriazine + N'-(3,4 dichlorophenyl)-N,N dimethyl urea) on three different stone types, i.e., limestone, sandstone, and granite, it is evident that they show a similar behavior to that of samples treated with a water repellent, with a significant decrease in both the absorption rate and the total amount of water absorbed (Tretiach et al., 2007). Biocides that provide some hydrophobicity to the stone, such as this one, have proved to slow down recolonization. For example, lichenocidal effects were still observable three years after treatments on some Angkor monuments (Uchida et al., 1999). However, it is unknown what produces the water-repellent action since for biocides, only the active ingredient is identified and not the eventual presence of other molecules that might provide other functions, such as adhesiveness or water repellency. As a matter of fact, commercial formulations include various substances, and among the most recent developments is the enclosing of the biocide within polymers so as to have it available on the surface "when needed" (Nugari and Salvadori, 2003b). This development, similar to the slow release of microencapsulated pharmaceuticals, would allow a reduction in the number of required treatments and provide a long-term protective action. For example, some skin disinfectants currently in use may contain, apart from the active biocidal principle, siloxanes or silicones that keep the active principle on the skin for longer times.

Among the recent compounds tested in the conservation field, titanium dioxide has been shown to act as both an algicide and a fungicide. Titanium dioxide, particularly in the anatase form, is a photocatalyst under ultraviolet light and is capable of oxidizing various organic compounds into water and carbon dioxide. On the basis of this property it could be expected that some materials activated by solar light could serve to destroy organic compounds present on their surface, such as pollutants and microorganisms, and would therefore act as "self-cleaners." In the study of Maury Ramirez and De Belie (2009), the reduction of the algal growth on concrete surfaces treated with TiO_2 was evident when compared to the untreated ones. On the other hand, Pinna et al. (2009), experimenting with ceramic materials in outdoor environments, found only a reduction in fungal growth and practically no influence on the colonization by cyanobacteria, green algae, and lichens. The photolytic degradation of microorganisms could, perhaps, be improved by increasing the amount of light or the relevant UV component of the light.

INTERACTIONS BETWEEN PRODUCTS

In general, conservation interventions are completed, after a biocidal treatment, with the application of a water repellent. The aim of the latter products is to reduce the uptake of water by the stone and, consequently, its biological recolonization. To date, no standard procedure has been developed that outlines the order and methodology to be followed for the different conservation operations, such as

- elimination of the biocide residues by thorough washing,
- application of a biocide after the stone object has been completely cleaned and before the application of a water repellent,
- application of a water repellent and then a biocide, and
- application of a water repellent mixed with a biocide.

Furthermore, it is known that the effectiveness of a water repellent is limited in time, depending on various factors (Charola, 2003), and the surface may therefore require regular maintenance with biocides if the aim is to reduce recolonization of the object. Although this is a key point, the effectiveness of biocides and that of water repellents are, in general, evaluated individually, and the result of combining both treatments has only been studied sporadically and to a limited degree (Malagodi et al., 2000; Moreau et al., 2008a). It is of fundamental importance to

develop guidelines for the evaluation of the combination of biocides with water repellents to determine how the effectiveness of the combined or sequenced application of products can be improved. For this purpose, an elucidation of the interaction of these products is necessary so as to be able to develop improved products and application methodologies.

Sequential application of two different products may result in negative interactions. For example, biocides appear to interact with water-repellent products, whether they are applied before or after them (Malagodi et al., 2000). Furthermore, the application of a quaternary ammonium biocide on limestone previously treated with an alkylpolysiloxane showed a decrease in the superficial water repellency and a modification of the capillary absorption of the stone (Moreau et al., 2008a). However, poulticing the surface was sufficient to eliminate the biocide and reestablish the original water repellency of the surface. In any case, this type of biocide would not remain long on the surface of the object if exposed to rain given its high solubility in water. These studies only evaluated the performance of the water repellent and did not assess whether the biocidal effect might be decreased or even eliminated if a water repellent was applied as well.

The hydrophobization effect has a variable duration, in most cases years, although in other cases it may be limited to months or even only weeks. The duration depends on the type of product, concentration, and amount applied for a given type of stone. On surfaces that have lost their water repellency or have not been treated with a water repellent, biological recolonization begins on those areas that are directly wetted by the rain, and these are correlated to the geometry of the object (Nascimbene et al., 2009), as shown in Figure 4. On surfaces that retain

FIGURE 4. Two limestone statues in different environments: (a) urban environment (Venice, Italy) and (b) rural environment in a park in Passariano, Italy, showing that the geometrical configuration of the statues favors wetting of some areas where biocolonization will develop preferentially and that the biocolonization developed is dependent on the environment.

water repellency, a striped pattern develops where dust accumulates and biocolonization develops first (Charola et al., 2008). As expected, these stripes develop on the areas that receive direct rain or water flow, as shown in Figure 5.

DEVELOPMENT OF NEW PRODUCTS

Over the past 10 years different products have been developed with the aim of retarding biological colonization for as long as possible. A few scholars have studied new repair mortar materials that prevent or retard biological growth, especially for application on archaeological sites. Mortars have a high bioreceptivity given their high porosity and surface roughness, which favors their colonization by microorganisms and plants, as shown in Figure 6.

Antimicrobial activity was obtained by adding different copper compounds, biocides, water repellents or water repellents plus biocide to mortars (Quaresima et al., 1997; Ferone et al., 2000; Nugari and Salvadori, 2003b). Mortars treated with water repellents are effective in preventing algae and lichen colonization. However, the development of fungi seems to be significantly favored by this treatment (Mansch et al., 1999). These results were confirmed by Urzì and De Leo (2007): fungi, in particular, black fungi, were the main colonizers of mortars treated with water repellents as they require less water to develop and can grow on various polymers (Koestler, 2000).

The application of water repellents and biocides in a single step (the products were mixed together before the application) is the most effective approach to retard biological growth in culture; it has been effective up to 15 months after the treatment, especially against algae and bacteria (Urzì and De Leo, 2007). However, there are no data to compare the effectiveness of each product separately. A commercial product, based on silane derivatives

FIGURE 5. (a) Detail of a limestone sculpture treated with a water repellent, showing the resulting streaked pattern where biocolonization develops (Villa Pisani, Stra, Italy). (b) Detail of the garden wall of the Palace of Belem (the residence of the president of Portugal) in Lisbon. Note that only the render is water repellent, showing the streaking effect. On nonhydrophobized surfaces, such as the stone in the arch and around the plaque, colonization is more diffuse.

FIGURE 6. (a) A brick wall near the archaeological area of Ostia Antica (Italy) where no capping was installed. The horizontal surfaces capture the water that is retained in the wall, favoring the development of the whole range of colonizing species. (b) The same site some years after the installation of the capping.

and a mixture of biocides, has been shown to provide effective protection even after five to seven years, with little biological colonization developing (L. Borgioli, C.T.S., Altavilla Vicentina (Vicenza), Italy, personal communication). Another example of good performance of an application of a mixed solution (alkylalkoxysilane plus a pyridine derivative) has been reported for sandstone where little biocolonization had occurred after six years (Gomez-Bolea et al., 1999). This same treatment displayed good results on bricks and mortars but not under continuous rising damp conditions (Ariño et al., 2002), confirming once again that the long-term effectiveness of these products depends on the microenvironmental conditions and, in particular, the water content of the substrate (Caneva et al., 2008).

The application of water dispersions of zinc- or tin-containing polymers follows the idea of the metal strips (Wessel, 2003). These form a polymeric film on the stone surface, where the slow release of the metal ions serves to prevent recolonization. So far, these products have only been tested in the laboratory, where their bactericidal and fungicidal properties have been demonstrated (Chernorukova et al., 2004).

A similar approach was used by the addition of metallic copper, either as fine fibers or as a powder, into the mortar mix. It was found that 0.35% by weight of this element was sufficient to retard colonization for more than nine years (Henriques et al., 2007). An important factor is the physical form since the fine powder was far more effective than the same amount of metal in fine fibers. However, it is important to point out that the mechanical properties of the mortar, such as compressive strength, flexural strength and, to a lesser degree, the elasticity modulus were reduced. The porosity and, consequently, the water absorption were also reduced, and there was a slight change in color of the mortar toward a bluer shade.

NEW TRENDS IN PREVENTING BIOCOLONIZATION

The European project Biofilm Inhibitors of Damage on Materials (BIODAM) (Alatomi et al., 2004; Young et al., 2008) proposed a multiphase approach to eliminate biofilms or prevent their formation. For this purpose biocides were paired with other chemical substances, such as permeabilizing agents, pigment and exopolymer inhibitors, and photodynamic agents, that increase the vulnerability of microorganisms. This approach would reduce the amount of biocide required, an important point that affects not only conservators but the environment as a whole. Permeabilizing agents serve to increase the permeability of the cellular membrane, thus increasing the penetration rate of the biocide. Several permeabilizing agents, e.g., polyethyleneimine, meso-2,3-dimercaptosuccinic acid, nitrilotriacetic acid, bis(2-ethylhexyl) sulfosuccinate, and ethylenediaminetetraacetic acid (EDTA), have been tested on biocide-resistant *Pseudomonas aeruginosa* successfully.

Pigment inhibitors serve to block the development of pigments in the microorganisms without causing them to die. Thus, compounds such as tricyclazole, arbutin, and carpropamid inhibit the formation of melanin and other dark pigments in fungi, and cerulein inhibits the synthesis of carotenoids in bacteria. This approach addresses the chromatic problem posed by various microorganisms but does not prevent the growth of the organisms. Furthermore, the presence of melanin serves as an efficient protective barrier to several environmental factors as well as to biocides. Thus, reducing the amount of this pigment will make the microorganisms more vulnerable to these agents.

It is known that the EPS play a crucial role in the formation and survival of the biofilm and its adherence to the substrate. Therefore, substances such as bismuth derivatives that inhibit the synthesis of exopolysaccharide synthesis may serve to actually prevent the formation of the biofilm.

Some photodynamic agents, once activated by light, can produce free radicals that can destroy any nearby cells or damage their cellular membranes. Among these, nuclear fast red and methylene blue are capable of annihilating cyanobacteria on stone, and since they degrade with light, they do not cause any staining problems on the substrate. Although laboratory studies proved encouraging, outdoor pilot testing on sandstone treated with these chemicals proved inconclusive (Young et al., 2008).

Antifouling agents, i.e., substances that prevent the establishment, adhesion, and growth of microorganisms under water, could also prove effective for preventing biocolonization. Cuzman et al. (2008) tested natural antifouling agents, some derived from plants, such as capsaicin and cinnamaldehyde, and some from marine animals, such as 3-poly-alkyl piridium salts, *Ceramium botryocarpum* extract, and zosteric acid. These were applied on marble or glass samples immersed in water to which a biological mixture of cyanobacteria, algae, fungi, and protozoa was added. The antifouling compounds were mixed with a silicone water repellent and an ethyl silicate consolidant. A significant reduction of colonization was observed in comparison to that obtained by treatment with either the water repellent or the consolidant alone, even when these were mixed with a commercial biocide (Algophase).

CONCLUSIONS

The new products being developed show promise. However, until these are available for general use and their effectiveness has been proven and their limitations defined, the best approaches to reduce biocolonization and the deterioration that it induces on the substrate are (1) modification of the environmental conditions, whenever possible, and (2) development of a regular maintenance program after the conservation or restoration intervention. This could be limited to the removal of any accumulation of dust or soiling, even without the application of a biocide.

It is obvious that further studies are needed with regard to the interaction between biocides and water repellents and mixtures thereof. These studies should focus not only on the initial hydrophobization or biocidal properties but also on their long-term effects as well as the interactions that may develop between the products with time.

Also, it would be desirable that any compound added to a commercial biocidal formulation that will affect the performance of the active ingredient should be listed. It is evident that this will require the development of a code of practice or a code of ethics for industry and commerce as well as international standards for product specifications. For this to occur, the importance of the issue has to be understood.

In conclusion, it is fundamental to be able to determine when biocolonization is desirable and does not pose a problem, when it is merely an aesthetic issue, and when it is a serious deterioration factor. This is probably the most difficult task yet to be solved.

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New Environmentally Friendly Approaches against Biodeterioration of Outdoor Cultural Heritage

Francesca Cappitelli, Federica Villa, and Claudia Sorlini

ABSTRACT. Microbial biofilms have a role in the weathering of stone exposed to open air. It is a common practice to use toxic products to control microbial fouling. The aim of the present research was the development of an innovative, green, and cost-effective technology for the prevention of biodeterioration on outdoor cultural heritage surfaces. Therefore, the effects of a synthetic analogue of capsaicin (from chili peppers) and zosteric acid (from eelgrass, *Zostera marina*) in preventing the development of detrimental biofilms were studied. In particular, the use of zosteric acid was an effective biocide-free strategy against both bacterial and fungal biofilms. Zosteric acid caused a more than 90% reduction of *Escherichia coli* and *Bacillus cereus* adhesion, whereas coverage of *Aspergillus niger* and *Penicillium citrinum* was reduced by 57%. Under different environmental conditions, zosteric acid reduced *Candida albicans* (the model fungus for fungal biofilm studies) biofilm formation by at least 70%.

THE NATURE OF BIOFILMS

Bacteria have two modes of growth: planktonic and sessile. It is believed that planktonic cells suspended or growing in a fluid environment are important for rapid proliferation and spread into new habitats, whereas slow-growing sessile cells grow attached to a surface and act in a multicellular organized manner. Biofilms are defined as sessile microbial communities embedded in self-produced extracellular polymeric substances (EPS) on either organic or inorganic substrata (Costerton et al., 1995). Biofilms are ubiquitous and can be found on practically any surface, including those of cultural heritage, such as buildings and monuments.

Biofilms include bacteria, algae, and fungi that are responsible for the microbial deterioration of cultural heritage. A further problem associated with biofilm formation includes biofouling, the undesirable accumulation of microorganisms, plants, and animals on a surface (Whelan and Regan, 2006). Bacteria are generally the first organisms to foul surfaces exposed to different environments, through adhesion and subsequent biofilm formation.

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The formation of microbial biofilms begins with the reversible adhesion of a small number of cells to a surface. On the abiotic surface, initial attachment between bacteria and the surface is governed by nonspecific interactions, such as electrostatic, hydrophobic, or van der Waals forces. Initial studies have proved that the rate of bacterial adhesion to a wide variety of substrata is responsive to some physical features, such as hydrophobicity. After binding to the surface through a gluelike matrix, bacterial cells begin the process of irreversible adhesion, proliferation, and accumulation as multilayered cell clusters. The attached cells synthesize new exopolysaccharide material in order to cement their adhesion to the surface and to other cells in the developing biofilm. These extracellular matrices, composed of a mixture of materials like polysaccharides, proteins, nucleic acids, and other substances, are considered to be essential in holding bacterial cells together in the biofilm structure, in helping to retain nutrients for cell growth, and in protecting cells from dehydration and other stresses. This phase is characterized by active binary division of attached cells and cell recruitment. Cell accumulation requires coordinated efforts from the microbial community to produce a well-organized structure. After having irreversibly attached to a subtratum, bacterial cells undergo phenotypic changes. Mature biofilms typically consist of differentiated mushroom-like structures of cells embedded within the extracellular polymer matrix. This contains voids open to the bulk fluid to allow the transport of nutrients and oxygen from the interface to the inner parts of the biofilm and for the removal of metabolic wastes. Bacterial cells detached from the biofilm reenter the planktonic state and may start a new biofilm formation cycle.

Microbial biofilms display discrete temporal and spatial organization (Pace et al., 2006). There are many microenvironments within a biofilm. The metabolic activities of the cells, together with diffusion processes, result in concentration gradients of nutrients, signaling compounds, and waste products within the biofilms. A variety of conditions are governed by local pH, oxidizing potential (redox), and other parameters. Cells close to the surface are exposed to high oxygen concentrations, whereas in deeper layers oxygen is hardly available. Gradients for either nutrients or metabolites from the biofilm create a heterogeneous environment even for a single-species biofilm. Within a biofilm community, cells with diverse genotypes and phenotypes follow distinct metabolic pathways, stress responses, and other specific biological activities, although they are located side by side (metabolic heterogeneity) (Stewart and Franklin, 2008). In order to develop preventive conservation strategies for biofilm control, it is necessary to fully understand the mechanisms involved in initial attachment, development of the biofilm phenotype, maturation and detachment, and the related regulatory processes.

THE ACTION OF BIOCIDES

There are no current indexes of microbial biodeteriogenic activity that are widely accepted by the scientific community involved in the conservation of stone objects or structures. Such indexes would be very helpful to plan a case-by-case conservation action compatible with the principle of minimal intervention (Gazzano et al., 2009). For decades, abatement of microbial growth has commonly been achieved by applying biocides to surfaces. Biocides are chemicals with an active, and in general toxic, effect on living organisms. European Community Directive 98/8/EC of the European Parliament addresses the issue of placing biocidal products on the market. In particular, it concerns the authorization for the marketing of biocidal products within the member states. At the European Community level this directive established a list of approved active substances that may be used in biocidal formulations. It aims to ensure that a high level of protection is provided for users, the public in general, and the environment since it has long been recognized that biocidal effects may extend beyond the target organism with resulting undesired adverse effects to man and the environment (Rasmussen et al., 1999). On the basis of the hazard assessment and risk characterization for human health, the European Commission has banned the use of some frequently used active substances, including some commonly used in conservation practice.

In recent years, the inherent resistance of biofilm microorganisms to biocides has been found to be generalized. Bacteria within a biofilm display a variety of phenotypes, providing the community as a whole with an enormous capability to resist biocides. Treatments with traditional doses of biocides are insufficient to destroy all of the biofilm population. Bacteria embedded in the biofilm matrix are remarkably more tolerant to biocides, up to 1,000-fold relative to planktonic cultures of the same bacterial strains, depending on the species-drug combination.

In addition, organic biocides might serve as nutrients; therefore, they must be used carefully in conservation practice (Krumbein and Gross, 1992; Leznicka, 1992; Warscheid, 2000). Dead cells may also provide nutrients for subsequent colonization; therefore, leaving dead cells on the surface is not advisable (Flemming, 1998).

Finally, after exposure to the killing effects of biocides, a small surviving population of persistent bacteria

can repopulate the surface immediately and can acquire enhanced resistance to further biocidal treatment.

A number of biofilm characteristics contribute to the biocide resistance of biofilm cells, such as the presence of a diffusion barrier provided by the EPS, the interaction with the exopolymers, the slow growth mode of sessile cells, the multicellular nature of the biofilm, and the possible genetic expression of certain resistance genes. A biofilm extrapolymeric matrix has the potential to reduce the penetration of biocides either by physically slowing their diffusion or chemically reacting with them. Sessile cells generally have low metabolic activity, thereby resulting in a reduction of the antimicrobial susceptibility. Genetic studies indicated that, in general, more genes are expressed in biofilms than by planktonic cells. Finally, it is thought that persisters, a subpopulation of extremely resistant cells, are responsible for biofilm regrowth when the treatment is discontinued (Pace et al., 2006).

CELL-TO-CELL COMMUNICATION SYSTEMS

Cell-to-cell communication systems regulate the expression of a panel of genes, allowing microorganisms to adapt to modified environmental conditions. Bacteria organize themselves structurally though the synthesis of, and response to, intercellular signal compounds (small diffusible molecules). The ability to coordinate gene expression in accordance with population density and hence to act as a group is a process termed quorum sensing (QS) (Balaban, 2008). "Quorum" is the number of individual cells needed to induce an activity. In this process bacteria produce diffusible hormone-like molecules (autoinducers) that interact with specific receptors on themselves and on neighboring cells, which, in turn, regulate the expression of specific target genes. By integrating this with other environmental stimuli, bacteria are capable of exhibiting complex responses and take part in sophisticated interactions, allowing them to survive in adverse environments. A large number of bacterial species are known to possess this communication mechanism, through which bacteria can sense changes in their environment and coordinate gene expression in favor of the survival for the entire community. To date, several types of QS systems are known: one for gram-positive bacteria relying on polypeptides and another for gram-negative bacteria mediated by *N*-acyl homoserine lactone (AHL) derivatives. A third type of QS system, AI-2, has been proposed as a global signaling system common to all bacteria (Pace, 2006). Quorum sensing in many bacteria regulates a number of physiological activities, including those that involve biofilm formation.

NEW STRATEGIES TO PREVENT BIOFILM FORMATION

Although it is evident that alternatives to the use of biocides for biofilm treatment are needed, very few studies in the conservation field have investigated them. The first step toward this direction was the EU project "Inhibitors of Biofilm Damage on Mineral Materials" (BIODAM). It evaluated the combination of biocides with (1) permeabilizers, (2) special slime (EPS) blockers, (3) pigment inhibitors, and (4) photodynamic treatments.

However, to the best of our knowledge, no study in the conservation field has investigated the prevention of bacterial cell adhesion to the substratum with nontoxic, environmentally friendly compounds and the disruption of cell-to-cell communication involved in biofilm formation through physical, chemical, and biological approaches, as proposed for other research areas (Flemming et al., 1996). The need for environmentally benign antifouling technologies has led to renewed interest in the ways that organisms protect themselves against predation. Therefore, natural nontoxic antifouling compounds represent an attractive strategy. These antifouling compounds have been isolated mainly from marine organisms that are not colonized by microorganisms. An interesting antifouling agent is zosteric acid (p-sulfoxy cinnamic acid), a natural extract from eelgrass (*Zostera marina*) that prevents biofouling by some organisms, such as algae, barnacles, and tubeworms, at nontoxic concentrations (Barrios et al., 2005). Most recently, with the further understanding of cell-to-cell communication in microorganisms, QS has emerged as one of the most important mechanisms for controlling the development of highly structured and cooperative biofilm consortia, on both organic and inorganic substrata. The QS systems offer three points of attack: (1) signal generation, (2) the molecule itself, and (3) the signal receptor. Steinberg et al. (1997) isolated halogenated furanones (secondary metabolites) from the Australian macroalga (seaweed) *Delisea pulchra*. These compounds interfered with bacterial signaling and displayed remarkable anticolonization activity (de Nys et al., 1993). Furanones protect the alga from colonization by both prokaryotes and eukaryotes and therefore are strong deterrents of the settlement and growth of both micro- and macrofouling (de Nys et al, 1993). Since this discovery, methods have been developed

to synthesize the natural antifouling molecules and their synthetic derivatives in laboratory.

The research goal proposed has been the development of this innovative, green, and cost-effective technology for the prevention of biodeterioration on outdoor cultural heritage surfaces. The results obtained so far indicate that this technology may be a powerful tool in the conservation field (Cappitelli et al., 2006; Cappitelli and Sorlini, 2008). In particular, effects of synthetic analogues to capsaicin (Villa et al., 2009) and zosteric acid in preventing the development of detrimental biofilms were studied. This is particularly relevant to materials in contact with water or exposed to outdoor conditions. Since antifouling agents play a strong and specific in situ effect against both micro- and macrofouling, one strategy considered for preventing biofilm formation is to coat cultural heritage surfaces with compounds capable of interfering with adhesion or signaling mechanisms. Antifouling agents affixed and immobilized on surfaces should inhibit bacterial attachment and retain activity for extended periods. Methods for binding antifoulants to surfaces of protectives or consolidants or for incorporating them into the products themselves are being investigated, as described in the next section.

METHODOLOGY TO SELECT POTENTIAL ANTIFOULING AGENTS

Bacteria and fungi used in the experiments were either pure or mixed cultures. It is advisable to employ microbial isolates from biofilms, especially those growing on a cultural heritage substrate (Figures 1 and 2).

All organisms require a source of carbon and energy for growth. A good candidate for an environmentally friendly antifouling agent should not be a carbon and/or energy source for the microorganisms tested. Mineral media that would not provide a source of carbon and energy were therefore prepared with the antifoulant added at different concentrations. Furthermore, the antifouling molecule was used at a concentration below the minimal biocidal concentration. To complement these studies, toxicity experiments were conducted in a rich medium to which the antifoulant was added at different concentrations. The specific growth rates with or without the antifoulant were determined via cultivation or by measuring the optical density spectrophotometrically. Alternatively, the viability of microbes was measured by staining with a LIVE/DEAD® BacLight™ Bacterial Viability Kit (Figure 3).

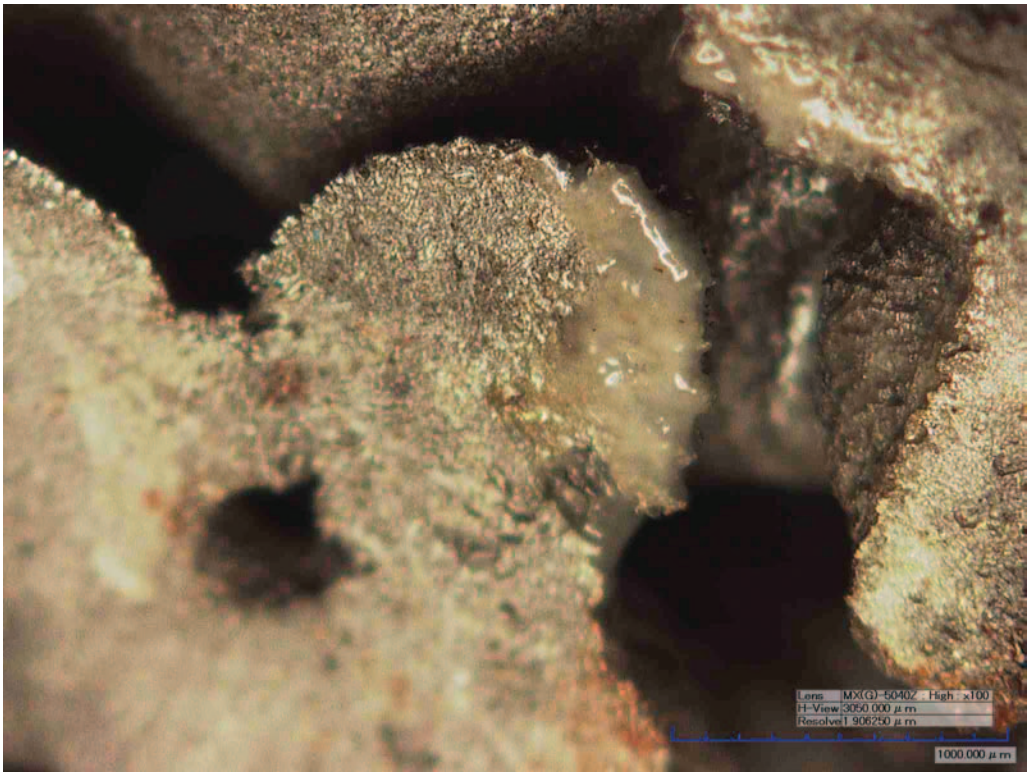

FIGURE 1. Bacterial biofilm on a metal surface (image taken with a 3D HIROX KH-7700 video-microscope).

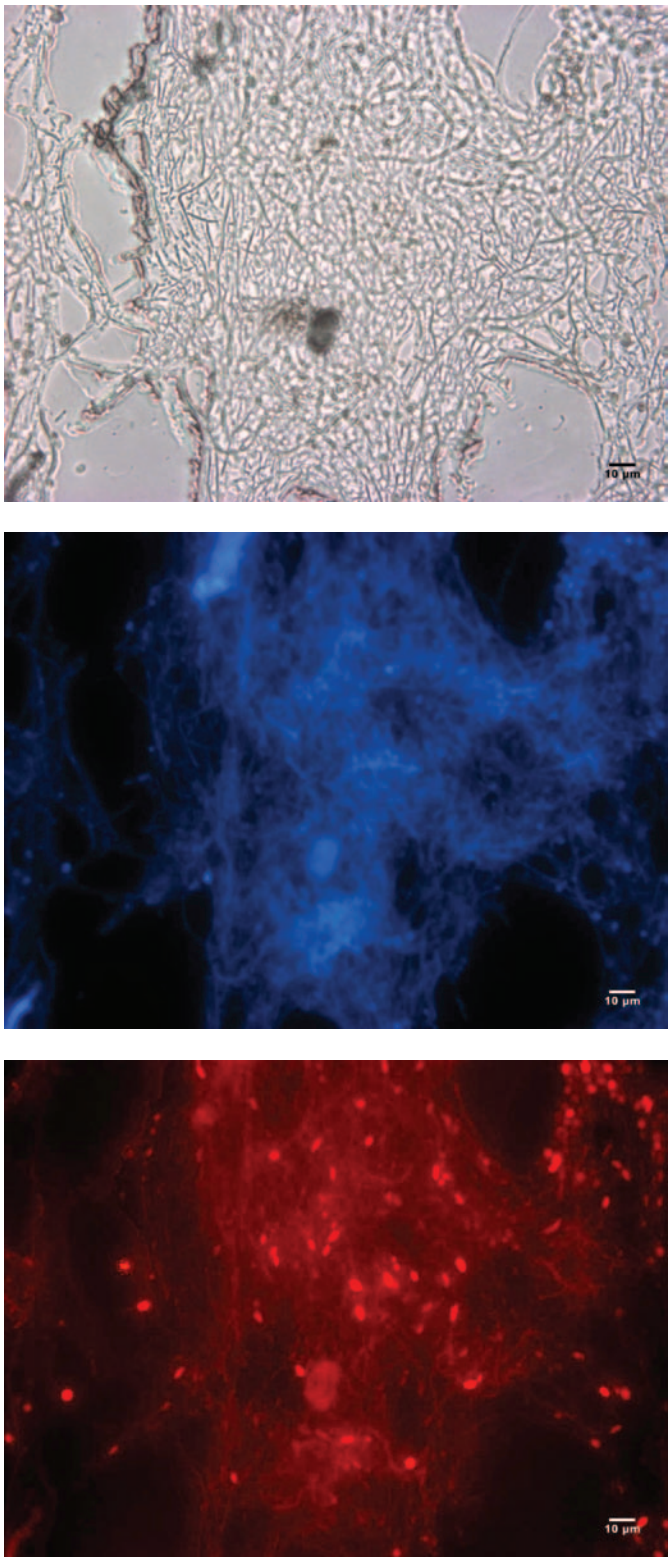

FIGURE 2. (Top) Phase contrast and epifluorescence images of biofilm on adhesive tape strips sampled from an outdoor wall showing (middle) DAPI staining and (bottom) hybridization with the universal Eukarya probe EUK 516.

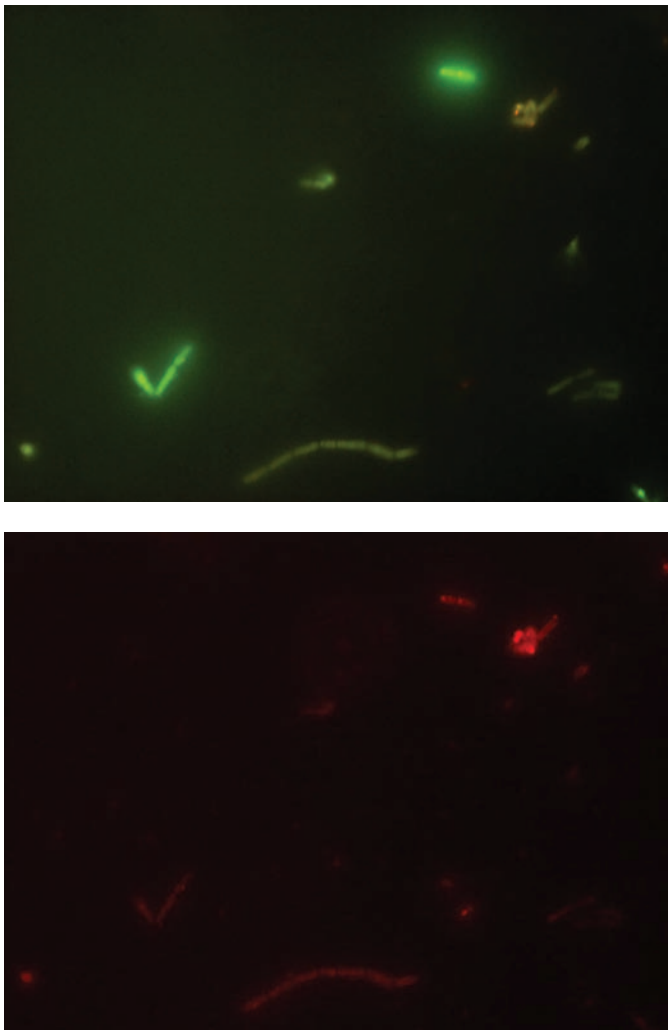

FIGURE 3. A LIVE/DEAD® BacLight™ Bacterial Viability Kit was applied to estimate both viable and total counts of bacteria. BacLight is composed of two nucleic-acid-binding stains: SYTO 9™ and propidium iodide. SYTO 9 penetrates all bacterial membranes and stains the cells green (top), whereas propidium iodide only penetrates cells with damaged membranes, producing red fluorescent cells (bottom). The pictures show that the tested molecule acted as a biocide, killing all cells.

To statistically validate the investigation, biofilm tests were carried out in 96-well microtiter plates. There are different kinds of microtiter plates in terms of their hydrophobic or hydrophilic character (Figure 4a).

Adhesion can be assessed quantitatively using fluorochrome-labeled cells incubated on various black microtiter plate test substrata. Fluorescence was measured using a plate reader (Figure 4b). For each experiment, a standard fluorescence intensity curve versus cell number (as measured by direct counting) and microorganism type was prepared.

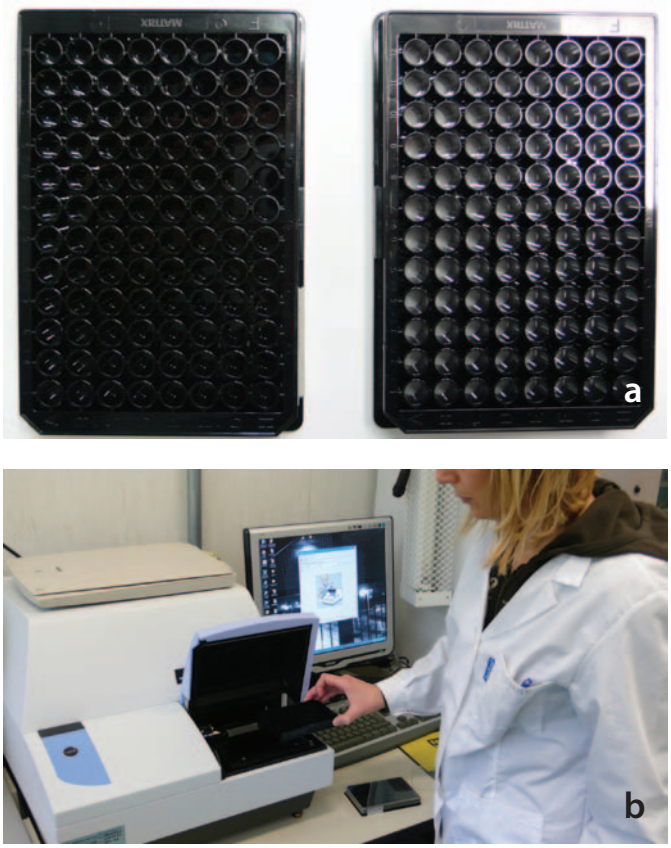

FIGURE 4. (a) The 96-well plates for (left) hydrophobic surfaces and (right) hydrophilic surfaces. (b) Multilabel plate readers by Perkin Elmer.

There are several parameters that should be taken into account to evaluate the efficiency of an antifouling molecule. The most important are the following: type of microorganism, type of surface (hydrophobic or hydrophilic), antifoulant concentration, pH, time, and temperature. In the past, the assay optimization process has typically taken between 4 and 12 months, using traditional one-factor-at-a-time experiments (Altekar et al., 2006). However, statistically designed experiments are a powerful tool for improving the efficiency of experimentation. The technique of design of experiments (DOE) is an important link between the experimental and the modeling world. It requires only a small set of experiments where all the variables are analyzed simultaneously within fixed ranges (selected so they do not inhibit cell growth) according to rigorously formulated mathematical protocols. Besides identifying the variables that most influence the results, DOE serves to identify possible interactions and synergies among factors. With this multivariate approach it is possible to obtain a model describing the performance of potential antifoulants in complex scenarios.

Although the microplate assay demonstrates the antifouling properties of a new compound, it does not predict its impact on biofilm architecture. Laboratory-scale biofilm reactors combined with microscopic techniques are extremely valuable for discovering the fundamental characteristics of biofilms (e.g., the spatial and temporal patterns of microbial colonization, specific biofilm components, interactions, and cellular activities), providing useful insights for developing effective antifouling technologies.

Once a compound has been proven to be effective against bacterial attachment, a delivery system must be found. This could be a protective or a consolidant, e.g., a synthetic polymer (e.g., Villa et al., 2009).

RESULTS

Using the above-mentioned techniques, the antifouling potential of *N*-vanillylnonanamide and zosteric acid was investigated for both bacteria and fungi. Before the adhesion tests, it was proved that neither the synthetic capsaicin nor zosteric acid was toxic or represented a carbon and energy source for all the tested microorganisms. Microbial attachment assays on glass and polysine slides (special glass microscope slides with a permanent adhesive) were carried out with *N*-vanillylnonanamide in dispersion and were applied onto the surfaces using a polymer coating (Villa et al., 2009). It required 205 µM (µmol/L) of *N*-vanillylnonanamide to inhibit *Bacillus* adhesion by 48% on glass slides. The same compound blended into or sprayed onto a polyurethane coating at 205 µmol/kg showed that this capsaicinoid did not have any substantial impact on preventing adhesion.

As the results from *N*-vanillylnonanamide investigations were not completely satisfactory, attention was focused on the new potential antifoulant zosteric acid. For the studies, zosteric acid was successfully synthesized by Domenico Albanese (Dipartimento di Chimica Organica e Industriale, Università degli Studi di Milano). Using the DOE method, it could be shown that the antibiofilm activity of zosteric acid is species specific, causing a more than 90% reduction of *Escherichia coli* and *Bacillus cereus* adhesion, whereas *Aspergillus niger* and *Penicillium citrinum* coverage was affected by 57% (Villa et al., 2010). Zosteric acid reduced *Candida albicans* (the model fungus for fungal biofilm studies) adhesion and subsequent biofilm formation by at least 70%. In addition, the mathematical models revealed that the molecule successfully

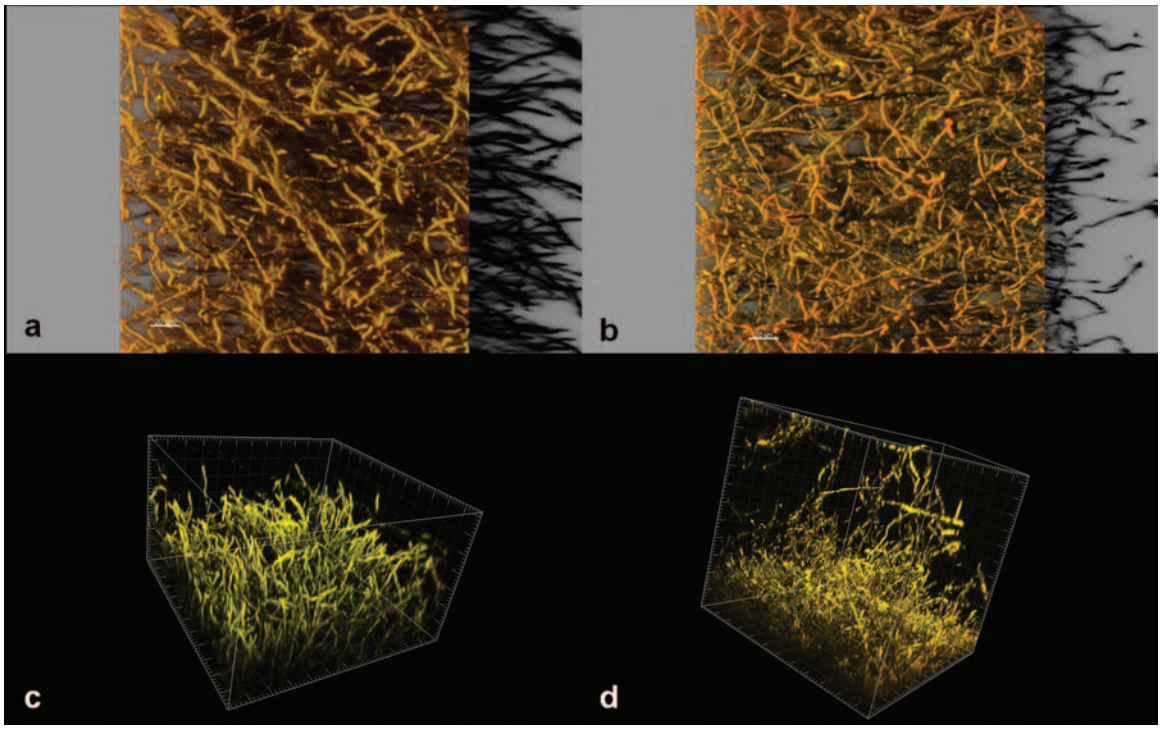

FIGURE 5. Three-dimensional projection of *C. albicans* biofilm (a, c) without and (b, d) with zosteric acid. The biofilm was visualized by confocal laser scanning microscopy after staining with the fluorescence probe FUN1. Images were analyzed with IMARIS software. Top is the view as seen from above; bottom is the view as seen sideways.

counteracted the effects of some colonization-promoting factors, such as time and temperature (Villa et al., 2010).

Experiments on *Candida albicans* showed that zosteric acid strongly impacted biofilm thickness and morphology, resulting from the inability of fungal cells to form filamentous structures while maintaining metabolically active cells (Figure 5). Finally, zosteric acid enhanced their sensitivity to different traditional biocidal treatments, causing a decrease in biofilm resistance of 0.5–8 log units.

By virtue of its ability to reduce microbial adhesion, modify the shape of fungal biofilm architecture, and enhance the performance of antimicrobial agents, zosteric acid should be further tested and developed as a means of preventing biofilm formation, by itself or integrated into a more complex protective system.

CONCLUSIONS

Although more research is still needed, environmentally friendly alternatives to toxic biocides to prevent biofilm formation represent a very promising method to control biocolonization. It is hoped that conservators will be eager to use these alternatives to biocides when they are technically available.

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Bioremediation of Algal Contamination on Stone

Eric May, Dania Zamarreño, Sarah Hotchkiss, Julian Mitchell, and Robert Inkpen

ABSTRACT. Green algae are common colonizers of stone throughout the world. Research suggests that algae do not cause biodeterioration directly, but the growth of such organisms facilitates colonization by other organisms. Standard treatment methods to date have centered on abrasive and chemical treatments rather than biologically based solutions. It is widely accepted that risk assessments for proposed treatments should include the potential for regrowth or accelerated growth and any impact upon the structure of the stone. In addition to these side effects, the use of chemical treatments is increasingly subject to tighter control from an environmental aspect. Significant research has been carried out on naturally occurring algicides for control of aquatic algal species. Work in the aquatic field suggests that some effective treatments for dealing with growths of algae exist, but caution and extensive testing are likely to be needed to assess the viability of these treatments to cross over to terrestrial heritage environments. In recent years, ecological studies of naturally occurring biological control of aquatic algae have focused on viruses, which cause lysis and subsequent death of the host organism. Viruses are found in sediments and are believed to be important in natural control of algal populations. This paper will describe pilot studies to assess the relative effectiveness and impacts of bioremediation by viruses for removal of growths of algae from Portland limestone.

INTRODUCTION

Green algae are common colonizers of stone throughout the world, and reports of the growth of this group of organisms on buildings and monuments come from across Europe (Rifón-Lastra and Noguerol-Seoane, 2001; Darienko and Hoffmann, 2003), southeast Asia (Uchida et al., 2000; Lee et al., 2005), Central America (Ortega-Morales et al., 2000), North America (Koestler et al. 1985), and South America (Crispim, 2004; Gaylarde and Gaylarde, 2005). Standard treatment methods to date have centered on abrasive and chemical solutions rather than biologically based solutions.

The factors affecting growth of algae and other phototrophic organisms on monuments were reviewed by Ortega-Calvo et al. (1995). They found no proof of biodeterioration directly caused by algae but point out that the growth of such organisms represents a significant input of organic matter to the stone, which would, in turn, facilitate colonization by other organisms. They stressed the importance of understanding the potential for synergistic interactions among the

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microbial communities within a stone ecosystem. Young (1997), in a study examining algal growth on sandstone, found that the change in composition of a "natural" soiling layer was leading to a change in the biological community colonizing the stone as resident pollution products such as sulfate were removed. Differences in algal growth were found to be related to the chemical cleaner used; in some cases a higher growth rate and greater algal growth were found on chemically cleaned surfaces than on untreated surfaces. MacDonald (1993) noted the retention of chemicals in the pores of the stone following cleaning. Young (1997) compared pore size and phosphate residues after treatment and concluded that the latter was responsible for increased algal growth but that the effect of phosphate varied between sandstones, depending on their mineral composition (see also Young at al., 1995). This suggests that any proposed treatment needs to be assessed over an extended period (two years or more) to assess the potential for both regrowth and accelerated growth. Additionally, any proposed treatment will need to be assessed for its impact upon the structure and subsequent behavior of the stone microflora (Tayler and May, 1994).

Significant research has been carried out on naturally occurring algicides for control of aquatic algal species. Sources for these algicides have included higher plants such as barley straw (Ball et al., 2001), *Juncus acutus* (DellaGreca et al., 2003), and *Potamogeton natans* (Cangiano et al., 2001); marine algae (König et al., 1999); and fungi (Jenkins et al., 1998). With respect to bacteria, two separate *Bacillus* species have been identified with antimicrobial activity against cyanobacteria (Reim et al., 1974; Nakamura et al., 2003). Work in this field suggests that existing effective treatments for dealing with algae growth exist, but caution and extensive testing are likely to be needed to assess the ability of these treatments to cross over to terrestrial environments. Some work has been carried out already on such "crossover" treatments. Choi et al. (2005) reported the use of *Streptomyces neyagawaensis* in suppressing the growth of cyanobacteria and a wide range of algae, including the epilithic green algae *Chlorella*, several species of which were found on granite walls in northwest Spain (Rifón-Lastra and Noguerol-Seoane, 2001) and on building facades by Schumann et al. (2005). Such studies are, however, limited, and no standard methodology for testing has been developed. In recent years, ecological studies of naturally occurring biological control of aquatic algae have focused on viruses, which cause lysis and subsequent death of the host organism. Viruses are found in sediments and are believed to be important in natural control of algal populations (Bratbak et al., 1996; Suttle, 2002). Numerous viruses have been identified that infect algae, including the chloroviruses that infect *Chlorella*-like green algae (Kang et al., 2005).

This paper describes attempts to investigate the relative effectiveness and impacts of bioremediation by viruses for the removal of algae growths on Portland limestone gravestones. The research strategy had three components: (1) to identify the nature of the problem on gravestones and other Portland Stone surfaces, (2) to isolate viruses with antialgal activity from mature biofilms, (3) to test the effectiveness of different viral treatments in laboratory pilot studies.

METHODS

Study Site and Sampling Regime

The study site was Brookwood Military Cemetery in Surrey, United Kingdom, which is administered and in the care of the Commonwealth War Graves Commission. Headstones were chosen from sections 11 and 12 (British and Australian) of the cemetery (Figure 1), where algal contamination was known to be a serious problem during the winter months. All headstones were of Portland limestone and had a similar aspect (facing northwest) but were influenced to varying degrees by overhanging or shading vegetation. Most headstones had splash plants in front of them, but these varied in size, shape, and form. All headstones

FIGURE 1. Study headstones at Brookwood Military Cemetery. All headstones are of similar aspect and known age, but environmental conditions vary. The oak tree to the right and stand of trees to the left were considered very likely to have an effect on algal growth.

were originals and therefore of a known age, but they were a mix of first and second World War headstones.

Image Analysis

High-resolution digital photographs were taken of the front and back of each gravestone (Figure 2). Three zones of biological contamination were identified on most stones, at the crown, central area, and base of the headstone (Figure 2). Using the Corel Photopaint v11 software package, the surface area of each zone of contamination was measured, and percent cover for crown, central area, base, and total area of contamination was calculated.

Biological Sampling

Biological material was removed from representative headstones using sterile razor blades and then placed in sterile containers for transportation to the laboratory. Small surface scrapes were taken where possible in all three algal contamination zones on back and front of headstones. Every effort was made to ensure that minimal physical damage was inflicted on the surface of the headstone and that no unsightly marks were left.

In the laboratory, a portion of each sample was suspended in sterile water and observed under 40× and 100× magnification using a Leitz Laborlux S microscope. Digital images were taken using a Leica IM1000 image capture system. Images were collected for all observed taxa from each sample in order to estimate the biological diversity of each sample. A digital record of diversity was prepared.

Culturing

Biological samples were cultured in the laboratory using modified Knops medium and an ambient temperature and

FIGURE 2. Image analysis of a representative second World War headstone. (left) A digital image of the back of the headstone with three zones of algal contamination at the crown, central area, and base. (right) Extracted zones of algal cover (using Corel Photopaint) from which the percentage area cover of algal contamination was calculated.

light regime. The medium was chosen for its suitability for culturing both algae and cyanobacteria and the growth regime, as it represented the most natural scenario. Algal cultures appeared to be reaching a level of growth suitable for experimental manipulation in approximately one month. Portland limestone test surfaces were inoculated with algal cultures for use in subsequent phases of the project. Solid and liquid cultures containing small Portland limestone discs approximately 15 mm in diameter and larger discs approximately 4 cm diameter and 1 cm thick were inoculated with purified algal cultures and virus dilutions.

Assessment of Algal Cover

The algal contamination percentage cover and the biological diversity data were analyzed using the PRIMER v6 software package. PRIMER is a multivariate statistical package specifically developed for the analysis of complex ecological data (Clarke and Warwick, 2001; Clarke and Gorley, 2006). The underlying strategy was to highlight any patterns of similarity or dissimilarity across samples of data on the basis of their biotic composition. The results were then compared with known or hypothesized interrelations between predetermined groups of samples. In this research, the predetermined groups were samples taken from groups of headstones influenced by different environmental factors. Age of headstone, front versus back of headstone, splash plant, proximity of tree cover and/or shading, and whether headstones were placed in singles, doubles, or triples were all investigated.

A typical output of this kind of analysis is an ordination (Figure 3) based on multidimensional scaling (MDS). The ordination gives a two-dimensional visual representation of how similar or dissimilar the groups of samples are and thus provides a tool for rapid analysis of biological patterns. The example given in Figure 3 shows two distinct groups of samples; in this case it shows a subsample of the complete Brookwood data set. Group 1 represents first World War headstones, and group 2 represents second World War headstones.

RESULTS

Surveys to Determine the Extent of Algal Cover

The aim of the surveys was to assess the effectiveness of image analysis as a tool for monitoring patterns of algal colonization on headstones and the effects of any treatment process. The technique proved to be extremely accurate and rapid, and high-quality digital images of selected headstones were archived. These were used for comparative studies with images taken during future surveys with the aim of monitoring any changes to seasonal patterns of algal contamination and also for comparing headstones that have and have not been treated using bioremediation.

During September 2006 there were distinct differences in patterns of algal growth on selected headstones, and these varied over broad scales throughout the study area. However, headstones experiencing similar environmental conditions had similar levels of algal contamination. The percentage cover analysis revealed distinct groups of headstones that reflected known environmental patterns. Figure 4 shows an MDS ordination in which groups of headstones are evident and have been highlighted by colored circles.

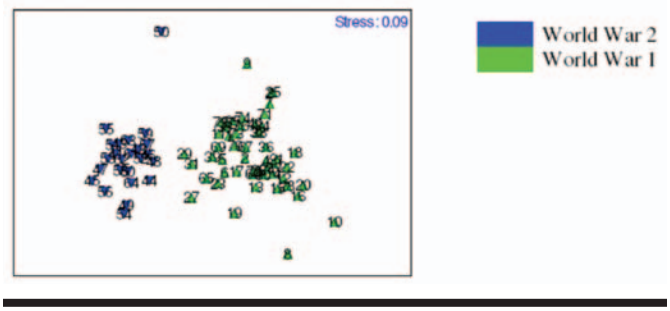

FIGURE 3. MDS ordination from PRIMER percentage cover analysis of algal contamination on headstones. Two distinct groups of samples can be seen in this ordination, although there are some outliers. Each number represents either a first or second World War headstone. The closer the samples group are together in the ordination, the more similar they are in biotic composition.

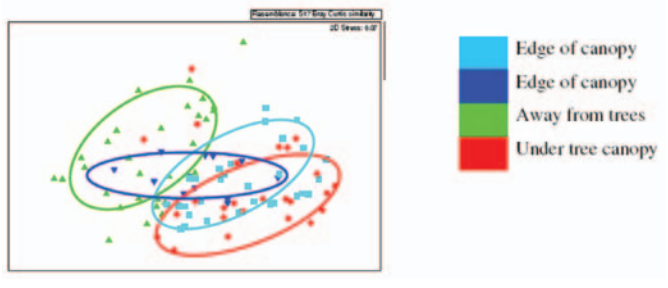

FIGURE 4. MDS ordination showing similarity in percentage cover of algal contamination between groups of headstones in September 2006. The four color-coded groups represent groups of headstones influenced by varying levels of tree cover.

As would be expected with any study of this kind, there was much variability, and some samples did not necessarily group with others, but essentially, the samples fell into groups representing headstones affected by varying levels of tree cover along a gradient from no tree cover (green group) to full tree cover (red group). The other groups (dark and light blue) were situated at the edge of a tree canopy and thus are intermediate in their physical environment and do not separate out into distinct groups but form a continuum. It must be noted that the groups highlighted in Figure 4 are preselected and represent groups of headstones with expected differences. Nevertheless, they clearly fall along a known environmental gradient that suggests that tree cover has a strong effect on patterns of algal cover on headstones.

Although proximity of tree cover was expected to have a major effect on patterns of algal colonization, further analysis of the data showed a number of other environmental factors could be influencing the patterns of algal growth on smaller scales. Age of headstone and aspect, i.e., front versus back of headstone, also appeared to have some influence.

A second survey confirmed that broad-scale patterns of algal contamination were evident in October 2006 (Figure 5) and that the effect of tree cover was still the most influential factor. Unlike in the initial survey, an additional group of headstones were investigated that were situated close to a stand of trees but not directly beneath the canopy. These headstones were shaded for parts of the day and were in full sunlight for parts of the day. Figure 5 clearly shows a gradient in patterns of algal cover that directly corresponds with a gradient in tree cover. Algal cover on headstones under a tree canopy (red group) is different from that on headstones not affected by tree cover (dark blue). Headstones situated at the edge of the canopy (light blue group) are somewhat intermediate in algal cover. The group of headstones situated close to a stand of trees has variable patterns of algal cover, and these are similar to a range of other headstones placed throughout the study area. Given that these headstones would be influenced to varying degrees by tree cover, this result is to be expected.

Further analysis showed there was little influence from the age of a headstone (Figure 6a) or the front versus back aspect (Figure 6b) of a headstone as suggested by the first survey. The presence of a splash plant (Figure 6c) also appears to have little effect on patterns of algal cover, although this would be expected to affect only the base and possibly the central area of the headstone, and further analysis is required to fully assess this.

Characterization of Algae

Biological samples were taken from Brookwood and Portsmouth limestone structures to assess the diversity of microorganisms present. In order to develop remedial

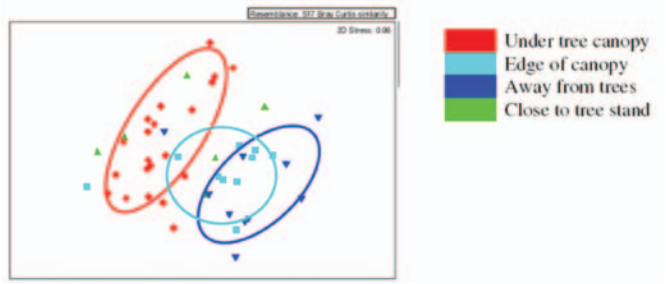

FIGURE 5. MDS ordination showing similarity in percentage cover of algal contamination in October 2006 between groups of headstones influenced to varying degrees by tree cover.

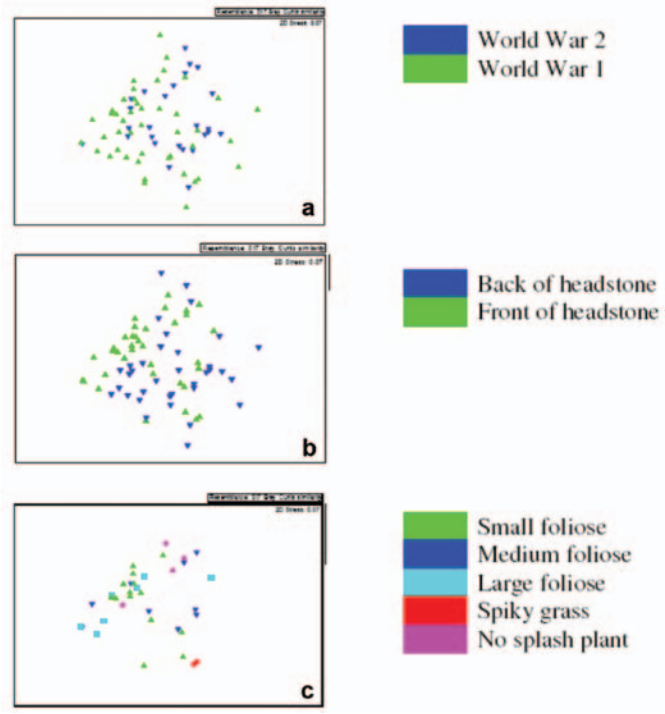

FIGURE 6. MDS ordinations of percentage algal cover data in October 2006. (a) Age of headstone, (b) front or back aspect, and (c) presence and type of splash plant appear to have little effect on patterns of algal contamination.

treatments, information was required on the number of different organisms present, their relative abundances, and their spatial variability. Microscopic analysis suggested the algal growth on the Brookwood headstones to be diverse. However, the microorganisms found in the three contamination zones on each headstone were different, but this difference was not quantified. The crown, which is the area that receives annual remedial treatment, appears to host the least diverse biological community and comprises mainly single-celled and colonial green algae, predominantly Chlorococcales, including *Chlorella* spp., *Chlorococcum*, and *Nitzchia*. The central and basal areas host far more diverse communities comprising green algae, cyanobacteria, diatoms, and golden brown algae.

Isolation of Algae and Detection of Viruses

Viral assay and growth requires individually pure cultures of significant density, and these were very difficult to obtain from the mixed algal communities isolated from stone. Stone algae grew very slowly in the laboratory, both in pure liquid culture and on stone, which impeded isolation and experimentation with viruses. Contamination of the algal cultures obtained from stone with bacteria and fungi was also a major technical problem and clearly made assay and amplification of viruses very difficult. To prevent contamination, antibiotics, ampicillin, streptomycin, and nystatin were added to the growth medium. Using this approach, environmental samples could be processed and any algae present could be grown successfully under laboratory conditions in Bold's Basal Medium (BBM) liquid and on solid media. Thirty purified algal cultures were isolated from only 1 of 70 mixed cultures obtained from Brookwood headstones growing under laboratory conditions.

In order to detect the presence of viruses in Brookwood samples, 1 mL of different algal suspensions, grown in Knop's liquid medium, was spread onto Knop's agar and incubated until an algal lawn developed, as shown in Figure 7. In some lawns, zones of clearance, or plaques, were detected, indicating the presence of virus. Some viruses were identified in the laboratory from plate cultures, producing obvious clearing, in algal lawns. This evidence further suggested that this might be a viable method for destroying algal biofilms on stone. When agar culture plates did show signs of plaques indicative of virus attack, the cleared zone was removed with a sterile razor blaze, suspended in sterile saline, microcentrifuged, and then filter sterilized (pore size of 0.2 µm) to remove cells. Suspensions were then stored at 4°C in the dark until needed.

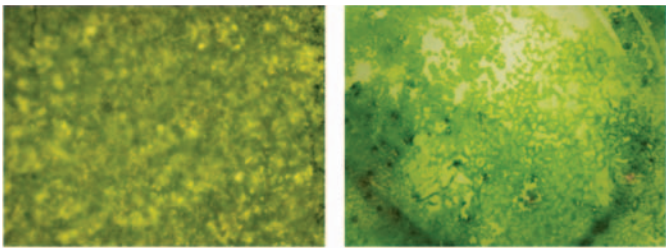

FIGURE 7. A typical view of (left) an uninfected algal lawn and (right) an infected lawn developed on Knop's agar.

Fluorescence Microscopy

Work with solid plate cultures of algae was the most promising in terms of recognition of virus activity. However, although no clear evidence of viral presence was detected in many of the mixed liquid cultures obtained from Brookwood samples, four pure cultures denominated K, I, L, and M showed some signs of viral presence in liquid cultures, indicated by reduced algal growth. Viral assay through the solid plate technique, in which the suspected liquid was added to a suspension of algal culture and poured over a solid agar surface, was unsuccessful with these four samples. However, the presence of virus particles was confirmed by fluorescence microscopy by staining with SYBR Green I stain, which targets nucleic acid and stains virus (Figure 8). By this means, virus presence was also identified in 9 out of 70 of the mixed algal cultures after centrifugation and filter sterilization, although the concentration of virus detected in the different samples seemed to be very low.

Viral Assays Using Aquatic Viruses

Since *Chlorella* was one of the predominant colonists on headstones and a common isolate from samples, pure cultures of three *Chlorella* viruses and their corresponding hosts, which had been isolated from aquatic environments, were obtained from James L. van Etten (University of Nebraska). The three different *Chlorella* viruses, PBCV-1, MT325, and ATCV-1, had *Chlorella* strains, NC64A, Pbi, and Sag 3.83, respectively, as algal hosts. The paired combinations made it possible to further develop methods for virus testing to test using these viruses against the algal isolates obtained from Brookwood headstones and investigate the concept of inhibition of algae on building stone.

In order to detect whether particular algal isolates were sensitive to virus, 100 µL of three mixed algal cultures,

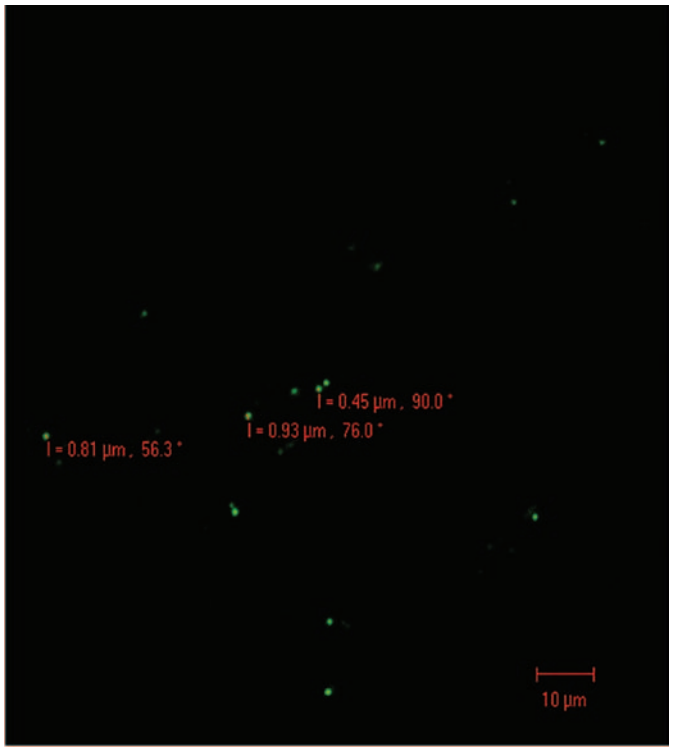

FIGURE 8. Viral particles (green dots) detected by fluorescence microscopy (SYBR Green I stain under immersion oil, × 400) in sample I3.

designated SAM1, SAM2, and SAM3, were plated onto Knop's agar, from which single colonies were developed after approximately three weeks. Eight single colonies were isolated from SAM1, denominated A to G; seven single colonies were isolated from SAM2, denominated H to N; and 12 single colonies were isolated from SAM3, denominated Ň to Y. All algal samples were inoculated into 10 mL of BBM liquid medium in plastic universal bottles and grown for one month. Viral assays to isolate the viral particles and detect sensitive algae were carried out with samples A–G and pure cultures of *Chlorella*. The viral assay carried out with *Chlorella* Sag 3.83 showed that algae did not grow at all on the plates with the highest viral concentration, producing confluent lysis, but with intermediate dilutions (8.1×10^6 plaque-forming units (pfu)/mL), viral plaques could be seen clearly (Figure 9).

Action of Viruses against Algae on Stone Discs

Small-diameter (1 cm) limestone discs were soaked in 9 mL of different viral dilutions (10^{-1}–10^{-13}) for three

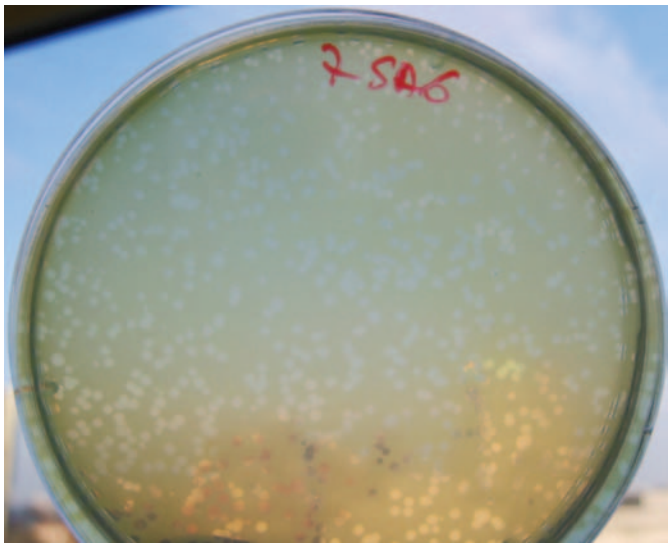

FIGURE 9. Viral assay plate prepared with *Chlorella* Sag 3.83 after four days of incubation at room temperature and natural light.

days. They were then inoculated with 100 µL of *Chlorella* NC64A and incubated in natural daylight at room temperature (25°C). Figure 10 shows the effects of virus on the growth of the algae. Whole plates were photographed 22 days after inoculation for NC64A. Algal growth could be detected in the positive control and on those discs treated with a low virus concentration. However, algae did not grow on those discs treated with high virus concentration even 22 days after inoculation.

A second series of experiments was done where large-diameter (4 cm) limestone discs were simultaneously inoculated with a growing culture of *Chlorella* NC64A and a series of dilutions of the appropriate virus and incubated on a bed of moist vermiculite without extra nutrients. It was observed that on those limestone discs that were treated with the highest concentration (dilutions 1–4) of viruses, algal growth was not established. However, when higher dilutions were applied, algal growth was established. Algal growth was not established on those discs treated with high virus concentration, even three weeks after treatment (Figure 11). On the first, second, and third week after the experiment started, the limestone discs were retreated with the corresponding virus dilutions. At this time, one of the positive control discs (showing untreated algal growth, disc g) was treated with virus (Figure 11). By comparing Figure 11a and 11b, it can be seen that (1) no algal growth occurred on discs h–k, (2) algal growth decreased in discs l–o, and (3) no effect was seen on discs

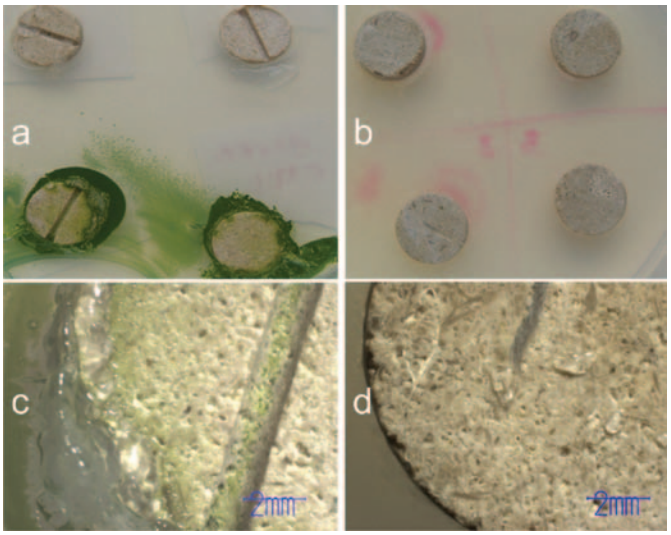

FIGURE 10. *Chlorella* growth on stone discs pretreated by submersion with different viral dilutions (PBCV-1) on Modified Bold's Basal Medium (MBBM) medium at 25°C: (a) control discs with (bottom) and without (top) algal inoculation; (b) after high virus concentration treatment, showing lack of algal growth; (c) disc treated with low virus concentration, showing algal growth on stone disc; and (d) disc treated with high virus concentration, showing complete inhibition of algal growth on stone disc. All discs were inoculated with *Chlorella* NC64A and incubated for 22 days after virus treatment.

FIGURE 11. NC64A growing on stone discs at room temperature (25°C) under natural light for (A) 2, (B) 3, and (C) 6.5 weeks after initial treatment. Discs a–c are the negative controls where no algal growth occurred, and discs d–f are positive controls. Discs h–k were treated with high virus concentration, discs l–o with intermediate virus concentration, and discs p–t with low virus concentration. The arrow corresponds to the limestone disc that was treated with a high virus concentration after algal growth was established.

p–t. Three and a half weeks after the last treatment, it was evident that algal growth had been completely inhibited and *Chlorella* NC64A had disappeared. Algal growth had also disappeared on discs k–m (high levels of virus) and was reduced in discs n–p and r (intermediate levels of virus). No effect was seen on discs q, s, and t, which had received low levels of virus.

Search for Viral Effect and Viruses on Algal Cultures from Brookwood Cemetery

Treatment of *Chlorella* growing on stone and pretreatment with viruses showed that they successfully damaged algal growth; research was directed to find out if *Chlorella* viruses were able to affect Brookwood algae. A microtiter plate technique was used to assess 25 pure or semipure algal cultures that had been isolated from Brookwood Cemetery headstones. This approach showed that six natural algal cultures were affected by the *Chlorella* viruses. When a two-phase separation technique based on PEG 6000 was used to assay 56 algal cultures from Brookwood Cemetery for viral samples, 48 samples formed an interphase, indicating the presence of virus. The different interphases were dialyzed, and the dialysis product was spread plated onto BBM agar plates to check for viral activity. Ten of these samples showed viral plaques, and these were collected and stored in Tris-HCl (pH 7.8) buffer at 4°C until use. Any sensitive algal cultures were grown in 10 mL of BBM liquid medium in 20 mL Universal plastic tubes. One of the Universal tubes was inoculated with 100 µL of the stored viral plaques. All the algal cultures grew without showing signs of viral attack.

Isolation of Viruses from Other Sources

The search for viruses was extended to pond waters taken from parks within Portsmouth that suffer algal blooms during the year. Water samples were tested for the presence of viruses that might attack algae on stone. Water samples were filtered through a 0.45 µm pore size Nucleopore filter to remove bacteria, algae, and other large particles. The different filtrates were dialyzed and stored at 4°C for further use. The filters were placed onto BBM agar plates and incubated at 25°C at constant illumination for one week. The heaviest algal growth was obtained from Victoria Park, and the lowest growth was obtained from Canoe Lake (Figure 12).

A microtiter plate technique was used to test the different water samples for the presence of algal viruses using algal isolates obtained from Brookwood headstones (Figure 13). A dilution series (tenfold) of each water type was inoculated with different algal species that had been purified from Brookwood samples. Plates from Victoria Park did not show signs of algal attack or damage, and for the microtiter plate set up with Baffins Pond filtrate, only sample K showed change or damage. However, plates prepared from Canoe Lake filtrate showed that two different algal samples, K and I, were sensitive, and dialyzed samples

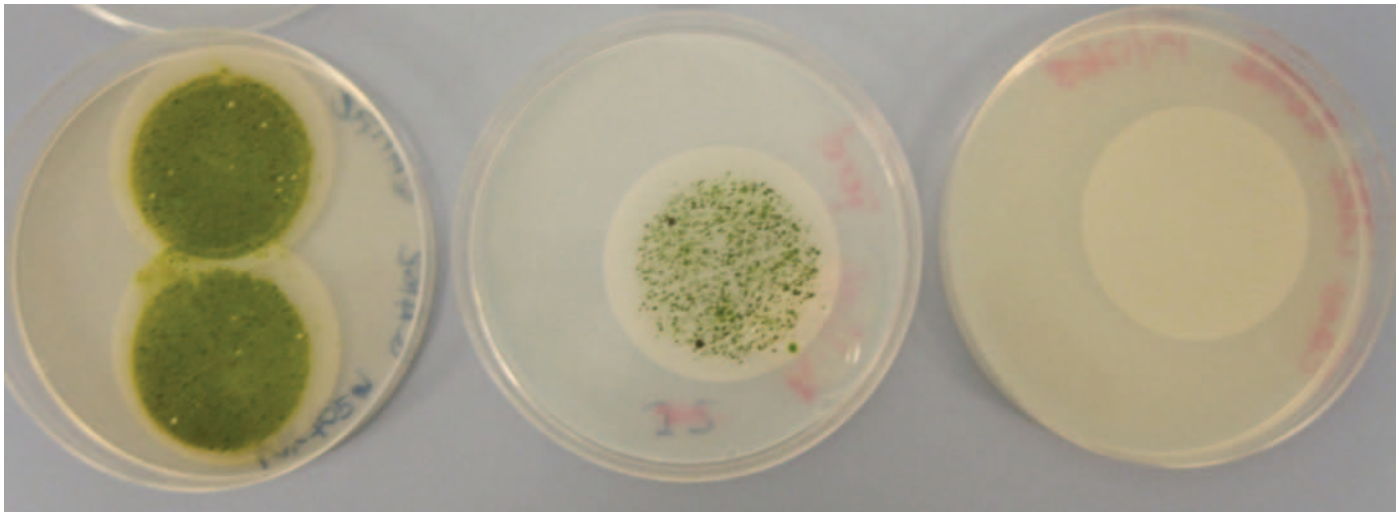

FIGURE 12. Algae growing on 0.45 µm nucleopore filters placed on BBM agar after one month of incubation at 25°C at continuous illumination from (right) Victoria Park, (center) Baffins Pond, and (left) Canoe Lake.

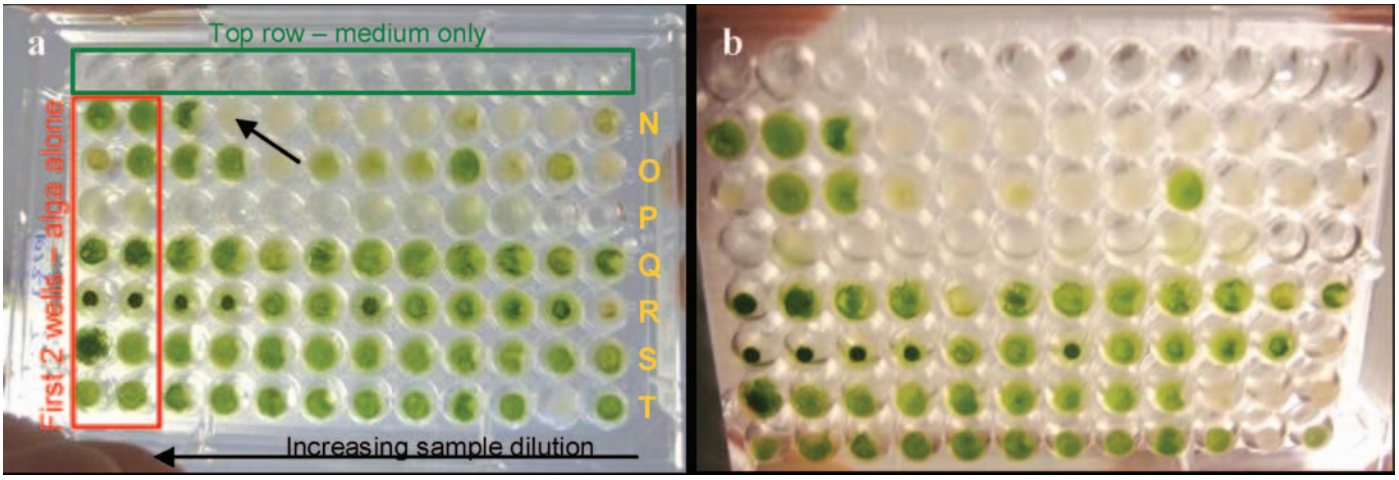

FIGURE 13. Microplate inoculated with purified cultures of environmental algae isolated from Brookwood headstones. Each row is a dilution series from left (high) to right (low), with the first two wells as alga-only control. The top row is an uninoculated, medium-only control, and subsequent rows (from second row to bottom) contain different algae samples N to T, treated with dilutions of dialyzed Canoe Lake water after (a) four days and (b) eight days. Samples N, O, R, S, and T were sensitive, showing clearing of growth (example well shown by small arrow) in wells, relative to the alga-only controls. Clearing occurred over a greater dilution range for N and, to some extent, O; it was very limited for R and T; a limited effect with S only became apparent after eight days; no effects were found for Q. Alga P showed poor growth, including the controls.

showed that samples N, O, R, S, and T were sensitive to varying extents. Eight days after the initial treatment, it was found that algal growth was still being inhibited in some of the wells, notably samples N, O, and S.

DISCUSSION

Two surveys were conducted at the Brookwood Military Cemetery in Surrey, and data were collected on patterns of algal cover on headstones. An image analysis technique was designed and tested for use in capturing data relating to patterns of algal contamination on headstones. The technique proved to be extremely accurate and rapid and will be used for any future surveys of headstone contamination. High-quality digital images of selected headstones have been archived for future use.

The image analysis technique chosen proved to be very suitable for surveys of algal contamination on headstones, and the data collected form a baseline study for future surveys. Such surveys of the headstones are essential in order to monitor the in situ effects of treatment and subsequent recolonization by microorganisms. Broad-scale patterns of algal contamination are evident, and a number of environmental factors that potentially affect these patterns have been identified. Algal cover is highly variable within the sections of cemetery sampled. Proximity of tree cover or shading vegetation, age of headstone, and front versus back aspect of headstone all appear to influence algal contamination. By preselecting groups of headstones for the second survey it was possible to highlight the influential environmental factors using statistical analysis. Although the results of the first survey suggested that age and aspect of headstone may influence patterns of algal cover, this was not the case in the second survey. However, growth conditions were very different between the two surveys, and it is possible that differences in seasonal conditions may have affected the observed patterns of algal cover. The first survey was carried out after a period of very little rain and high temperatures, whereas the second survey followed a period of heavy rainfall and a time when daylight hours were decreasing.

Algae and cyanobacteria will grow under a wide range of environmental conditions but tend to be sensitive to high light (i.e., high UV) levels. It is possible that algae growing on the backs (facing SE) of the headstones were experiencing high UV conditions during the summer and hence that their growth was affected. Observation (not quantified) of algal growth in laboratory cultures showed a rapid increase in algal growth as light levels and temperature fell. It was possible that by the second survey, environmental conditions were approaching those necessary

for optimal algal growth, and an increase in cover would have obscured any small-scale differences in patterns of algal contamination, i.e., between headstones of different ages and aspects. These observations highlight the importance of seasonal effects on biological communities and their consideration when developing treatments for biological control of populations that potentially change throughout the year.

Three zones of algal cover are evident on headstones, at the crown, the base, and the central area of the headstone, and these zones are common to both faces. The analysis of microorganism diversity suggested that the diversity of each of these zones is different. Patterns of microorganism diversity suggested that a number of different taxa are common across groups of headstone samples from Brookwood. Obviously, this is encouraging as a lower diversity and variability of microorganisms present will potentially reduce the number of antialgal microorganisms required for the remediation treatment. The pure cultures of algae obtained from the Brookwood samples have a high incidence of *Chlorella* species.

The initial work carried out to detect viral particles in the natural environment with fluorescence microscopy proved to be a successful technique. Viral particles were detected not only by plaque development in agar plates but also by microscopy. A two-phase separation technique was successfully used to concentrate viral particles. Algal growth, actively growing or established, was then treated with virus suspensions to test whether algal growth on limestone could be inhibited or cleared. All the different experiments showed that viral attack could decrease or inhibit algal growth on different media or surfaces. On agar plates, limestone discs, and slabs, high virus concentration inhibited algal growth over an extended period of time. The most marked effect was found when the highest virus concentration was used, as in the experiments carried out with *Chlorella* NC64A.

The experimental design also showed that viral treatment could be applied before, after, and during the period of algal growth. The number of applications will be further investigated by fieldwork. Treatment of environmental samples with the algal viruses acquired from James L. van Etten showed the high specificity of a particular virus for the algal host. A high viral concentration is required for elimination of algal growth, and this fact, taken together with the high specificity, could explain why early experiments carried out with environmental samples were unsuccessful. In addition, the concentration of viruses in the environmental samples, as detected by fluorescence microscopy, seemed to be quite low. The high specificity of virus for the host makes this work more challenging. Any treatment process will be based on a cocktail of viruses against the predominant algae, together with the application of chemical synergists to facilitate virus infection. As algal populations fluctuate during the year, virus concentrations in the natural environment are changing naturally; thus, there is a higher probability of finding appropriate viruses as the populations decline, as in aquatic ecosystems. The laboratory trials have shown that it is possible to eliminate a particular species of algae using viral remediation, but a much wider range of viruses needs to be isolated so that field trials tests against the natural algal populations identified can be done.

CONCLUDING REMARKS

Much of the work that has been done on algal viruses has been for aquatic systems. Our research was concerned with testing the feasibility of using viruses that pose no health threat to humans to control natural algal populations on stone. The presence of naturally occurring viruses in isolation plates obtained from Brookwood headstones illustrates the impact they can have on the algal communities. Although we were able to identify viruses using fluorescence microscopy, we were not able to isolate and amplify these viruses in the laboratory. Nevertheless, using paired algal hosts and viruses from aquatic systems, we obtained evidence that they can inhibit algal populations on stone in laboratory culture.

Isolation of the components of the algal populations proved to be very difficult and slowed progress on isolating natural viruses from stone:

- Stone algae grow very slowly in the laboratory in pure liquid culture and on stone, and this has impeded experimentation.
- Viral assay and growth requires pure cultures of significant densities, and these were very difficult to obtain from stone algal communities.
- Contamination with bacteria and fungi was a major technical problem.

Our results demonstrate proof of principle that algal types that are commonly found on the stones can be inhibited by viruses on stone in the laboratory. Clearly, at this stage, although it has been possible to show limited effects in the laboratory, the essential components of any treatment system, i.e., a range of viruses reflecting the variety of algal types, have not been obtained so far. They

were clearly present and were detectable but could not be isolated into laboratory culture. The answer may lie in our ability to control the different factors that affect the expression of activity of those viruses that affect algal populations on stone.

Acknowledgments

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Recolonization of Marble Sculptures in a Garden Environment

José Delgado Rodrigues, Marta Vale Anjos, and A. Elena Charola

ABSTRACT. The usual practice for eliminating biocolonization from stone statues in outdoor conditions has been the application of a biocide and subsequent cleaning. However, the cleaned condition is a transient state, and recolonization is inevitable. The recolonization rates can vary significantly as a function of the local and regional environmental conditions, and the respective patterns may exhibit strong variations at the microscale of the object. Given the variable time required for recolonization, the present study was undertaken over several years to collect data that although particularly relevant to the substrate considered and its environment, could shed some light as to which measures were the most effective in eliminating and preventing biocolonization. The paper focuses on the evaluation of various treatments applied in the past to the ornamental marble statues in the gardens of the National Palace of Queluz, Portugal.

INTRODUCTION

The National Palace of Queluz, as seen today, dates back to the seventeenth and eighteenth centuries. It is located some 12 km west-northwest of Lisbon, half way to Sintra. The baroque palace includes several buildings of high architectonic quality and is particularly known for the intimate relation between the buildings and the extensive contiguous gardens that are decorated with over 100 statues and busts, bases and pedestals, vases, balustrades, and over 20 marble fountains and a large limestone cascade. The larger part of the 15 hectare grounds is a park. The two formal gardens, the Malta Garden and the Hanging Garden, where most of the statuary is concentrated, are right next to the palace, and many French doors open directly into them.

The Robillion wing, which was annexed to the original building, bridges the level change from the Hanging Garden constructed over a water reservoir to the ground level, where a creek runs through the park. The west side of this wing faces the creek, which in this section flows in a highly decorated azulejo-lined canal. It has two terraces; the lower having a magnificent staircase leading down toward the promenade next to the canal.

The sculptures and decorative elements are distributed throughout the gardens, many of them under or close to high trees and dense bushes, where higher relative humidity and lower sun exposures are to be expected. Most stone elements are in a reasonable state of conservation, particularly those carved in

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marble, although some degree of deterioration can be identified in most of them. Minor scaling and fissures as well as sporadic fractures can be seen, but the major degradation that affects these decorative elements corresponds to surface erosion mostly caused by direct rain flow and/or biological activity.

Biocolonization is widespread all over the gardens, and it can reach nearly total and dense coverage, which may completely disfigure the decorative elements (Figure 1a). Green algae appear frequently during the wet season and turn dark grey when the dry season comes. In many places, this diffuse dark colonization constitutes the main colonizing component. Lichens widely proliferate on some of the stone elements and may constitute a cover so dense as to entirely conceal the stone surface, as illustrated in Figure 1.

In the course of the recent multiyear conservation and restoration project of the gardens of the palace (Charola et al., 2007), the opportunity was presented to evaluate the condition of the statuary, mostly marble and limestone, in the gardens. A study was carried out to compile all the information dealing with the cleaning and subsequent protection treatments from the reports and records kept by the palace staff for the past quarter century (Vale Anjos, 2006). In older records, some dating from 1820, it was confirmed that regular cleaning was considered normal practice in the early times of the gardens' history. Around 1948, some restoration actions were reported, and after a long period where no actions were recorded, documented interventions reappeared in 1977. Since then, the sculptures have been submitted to sporadic and variable interventions following different and even antagonistic approaches, facts that lead to a quite inconsistent situation in terms of appearance and conservation condition. Different methods for elimination of the dense biocolonization have been used, ranging from an intensive grit blasting, a

FIGURE 1. Two marble sculptures in the Queluz gardens, both of which are heavily colonized by lichens. Note the different types of lichen colonization on the sculptures as a function of their local environments.

method that is ethically unacceptable for carved elements, to the application of inappropriate sodium hypochlorite, and to biocides such as Preventol R80. Different concentrations and numbers of applications of the biocides are reported, reaching the extreme of 5% volume per volume (v/v) concentration applied five times.

Analyzing these data in conjunction with several years of photographic records, correlations could be drawn between the cleaning and hydrophobization treatments undergone by the statues and their condition after a given number of years.

The study also evaluated new approaches for eliminating visible biocolonization and controlling the subsequent recolonization (Vale Anjos, 2006). Effectiveness of biocides and recolonization were monitored by using a portable fluorometer prototype (Delgado Rodrigues et al., 2004).

The results obtained helped to define the strategy for the maintenance plan that was subsequently developed and is described in detail elsewhere (Charola et al., 2007). In brief, this approach is based on using low concentrations of Preventol R80 (of the order of 1.5% v/v) and leaving the object for several months without any additional action. This approach resulted in the elimination of any dead organisms by rain and wind, leaving a surface that looked naturally aged, which is considered highly appropriate for attaining a coherent integration of stone surfaces and the surrounding vegetation-based landscape.

MONITORING BIOCOLONIZATION

The study aimed to determine how difficult it was to remove existing biocolonization, to find the simplest way to apply the biocides, to define the minimum effective concentration required, and to outline the protocols to be implemented at a large scale in the future maintenance of the garden sculptures and decorative elements. It focused on visible biocolonization as compiled from the analysis of the historic data and field evaluation of the condition of the sculptures; it was evident that the most eroded areas corresponded to those that had been colonized by visible microorganisms, such as algae and lichens. Areas that showed no visible colonization were far less eroded, and this minimum erosion could be attributed to chemical attacks from rainwater combined with any corrosion induced by nonvisible microorganisms, which can be considered normal weathering of the stone. Since this weathering is minor compared to the damage induced by visible microorganims and the subsequent attempts to remove them, this deterioration was not considered of practical importance.

For this study an on-site testing program was carried out where the biocidal action of the tested biocides was monitored with a portable handheld fluorometer, available as a prototype, called "Biofinder", developed under the European research project "ONSITE—On-site monitoring of biological colonization on stone and plaster surfaces using field portable fluorescence based techniques." The prototype consists of an LED light excitation source in a defined wave length and a CCD for photon detection of the fluorescence emission in the characteristic wave lengths of chlorophyll pigments. The exciting and emission wave lengths were optimized to detect both chlorophyceae algae and cyanobacteria. It had been demonstrated that the instrument is capable of detecting on-site colonizers on a surface before they can be visually observed and is particularly well suited for the evaluation of biocide effectiveness. This project was based on earlier work that tried to use quantitative field techniques, namely, color analysis (Young et al., 1995), laser-induced fluorescence (Cecchi et al., 1996), and LED-induced fluorescence (Brechet et al., 1997). This last technology was the base technology applied in the Biofinder instrument, and once it was successfully improved, it served to fill the need that existed between on-site detection and quantification of biodeteriogens. The instrument was successfully tested in several field studies and proved to be particularly helpful for monitoring the biocidal action on all the lichen species found in the different case studies (Delgado Rodrigues et al., 2004).

The first phase of the study carried out at the Queluz gardens tested two biocidal products: Preventol R80, a biocide based on a quaternary ammonium salt, and zinc chloride. Concentrations of 1.5%, 2.0%, and 3.0% (v/v for Preventol R80 and weight per volume (w/v) for zinc chloride) were applied in one to four applications with about a one week interval between them. The biological activity was monitored with the portable fluorometer (Biofinder) before application and after each successive treatment. Figure 2 illustrates the type of data gathered in this study. The emission values can be used directly as measured or transformed as the ratio of the values measured in wet conditions versus those measured in dry conditions. The experience acquired in the ONSITE project showed that this ratio is especially relevant to discriminate biocolonization from high natural background fluorescence stones as well as live colonizations from dead ones. In fact, this ratio is close to 1 for noncolonized areas and for dead organisms, whereas its value clearly differs from 1 when live chlorophyll-bearing specimens are present.

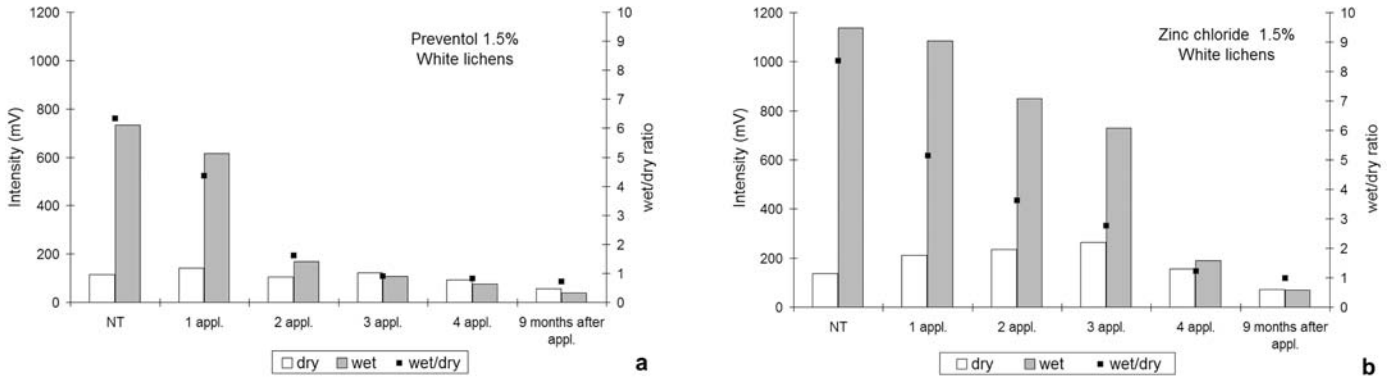

FIGURE 2. Examples of measurements taken with the fluorometer in successive applications of (a) Preventol R80 1.5% v/v and (b) $ZnCl_2$ 1.5% w/v to areas with white lichens. Bars represent data measured in dry (white) and wet (grey) conditions. Squares represent the ratio wet/dry values. Values from left to right correspond to nontreated (NT); one, two, three, and four applications spaced one week apart; and 9 months after the last application (data are from Vale, 2006).

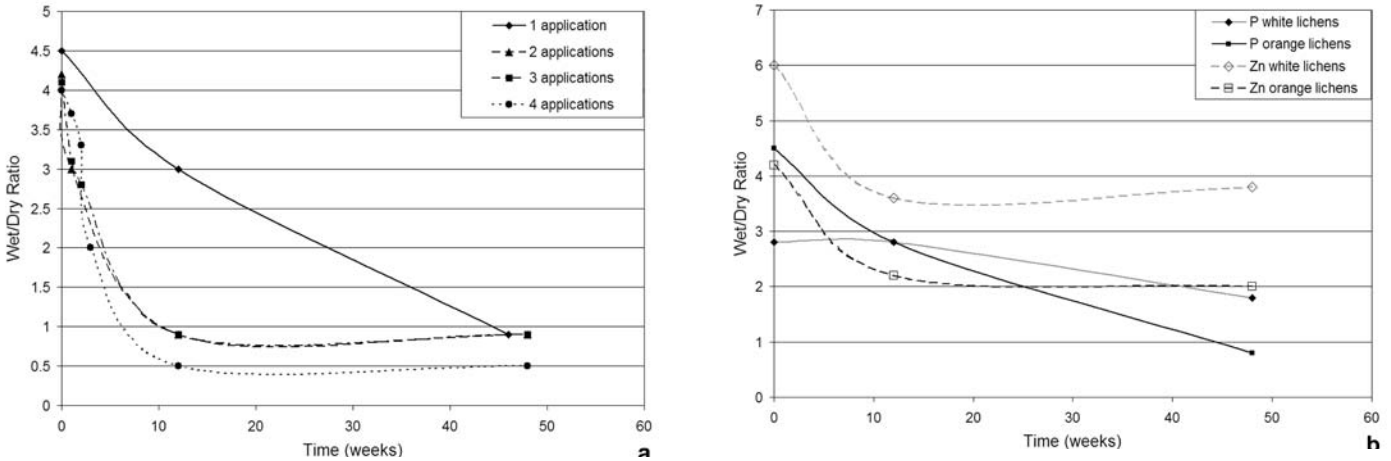

FIGURE 3. (a) Wet/dry ratio obtained from fluorometer readings for four successive applications of Preventol 1.5% to areas colonized with orange lichens. (b) Wet/dry ratio obtained after single applications of dilute biocide solutions (1.5%) of Preventol and zinc chloride on two different types of lichens, where the higher effectiveness of the Preventol is evident (data are from Vale, 2006).

For the specific conditions illustrated in Figure 2, it is clear that Preventol R80 is slightly more effective than the zinc chloride since the wet/dry ratio is very close to 1 after the second application. It was also interesting to note that biocides continue to be effective even after a last application, as seen in the zinc chloride data. This result leads to the conclusion that leaving the treated areas alone for some time might be a more efficient way of eliminating biocolonization than trying to remove it all at once.

Figure 3 shows the plots for the wet/dry ratio obtained from measurements taken during and after four applications of Preventol 1.5%, one per week, to areas colonized with orange lichens. Figure 3 also shows the effect of the application of two different biocides, Preventol and zinc chloride, at the 1.5% concentration to two different types of lichens, identified as white and orange lichens.

EFFECTS OF PAST CLEANING INTERVENTIONS ON RECOLONIZATION

Inspection of the sculptures that had recently undergone cleaning intervention showed that this procedure is usually pushed to extremes, particularly when grit blasting

FIGURE 4. Surface conditions left by two different cleaning methods: (a) application of a biocide and gentle brushing (roughly 30 × 40 cm area) and (b) aggressive grit blasting (area about 40 × 50 cm).

is used to eliminate biocolonization. In these cases, the stone surface acquires an evenly rough surface as compared to the uneven and pitted surface left behind by gentler cleaning methods (Figure 4). Furthermore, the latter do not induce the brilliant whiteness that results from aggressive grit blasting.

The surface roughness resulting from a cleaning intervention will affect the subsequent recolonization. The relatively smoother surfaces left by grit blasting are more resistant to the installation of new colonies, but this fact should not convey the mistaken notion that this unacceptable cleaning method may have some advantages in terms of maintenance.

The colonization rates that could be observed show a very high dependence on the local environmental conditions, with the fastest rates being observed on sculptures

that are under or very close to large trees. This is illustrated by four busts that decorate a balustrade surrounding a lead sculpture group and a fountain. Two of them in particular are directly under a large sycamore tree that provides a very favorable environment for biocolonization. The busts were cleaned in 1993 by grit blasting. Two years later, they underwent low-pressure water washing, and a biocide was applied. Four years later, in 1999, they needed to be cleaned again, this time with sodium hypochlorite poultices and the application of both a biocide and an aqueous dispersion of a water repellent. The repeated cleaning required clearly shows how fast recolonization can take place in this sheltered area. Figure 5 shows the appearance of these busts four years after cleaning.

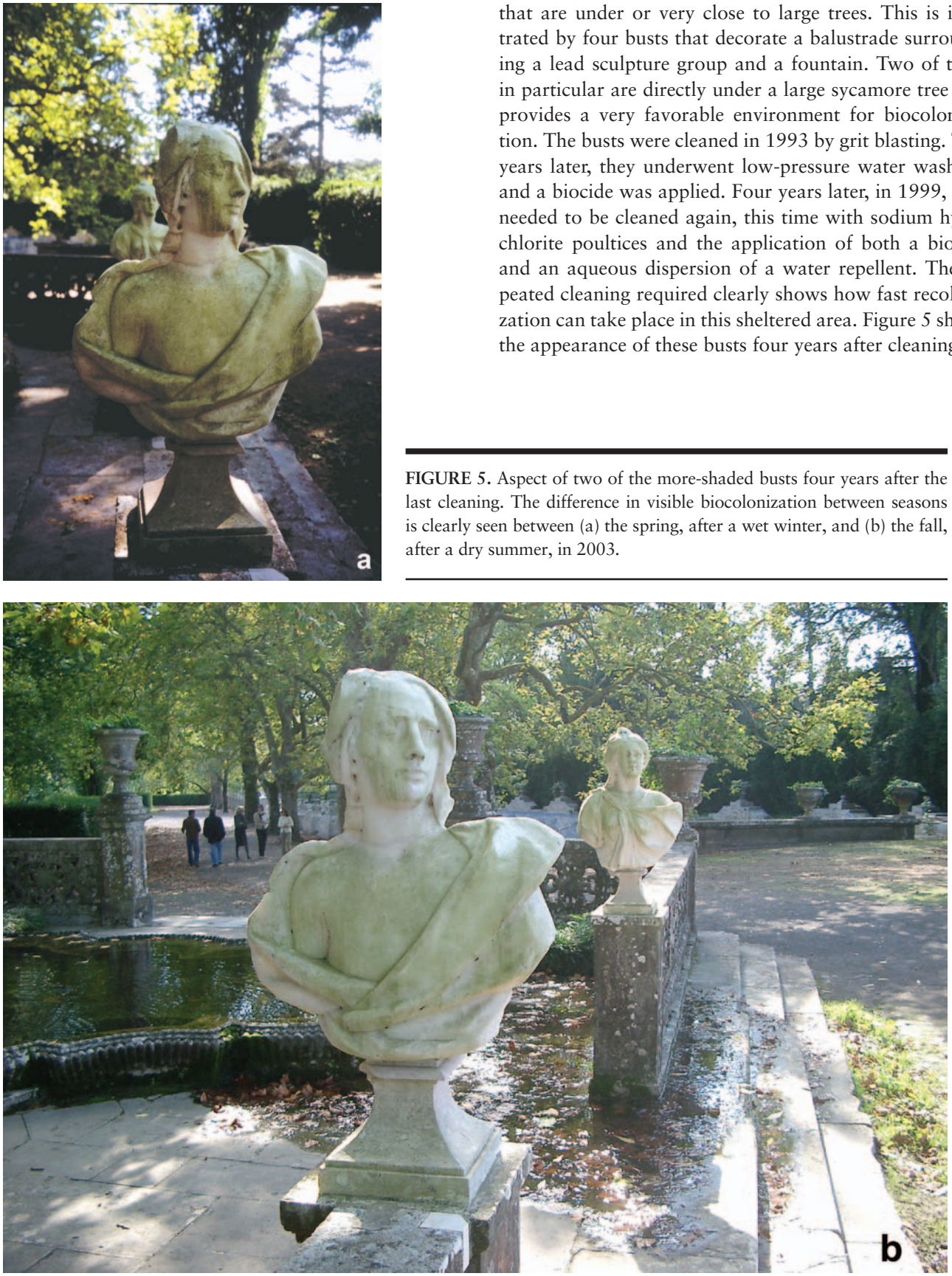

FIGURE 5. Aspect of two of the more-shaded busts four years after the last cleaning. The difference in visible biocolonization between seasons is clearly seen between (a) the spring, after a wet winter, and (b) the fall, after a dry summer, in 2003.

When the objects are located in areas exposed to sunlight for only part of the day, their orientation influences the recolonization location, as observed for a group of four sculptures representing the arts. The statues, *Painting*, *Music*, *Sculpture*, and *Architecture*, are located in the Malta Garden around a small fountain with no large trees in the neighborhood and are right next to the palace building. Figure 6 gives the location of the statues and the palace building as well as its orientation.

These sculptures were cleaned in 1996 and 2003 and were inspected in 2003, 2006, and 2008. The 1996 cleaning was based on biocide poultices and low-pressure washing followed by the application of a water repellent. The first observation was carried out in 2003, seven years after the first cleaning and prior to the second one (Figure 7) and showed a fairly severe recolonizaton of the surfaces.

The aggressive cleaning intervention carried out in 2003 left all the sculptures a brilliant white. Interestingly, dry grit blasting was used for two statues, *Painting* and *Architecture*, whereas pressure washing was used for the other two, *Music* and *Sculpture*, but no significant differences in their appearance could be noted. Nonetheless, two years after this drastic cleaning, the first signs of recolonization could be observed. Five years later, green algae

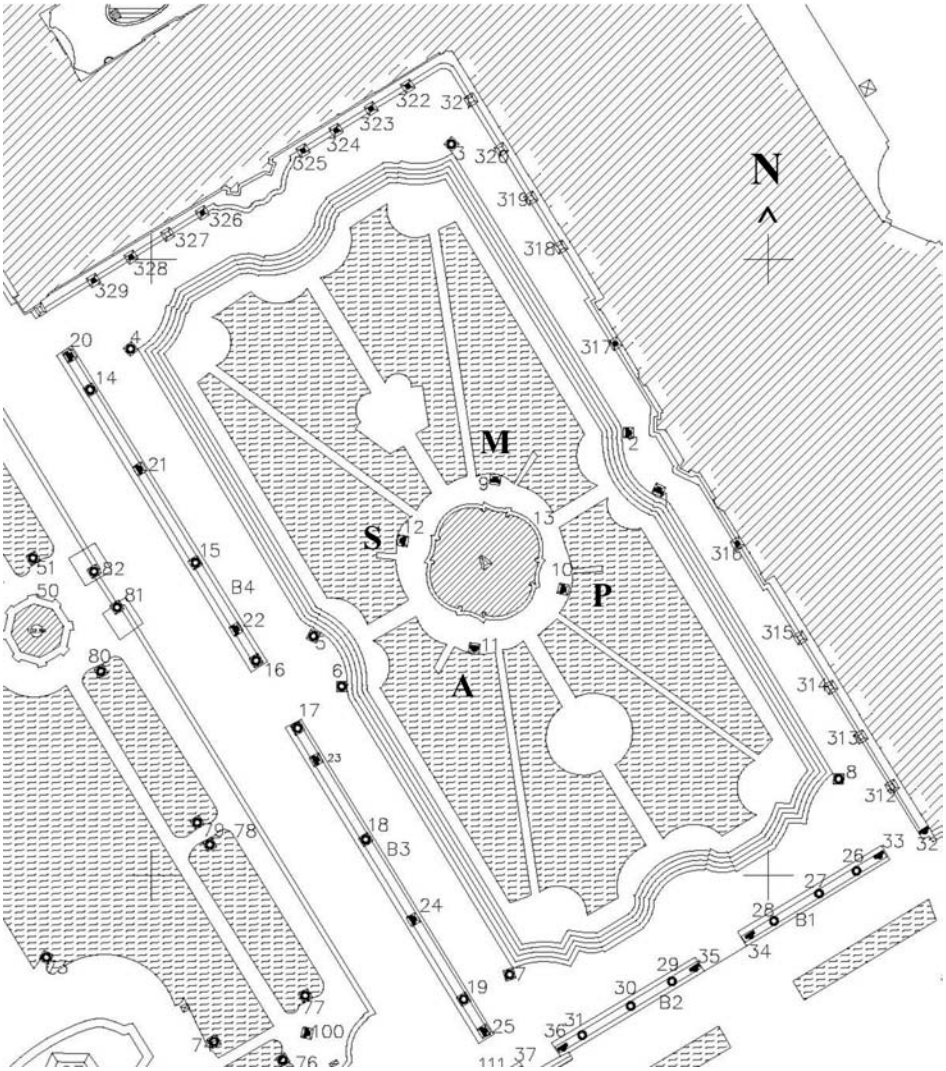

FIGURE 6. Diagram of the Malta Garden, showing the fountain in the center and the four sculptures, identified by their initials M, P, A, and S, around it. Hatched areas represent parts of the palace building. Black arrow points north.

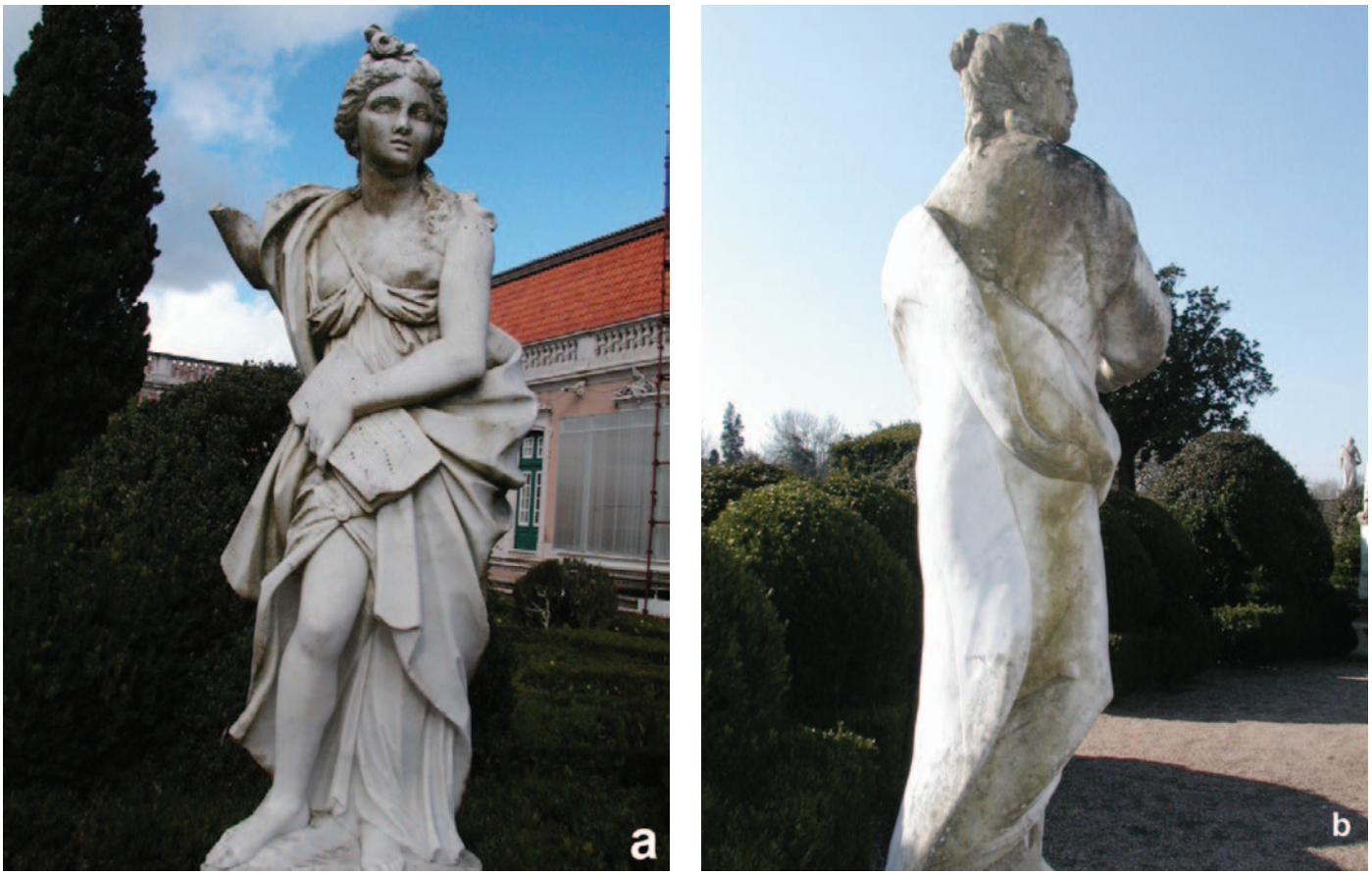

FIGURE 7. Recolonization condition of the arts statues in 2003, seven years after the 1996 cleaning: (a) *Music* and (b) *Painting*. Cleaning had been carried out with biocides and low-pressure washing, followed by the application of a water repellent.

were widespread on the north- and northeast-facing areas, and lichens were already present on the south-facing areas (Figures 8 and 9).

After the fall 2003 cleaning, yearly measurements were taken with the Biofinder on different areas of the statues, e.g., north and south facing, until biocolonization became evident three years after cleaning. The wet/dry ratio of selected site measurements, representative of the increase in recolonization that took place over those three years, is shown in Figure 10. These measurements confirm the visual evaluation of the appearance of the first signs of recolonization, and although the positive trend is not very sharp, it indicates that recolonization is well established in the sculptures. Nonetheless, it is worth mentioning that monitoring recolonization on a complex figure such as these sculptures presents some difficulties as colonization patterns are spatially heterogeneous, with areas where recolonization is dense and visible and others where virtually no recolonization is present. Consequently, no single graph will fully represent the reality of any sculpture, and Figure 10 is obviously just an illustration of the kind of data that can be gathered during the recolonizing phase.

Another example is from a group of six sculptures (putti groups) located on the south balustrade of the Malta Garden near the palace building (numbered 32 to 37 in Figure 6). These were cleaned in 1996, with the application of a biocide (a quaternary ammonium salt formulation) and a water repellent. By 2003 their appearance had significantly changed, and three years later, in 2006, the biological cover was already very noticeable (Figure 11). The striped pattern of the recolonization can be attributed to the water repellent because of the changes this product induces in the way water runs down the treated surface (Charola et al., 2008a, 2008b).

On the other hand, the sculptures shown in Figure 12 are located on the balustrade between the Malta and the Hanging gardens. This is a totally unsheltered space where they are exposed to direct sunlight for most of the day.

FIGURE 8. The arts statues in 2008, five years after cleaning with abrasive methods, showing recolonization of green algae in the northward-facing areas: (a) *Painting* and (b) *Music*.

According to information provided by museum staff, these statues received a biocide spray application (a quaternary ammonium salt) in 1999. No written records for this intervention are available. In 2003 they already showed evident signs of recolonization that evolved in the subsequent three years to a particularly striking increase in the lichen recolonization.

Finally, the two groups of four sculptures placed on the upper and lower balustrades, respectively, of the west façade of the Robillion Pavilion illustrate yet another set of conditions that influence the recolonization rates. These sculptures were cleaned by grit blasting, leaving them a brilliant white and deprived of the natural patina. Some six years after cleaning, the sculptures showed incipient (upper balustrade) to moderate (lower balustrade) recolonization, so the application of a biocide was carried out. In a recent inspection, some eight years after the biocidal treatment, recolonization had again reached an incipient level in the sculptures of the upper balustrade and was already intense in the sculptures of the lower one (Figure 13).

The clear difference in the microclimatic conditions of the upper and lower balustrades has an evident impact on the recolonization rates. Whereas the upper balustrade has full western exposure and not much shading from the building on the east, the lower balustrade is shaded both on the east, by the palace building, and on the west, by tall trees. Consequently, the environment on the lower balustrade tends to favor a faster recolonization rate. The recolonization pattern is relatively independent of the exposure

FIGURE 9. The face of *Music*, showing the first occurrences of lichens on the sunny south-facing areas. (a) Lichens and growth of algae on the relatively protected underside of the chin. (b) A detail of the lichen growth.

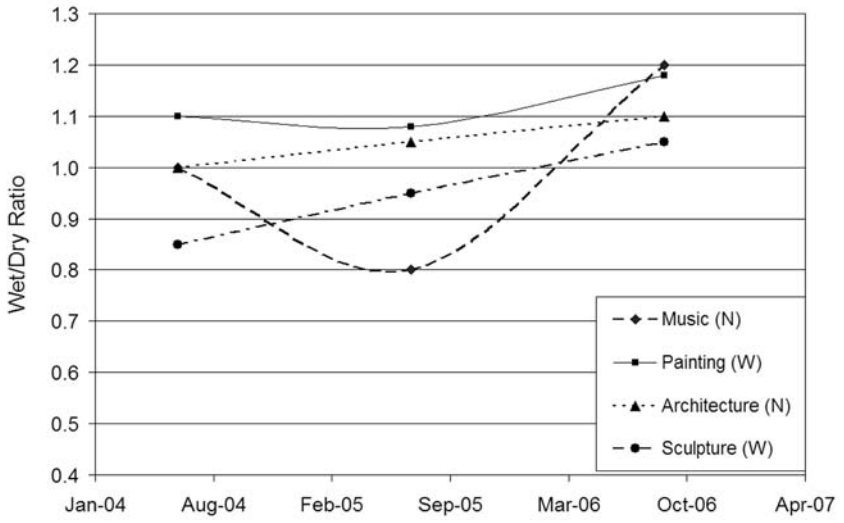

FIGURE 10. Wet/dry ratio of the measurements taken with Biofinder for the arts statues one, two, and three years after cleaning. The positive trend of the graphs indicates that recolonization is clearly in progress. The local decrease is certainly due to the spatial heterogeneity of the recolonization patterns.

orientation, suggesting that the presence of the shadowing trees overrides the effect of the orientation identified in the unsheltered sculptures.

Table 1 summarizes the main data collected during this study, including two fountains that were relocated in the garden but were not connected to the hydraulic system. These are located in a rather sheltered area in the park and are referred to as "dry fountains" in Table 1.

DISCUSSION

The many stone sculptures in the gardens of the National Palace of Queluz, the diversity of environments to which they are subjected, and the cleaning and protective interventions they suffered provided a wealth of information that served to evaluate the effectiveness of biocides and the performance of past interventions. The study corroborated

FIGURE 11. Recolonization of one of the putti groups cleaned in 1996. Appearance is shown (a) in 2003 and (b) in 2006. Note the well-defined dark green stripes in (a) as a result of the water-repellent treatment and their subsequent evolution to heavier features over time.

FIGURE 12. Sculptures located in open, unsheltered spaces. Recolonization is shown (a) four years after cleaning and (b) seven years after cleaning. Note the initial relatively slow increase in biocolonization, with the subsequent appearance of lichen colonization.

FIGURE 13. Appearance of the sculptures from the (a–c) upper and (d–f) lower balustrades eight years after application of a biocide. Note the significant increase in average colonization of the lower balustrade, where the statues are shaded on the east by the palace building and on the west by tall trees.

the well-known relationship between environment and the type and rate of recolonization. In highly exposed and unsheltered areas, recolonization by green algae occurs within two years after cleaning, whereas it takes lichens between four and five years to make their appearance. Meanwhile, in shaded areas protected by large trees, it takes slightly over one year to trigger a significant recolonization, particularly by green algae. Occasional shade, such as provided by buildings and/or nearby trees, results in disparities in the colonization rates according to the orientation of the sculptures, as evidenced by the sculpture group of the arts. Differences in recolonization rates for objects located with the same orientation but with different shading were observed between the upper and lower balustrades of the Robillion wing.

The application of water repellents after cleaning has been a common practice for sculptures in gardens,

TABLE 1. Summary of recolonization rates for some of the decorative marble sculptures in different locations in the garden of the National Palace of Queluz. WR indicates the application of a water repellent; degrees 1–5 indicate the degree of recolonization, with 1 being none to incipient and 5 being the highest degree.

Statues	Exposure	Treatment	Comments
Busts	Fully sheltered	Grit blasting and WR	One year to reach degrees 2–3. Four years to reach degree 5. Green algae are the dominant type within these periods.
Dry fountains	Protected	Biocides	Five years to reach degree 3.
Painting and *Architecture*	Partially protected	Grit blasting	Five years to reach degree 2. Algae predominate in NW and NE faces. First lichens appear in SE and SW faces.
Music and *Sculpture*	Partially protected	Pressure washing	Five years to reach degree 2. Algae predominate in NW and NE faces. First lichens appear in SE and SW faces.
Putti groups	Exposed	Biocide and WR	Six years to reach degree 2.
Robillion upper balustrade	Fully exposed	Grit blasting	Five years to reach degree 1.
Robillion lower balustrade	Protected	Grit blasting	Two years to reach degree 2. Sheltering effect reduces the influence of orientation.

irrespective of their location or the methods used for cleaning them. The analysis carried out showed that for this specific situation the negative effects induced by these products, i.e., the development of unsightly biocolonization patterns, were not compensated for by the protection they provide against recolonization. These patterns develop on hydrophobic surfaces because the water repellent inhibits the spreading of dew or initial rain drops, thus favoring the creation of water stripes as the drops roll down the stone surface. The wet stripes, apart from accelerating soiling and particulate deposition, constitute a water reservoir for the fast-growing initial colonizers, such as green algae and cyanobacteria, that eventually develop into well-colonized stripes on the stone surface (Charola et al., 2008a, 2008b). In addition, water repellents may also serve as a nutrient to colonizers (Koestler and Santoro, 1988).

Water repellents applied to a surface form a physical barrier between the water droplets and the stone substrate, thus preventing the droplets from dissolving and incorporating any traces of the applied biocides after the cleaning process, should they have a residual action. Consequently, under this hypothesis, the application of a water repellent after a biocidal treatment is not only ineffective but could also be counterproductive in terms of the subsequent recolonization. Furthermore, the biocide may interfere with the hydrophobicity that the water repellent should impart (Malagodi et al., 2000), a point that has been confirmed recently in a study verifying the negative impact of biocides on the effectiveness of a water repellent (Moreau et al., 2008). This result validates the need for a careful assessment of the interaction between the two types of products before any decision of their use can be made.

CONCLUSIONS

The history of conservation interventions carried out in the Queluz gardens shows that practically all cleaning methods have been used, ranging from gentle to very aggressive ones, reflecting a lack of clear guidelines for cleaning interventions and the erratic approach to maintenance over time. Also, long periods may have elapsed where no relevant conservation interventions were carried out, whereas at other times interventions were repeated within short intervals. As a consequence, many sculptures show loss of detailing, erosion from water paths concentrated by the application of water repellents, and rather smoothened surfaces in the case of those cleaned with grit blasting. It is therefore fundamental that a regular and long-term conservation approach be implemented to minimize these problems.

The conservation approach suggested for the Queluz gardens is that the sculptures should be treated only with biocides, left untouched for several months, and then lightly brushed to remove any detaching vegetation remnants. In most cases, brushing is not even necessary to remove the dead colonizers (Charola et al., 2007). Thus, the intervention is reduced to a minimum, with practically

no cleaning actions required. Since the application of the biocides should only be carried out when visible recolonization changes from incipient to moderate intensity, the number of required applications over time is reduced, thus diminishing the probability of inducing the appearance of biocide-resistant species.

Since there are many potential new biocides being developed, it is obvious that further on-site studies will have to be carried out in the future (Nugari and Salvadori, 2003). This study focused on finding a practical methodology to reduce the negative impact induced by cleaning actions while achieving an acceptable solution for the presentation of marble sculptures in historic gardens.

So far, the sculptures treated under this new approach show equal if not slower recolonization compared to more aggressive cleaning methods. Additionally, it should be noted that biocolonization is inevitable in outdoor environments and that the deterioration induced by incipient biocolonization is less than that resulting from regular cleaning over the same time period. Finally, the overall aesthetic aspect is by far more harmonious and compatible with a garden environment. The overall analysis of the data gathered in this case study leads to the following conclusions:

1. It is undesirable to have all sculptures and other stone objects cleaned at very close intervals because of the inevitable damage that can be induced.
2. The highly diverse environmental conditions, e.g., sheltering effects and exposure orientation, lead to an endless number of microenvironments and recolonization rates.
3. A certain degree of recolonization is acceptable for the specific cases of sculptures in gardens since this contributes to a coherent aesthetic presentation of the site.
4. Localized biocide application is recommended whenever recolonization changes from incipient to moderate levels.
5. The application of water repellents is not recommended for sculptures in gardens.
6. Further studies of the interaction of water repellents and biocides, including the order in which they are applied, are necessary.

The general conclusion that can be drawn from the present study is the importance of defining the aim of any intervention and of understanding the nature of the deterioration factors and their interaction with the substrate. This study also emphasizes the well-known fact that regular and long-term maintenance is the only way to preserve our heritage.

Acknowledgments

The director and staff at the National Palace of Queluz were very helpful in providing access to their records and in authorizing the experimental tests carried out on some of the decorative elements in the gardens. The National Laboratory of Civil Engineering provided technical assistance in the development of the Master's thesis for one of the coauthors on this topic. This study is a product of the Conservation/Restoration Project of the Gardens of the National Palace of Queluz that was made possible in part by the World Monuments Fund's Robert W. Wilson Challenge to Conserve Our Heritage.

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Case Study: Red Staining on Marble

Claire Gervais, Carol Grissom, Christopher McNamara, Nick R. Konkol, and Ralph Mitchell

Red stains were observed on the marble of the Certosa of Pavia in Italy as early as 1844 (Realini and Sorlini, 1988), and they have since been found on other marble monuments, such as the Cathedral of Orvieto, the *Fountain of Galatea* at Villa Litta north of Milan, the *Fountain of the Labyrinth* in Florence, and the pedestal of an equestrian statue in Copenhagen. Microbiological analyses carried out on stained marble samples have sometimes detected carotenoids and red-pigmented organisms. On the other hand, the presence of minium (Pb_3O_4, with a bright red color) on the aforementioned monuments suggests that these red stains can also be caused by the corrosion and oxidation of lead present in gutters, fountain plumbing pipes, or between marble blocks. Up to now, there has been no clear explanation of how the corrosion of lead and the formation of minium occur. Hypotheses include corrosion of lead gutters by acidic water (Zanardini et al., 1994), oxidation of lead salts derived from atmospheric attack of lead building components (Realini and Sorlini, 1988; Bruni et al., 1995), attack of lead by alkaline water that percolated through noncarbonated mortar (Bredal-Jørgensen et al., 2008), production of hydrogen peroxide by microorganisms (Petushkova and Lyalikova, 1986), and possibly oxidation of lead by bacteria (Realini et al., 2005).

The Memorial Amphitheater at Arlington National Cemetery, made of Danby Vermont marble (Mountain White grade) and constructed between 1915 and 1920, presents another example of red staining. Preliminary microbiological analysis resulted in the isolation of a red-pigmented bacterium (Figure 1). However, in all cases the stains have been found to contain lead corrosion products, in particular, the bright red minium. Scanning electron microscopy (SEM) accompanied by energy dispersive spectrometry (EDS), X-ray diffraction (XRD) analyses, X-ray fluorescence spectroscopy (XRF), and Raman spectroscopy revealed considerable diversity of lead compounds in the stains as well as in their shapes, sizes, and distribution.

The staining at Arlington National Cemetery, which also varies significantly in color and appearance (Figure 2), occurs at discrete locations. Most often, it is found on marble paving blocks between external columns of the amphitheater, particularly between the east and north entrances. Located on contiguous horizontal and vertical surfaces of the same blocks, these stains generally appear as agglomerations of small red spots on horizontal surfaces, brown-purple stains

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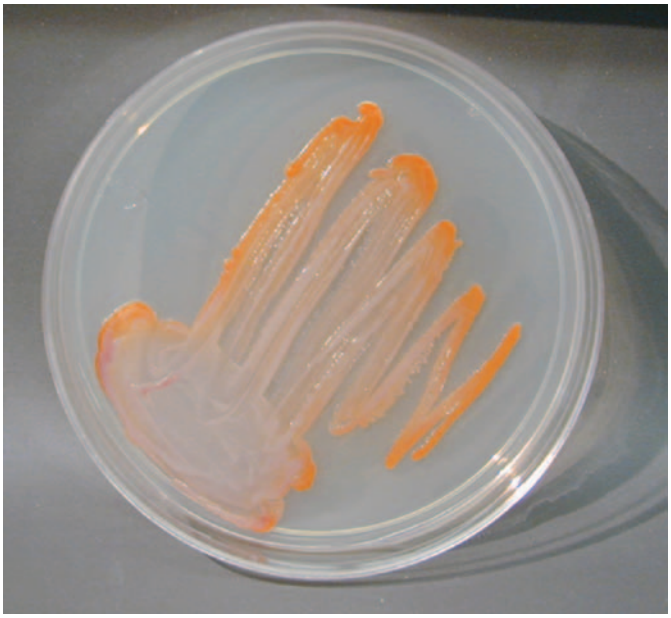

FIGURE 1. A red-pigmented bacterium isolated from stained areas in Memorial Amphitheater.

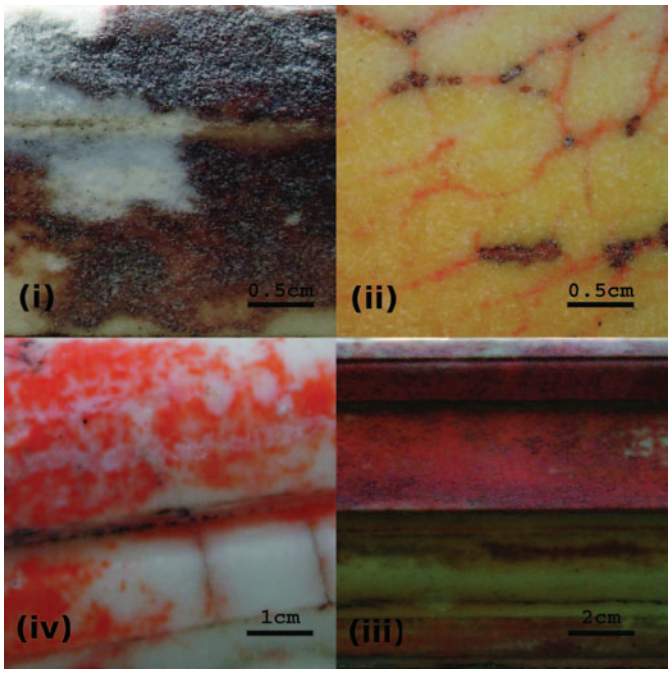

FIGURE 2. Typical colors and textures of colored stains observed at the Memorial Amphitheater (scale is approximate). (i) Purple stains, sometimes very dark and almost brown, often thickly encrusted, found mainly between the columns; (ii) yellow thin washes on a stairway corner post with red stains along fissures (note the presence of black Verrucaria lichens); (iii) typical staining of vertical areas on marble blocks between columns, with the presence of dark crusts, red stains, and yellow washes; and (iv) patchy orange-red stain on the stairway corner post.

on vertical edges, and yellow washes underneath. In situ XRF analysis confirmed that lead is present in all stained areas, with higher concentrations in purple areas. Staining is mainly concentrated in the middle of the blocks and never continues over vertical joints, as staining of biological origin likely would.

A second striking example of staining is located on the outer corner of a column base to the left of the amphitheater's stage. This area features a Liesegang-ring-like pattern, with purple, coral red, orange, and yellow areas located sequentially outward from the white corner. Comparison of photographs taken in 2004 and 2008 shows a net progression of staining, with migration away from the corner and expansion of the area of yellow washes (Figure 3). In situ XRF analysis confirmed the presence of lead in all pigmented areas as well as in the white area between the missing corner (apparently replaced with a mortar repair, now also missing) and purple stain (Figure 4). The largest quantity of lead was found in white and purple stains (regions 2 and 3).

Finally, a stairway post behind and to the left of the stage exhibits bright red spots around partially spalled-off corners and along crevices as well as yellow washes. A lead sheet was detected in the joint above the stained block. The XRF analysis confirmed lead in all pigmented areas, and both Raman spectroscopic and XRD analyses found minium in one of the bright orange-red corners. Identification of compounds in the yellow washes proved more difficult. Samples contained a mixture of red minium particles and tiny yellow particles that did not yield identification by XRD or Raman spectroscopy (possible causes could be a poor crystalline state or a scarce amount of corrosion products for XRD and low scattering of lead oxides for detection by Raman). The SEM-EDS also showed two lead-containing compounds with different crystal sizes and shapes. Although precise characterization of the two compounds is not possible because of topographic and thickness variations, one might reasonably assume that the compounds correspond to the red Pb_3O_4 and to one of the two yellow polymorphs of PbO (either litharge or massicot) on the basis of their gray values in secondary electron SEM images (Aze, 2005).

Possible sources of lead in stained areas include the lead sheet found in the joint above the stairway block and

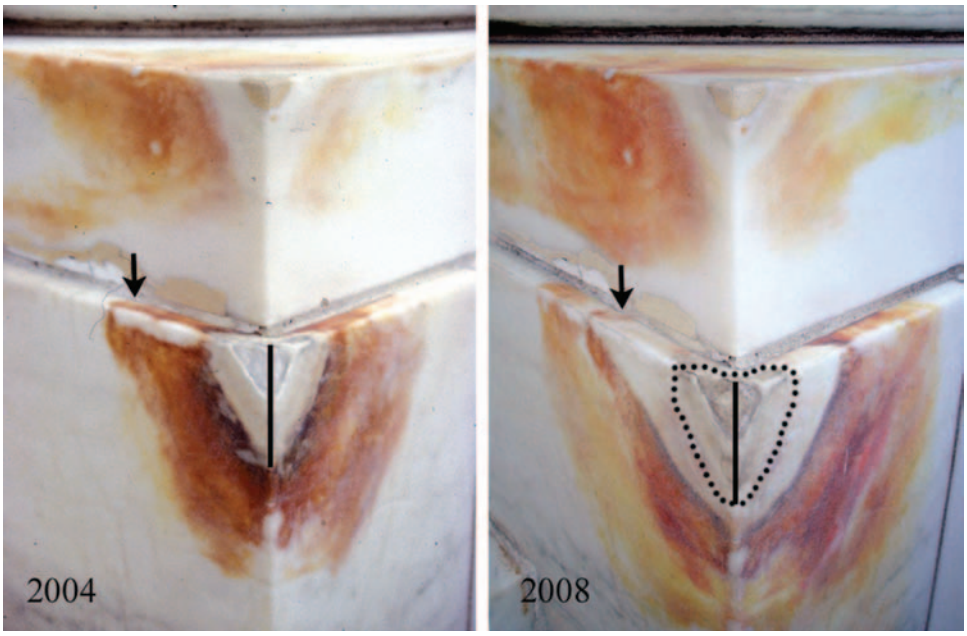

FIGURE 3. Evolution of the red staining between 2004 and 2008. The dotted line indicates the inner edge of the 2004 stain, and the black arrow indicates its outer limit. Vertical black lines reflect the same locations in each photograph.

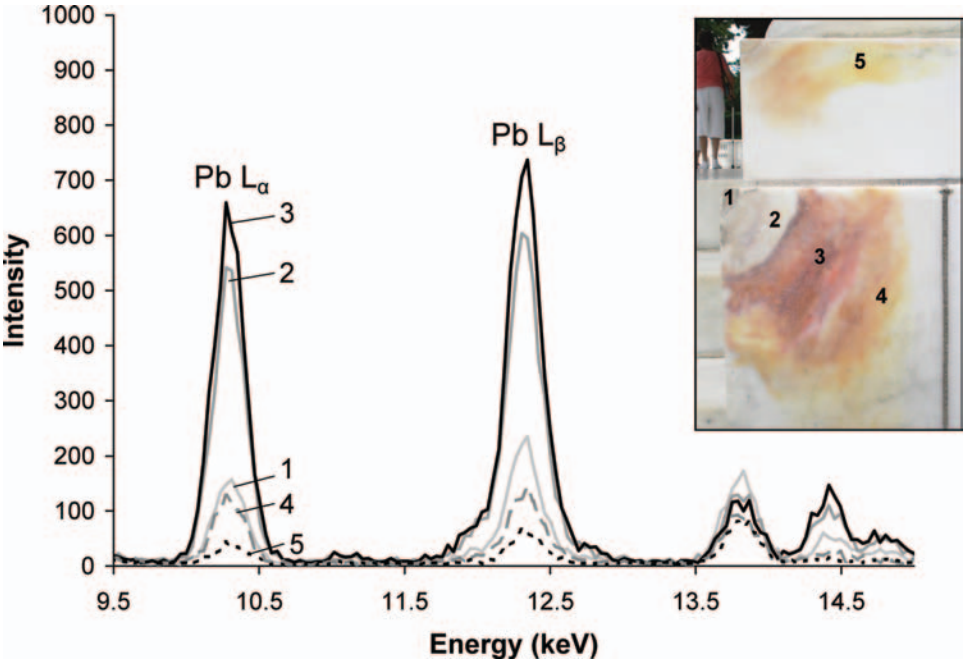

FIGURE 4. X-ray fluorescence analysis of the corner, showing highest concentrations of lead in white and purple areas. For regions 1, 2, and 3, spectra exhibit peak height ratios L_β/L_α of slightly more than 1, which indicates that the concentration of lead is higher inside the marble than at the surface.

a lead drip edge observed to the right of the stage on the interior of the amphitheater. The drip edge is heavily corroded, with minium found in red corrosion products by XRD analysis, and the vertical wall underneath also presented yellow washes similar to those observed on stained corners. In this case lead sheets were almost certainly used to prevent water penetration into basement rooms. Finally, lead sheets are likely to be present at floor level under the balustrade between the exterior columns, although none were observed. Regular water washing of the pavement in combination with rain has likely contributed to the severe red staining of the external paving blocks between the columns.

Lead sheets corrode rapidly in the presence of Portland cement and lime water (Brady, 1934). Typically, fresh Portland cement can give rise to pH values in the pore water up to 13.5 (in comparison, saturated lime water has a pH of 12.5). At this very high pH, PbO becomes a stable oxide, as does Pb_3O_4 when the environment is slightly oxidizing (i.e., in the presence of oxygen). Although it has not yet been confirmed, it appears that Portland cement may have been used for both pointing and mortar repairs at the Memorial Amphitheater. With that in mind, it is not surprising to find examples of red staining consisting of lead corrosion products.

Despite the presence of red-pigmented bacteria isolated from the Memorial Amphitheater, the staining observed on the marble is caused by lead oxides. In this case, we believe that the pigmented organism comprises a relatively small percentage of the bacterial community on the stone and that biases introduced by culturing caused large numbers of this bacterium to be isolated. However, the etiology of red staining on stone structures does not appear limited to lead oxides. Konkol et al. (2009) found red stains on a marble sculpture produced by *Serratia marcescens*. The stains were caused by an organic pigment, and no lead was used in construction of the sculpture. Careful analysis of red stains is needed to ascertain the cause of the staining and to determine appropriate solutions.

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Case Study: Biocontrol Testing at the San Ignacio Miní Jesuit-Guaraní Mission, Misiones, Argentina

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In the early seventeenth century Jesuit missionaries established some 30 missions to settle the nomadic Guaraní tribes in the area that now corresponds to southwest Brazil, east Paraguay, and northeast Argentina. After expulsion of the Jesuits in 1768, the missions were taken over by different religious orders, such as the Dominicans and Franciscans. Under the Jesuits, the missions had worked on an association system, therefore the attempt to have them work as separate units by the different orders was completely unsuccessful. Thus, the missions were abandoned, suffering vandalism and neglect, and were in most cases taken over by the jungle. The San Ignacio Miní mission, the first mission established in what is now Argentina, is located some 60 km (36 miles) northeast of Posadas, the capital of the province of Misiones. The site, some 70,000 m^2 (17.5 acres) in extension, was restored in the 1940s (Onetto, 1999) and was listed by the United Nations Educational, Scientific and Cultural Organization as a World Heritage Site in 1984.

In 2002, the Jesuit-Guaraní Missions Program of the province of Misiones was established as part of an international campaign to link the missions of the three countries in a tourism circuit that would help make them self-sustainable while developing uniform criteria for their conservation. Within this program, several restoration and conservation activities were carried out, among them that of the main portal of the church in the San Ignacio Miní mission. This intervention was carried out between 2006 and 2007 and provided an opportunity to test passive biocontrol systems (Magadán et al., 2007).

Passive biocontrol systems have been applied successfully in the past (Wessel, this volume). The system relies on the slow leaching of metal ions, such as copper or zinc, from metal strips by rainwater that then flows over the surface to be controlled, a point that can be observed on any structure that has bronze, brass, or zinc elements present. It is best observed in cemeteries, where metal lettering leaves a clean stripe in an otherwise biocolonized surface. The leached

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ions can act as a long-term biocide or as a fungistat to prevent new biogrowth if the metal strips are appropriately placed (Wessel, 2003). Although the implementation of these systems can be considered relatively straightforward on buildings of regular geometric shape, it is a challenge when dealing with ruins at archaeological sites. Therefore, a test site was established in the San Ignacio Miní site to evaluate performance.

For this purpose, a wall in one of the housing rows of the site was selected. The wall is easily accessible but not too visible to visitors, so as to avoid any tampering with the test site. Figure 1 identifies the location of the test area on a plan of the San Ignacio Miní site.

The wall, some 2 m high and nearly 1 m wide, is 6 m in length, and the test area occupied about half of the wall on its west end. Only the top and the north side were

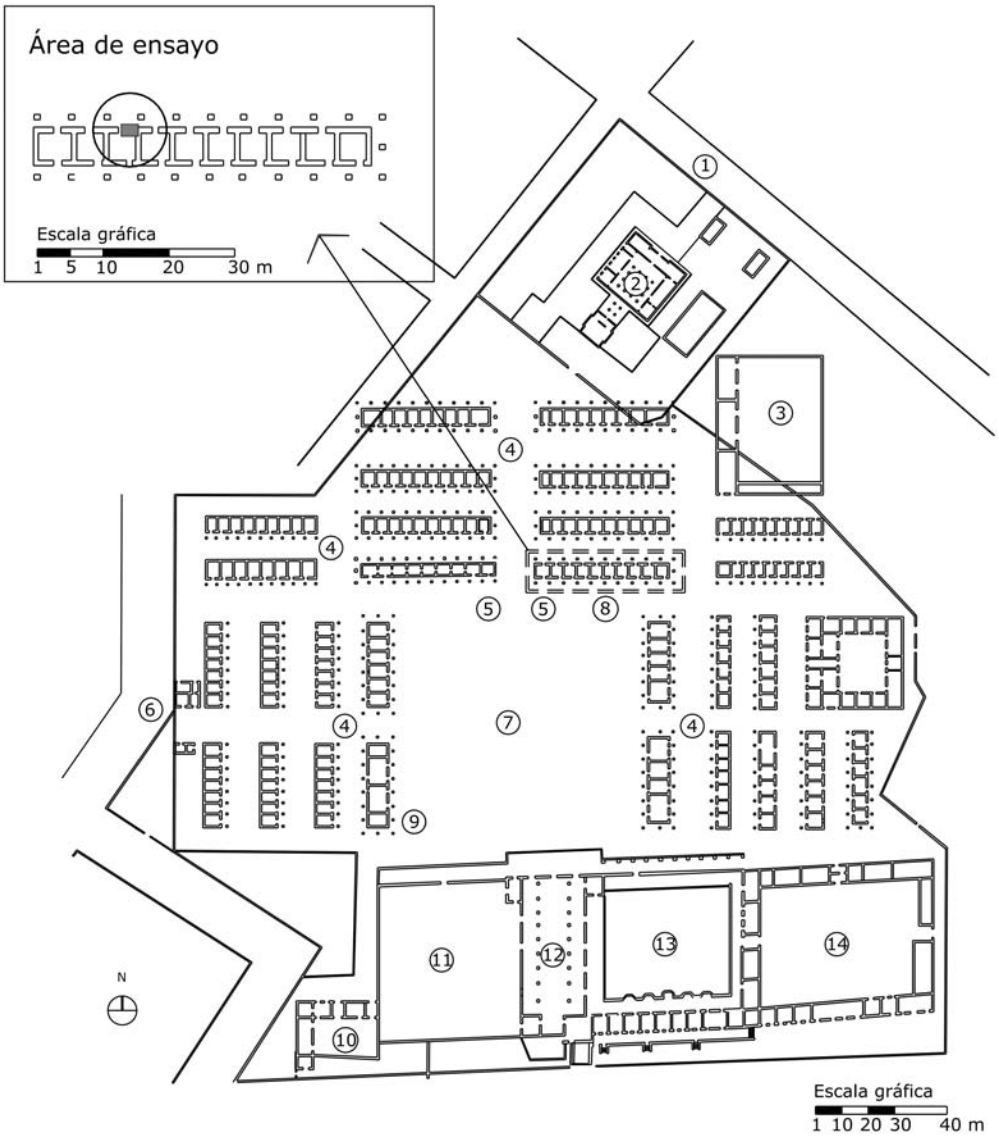

FIGURE 1. Site plan for the San Ignacio Miní mission showing the test wall location, circled and shaded, in the upper left corner. (1) Current entrance to site. (2) Interpretation center. (3) Former hostel for visitors. (4) Former living quarters for the Guaraní. (5) Former chapels. (6) Current exit from site. (7) Central plaza. (8) Living quarters row where the biocontrol tests were applied. (9) City Hall. (10) Home for widows and orphans. (11) Cemetery. (12) Church. (13) Cloister and living quarters for the Jesuits. (14) School and workshops.

treated. First of all, the thick colonization on the top and upper part of the north wall, consisting of some shrubs, higher plants, and a mat formed by the growth of liverworts, mosses, and ferns, was sprayed with an herbicide (Tordon, active ingredient picloran, i.e., 4-amino-3,5,6-trichloropicolinic acid) that is absorbed through the leaves. After three days, the vegetation had wilted, and it was removed by scraping with wooden spatulas. The larger bushes were cut, and any remaining intermediate plants were mechanically removed by pulling them out.

Three sections, each about 1 m in length, were laid out on the top of the wall to test the three different metals selected, lead, zinc, and brass (58% copper, 40% zinc, and 2% lead), to allow a comparative evaluation of their effectiveness as passive controls. These areas were covered, respectively, with (1) six lead strips (10 cm wide, 1 mm thick, and most of them nearly 1 m long, with some shorter pieces added to supplement them), (2) zinc mesh made of 2 mm thick wire (2 × 2 cm opening), and (3) hand-braided brass mesh made of 2 mm thick wire (2.5 × 2.5 cm opening) topped by four brass strips, about half a meter long (see Figure 2). In the middle section the whole width of the wall was covered by the zinc mesh; in the other sections only half the width of the wall was covered.

FIGURE 2. View of the top of the wall with the passive system in place. From the front to the back are brass mesh and strips, zinc mesh, and lead strips.

The north side of the wall was divided into the three sections corresponding to the sections on top of the wall where the metals had been installed, as shown in Figure 3. The right side of each section was left uncleaned as a control, whereas the left side was cleaned. First, each section was sprayed with a biocide (7.5% volume per volume (v/v) of the active ingredient, benzalkonium chloride, in water), and then the moist surfaces were brushed clean. A rather high concentration of biocide was used because the stone contains clays (a quartz arenite cemented by iron oxides and ferruginous clays), and it is known that quaternary ammonium salt biocides are strongly adsorbed by these minerals (Young et al., 1995). This work was carried out in the fall season in May 2007. Although the elimination of existing biocolonization with this passive control system is very slow, requiring several years (Wessel, 2003), the purpose of having control areas was to check how many years it would take to remove the existing layer of mixed biocolonization, including algae, lichens, mosses, hepaticas, and ferns, and which of the three metals would be more effective for this purpose.

As can be seen from the control areas in Figure 3, biocolonization of the structures in the site is a constant concern and requires regular maintenance, i.e., mowing the

FIGURE 3. View of the north side of the wall where the passive control systems were installed on the top. The left area is below lead strips, the middle area is below zinc mesh, and the right area is below brass mesh (photo taken in May 2007). The left half of each area had been cleaned at the beginning of the experiment; the right half was left uncleaned and served as a control. The upper orange arrow points to a small plant, and the lower one points to a hepatica. The blue arrow points to a moss- and hepatica-covered block. The red arrow points to a detaching lichen.

grass and periodically cutting down bushes that start to grow in the masonry structures of the site, so as to prevent them from mechanically damaging the structures with their roots as they develop into trees. Therefore, the idea of a passive control system that would reduce the required maintenance was gladly accepted by the authorities who allowed the installation of the test site.

Figure 4 shows the appearance of the test wall after 16 months, in late winter (August 2008). Most apparent is the regrowth of shrubs that had been cut, such as the *Manihot tweediana* (known locally as "falso café"), visible near the top of the uncleaned lead test area on the left. On cleaned areas no new biocolonization is visible except for some algae on the cleaned lead section. On the uncleaned control areas some vegetation even seems to have disappeared. In the lead section, for example, the upper orange arrow indicates the former location of a small plant, and the lower orange arrow indicates the area covered by liverwort or hepatics (Marchantiophyta division), where growth has decreased. In the zinc control section in the center, mosses (Bryophita division) appear to have been lost, but liverwort coverage appears to have increased, indicated by the blue arrow. In addition, dead brush between the first two rows of blocks has fallen off.

FIGURE 4. Same view as the one in Figure 3 16 months after the installation of the passive control system (photo taken in August 2008). The upper orange arrows point to the area where the small plant noted in Figure 3 has disappeared, and the lower orange arrow points to a decrease in hepatica cover. The blue arrow points to a diminished moss and hepatica cover. The red arrow points to the area where the detaching lichen was lost.

In the brass control area on the right, the red arrow indicates the location of the partially detached lichen present in 2007, which has since fallen off. However, some plants started to grow in the open joints (see cleaned block to the right of the blue area), and in contrast to the somewhat diminished growth on surfaces of the north side of the wall, new growth appeared on the top of the wall (see Figure 5), apparently unaffected by the passive controls.

One month later, as vegetation increased with the beginning of spring (September 2008), the wall was cleaned because it had been expected that the system would prevent this regrowth. Plants were removed manually on the top of the wall without disturbing the mesh or metal strips. On test areas on the side of the wall, smaller plants were removed by hand and with soft brushing on both previously cleaned and uncleaned control areas. The appearance of the wall by the beginning of summer (December 2008), three months after this cleaning, is shown in Figure 6. The result of the second cleaning campaign is best reflected in the loosening of some lichens that were starting to flake off, as can be seen in the top right hand block in the control area below the brass mesh.

Figure 7 shows the appearance of the wall in late summer (February 2009, five months after the second cleaning). The *Manihot tweediana* bush, ferns, and other smaller plants have reappeared. No obvious recolonization has occurred on the originally cleaned areas.

To date, this experimental setup has performed as expected (Wessel, 2003). Cleaned areas may have shown a slight increase in blue-green algae colonization. Continued monitoring is important to determine the length of time required to eliminate existing growth from control areas

FIGURE 5. View of the center section of the top of the wall, covered with the zinc mesh. Note the abundant growth of higher plants. Similar growth was found in open joints of other sections as well (photo taken in August 2008).

FIGURE 6. View of the side of the wall three months after hand removal of higher plants and brushing on all test areas (photo taken December 2008).

FIGURE 7. Same view as in Figure 6, five months after the September 2008 cleaning (photo taken in February 2009). Ferns, smaller plants, and the *Manihot tweediana* bush have regrown on the originally uncleaned control areas. No obvious recolonization has occurred on the originally cleaned areas.

and to determine which metals and which form of metals (strips or sheet) are most effective. Finally, and with hindsight, it is evident that some actions should have been implemented at the time the metals were installed. For example, an herbicide or biocide should have been injected into the cut stump of the bush, and there should have been a control area above with no metal was installed. Sealing the top of the wall with an appropriately formulated hydraulic mortar capping, perhaps including some metal powder (Henriques et al., 2007), would have prevented recolonization, as was done on top of the walls of the main portal and the lateral portal at the time of their restoration (Magadán et al., 2007; Magadán, 2008).

Acknowledgments

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Case Study: Comparative Study of Commercially Available Cleaners for Use on Marble Veterans Affairs Headstones

Jason Church, Mary Striegel, Christopher McNamara, Kristen Bearce Lee, and Ralph Mitchell

A variety of commercial products are available for use in cleaning stone surfaces contaminated with microbiological growth. The effectiveness of many of these products is questionable, however, and direct comparison of some commonly used products would be of significant interest to conservators. Therefore, a study was carried out to compare commercially available cleaners for the removal of soiling and biological growth from federally issued headstones. Specific goals were to test cleaning products for effectiveness to recommend those products and methods best suited to clean and preserve headstones.

The study focused on five national cemeteries: Alexandria National Cemetery in Pineville, Louisiana; Bath National Cemetery in Bath, New York; Jefferson Barracks National Cemetery in St. Louis, Missouri; San Francisco National Cemetery in San Francisco, California; and Santa Fe National Cemetery in Santa Fe, New Mexico. These cemeteries were chosen to represent the various regions of the National Cemetery Administration (NCA) as well as different climatic zones, including subtropical, temperate, continental, semiarid, and oceanic climates. Stones that were tested in the cemeteries were carved from the Colorado Yule marble and Georgia White Cherokee marble used for the majority of both modern and historic federally issued headstones.

Prior to cleaning, baseline biological activity was documented on test areas in the fall of 2005. Headstones were then evaluated 6 and 12 months after cleaning. Tap water from the site and five commercially available cleaners were selected for application to test areas on 48 headstones at each cemetery. Products were chosen to include cleaners that are frequently used, environmentally friendly, user friendly, and unlikely to damage the stone. Daybreak (NCH Corp., Certified Labs), based on sodium hypochlorite, is the most commonly used cleaner within the NCA. Kodak Photo-Flo (Kodak Corp.), a mixture of p-tert-octylphenoxy polyethoxyethyl alcohol and propylene glycol, has been

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commonly used to clean headstones following the recommendation by Strangstad (1988), presumably because it promotes faster drying. The three remaining cleaners were chosen to provide a range of compositions. $H_2Orange_2$ Grout Safe (Proven Solutions Inc.) contains hydrogen peroxide in a slightly acidic solution. D/2 Architectural Biocide (Sunshine Makers Inc.), mainly containing quaternary ammonium compounds, has antimicrobial properties. Marble Cleaner (World Environmental Group) has cleaning properties based on the action of surfactants and chelating agents. Cleaners were spray applied to test patches measuring approximately 6 × 6 inches (approximately 15 × 15 cm) on 20 headstones at each site. Each solution was applied to stones in both sun-exposed and shaded locations to account for possible differences arising from local environmental variations.

Headstone test patches were evaluated for changes in appearance after 6 months and biological activity after 6 and 12 months. Appearance changes were documented using photography and color measurements. The color data were collected with a Minolta CR-400 Colorimeter (Figure 1), measuring three spots on each test area and averaged for a total of 18 measurements per headstone. The same areas were measured in each case and evaluated by calculating the number of color changes where the total change (D*E*) was greater than 5 points, which is perceptible to the human eye.

A 3 × 3 cm area of the headstone surface was sampled for microorganisms using BBL Liquid Amies Culture Swabs (Becton-Dickinson). To quantify microbial growth, heterotrophic bacteria and fungi were enumerated by plating samples on solid media (nutrient agar and malt extract agar, respectively). Plates were incubated at room temperature for two days, and colonies were counted. Cyanobacteria and algae were enumerated using a hemocytometer. Performance of test cleaners was compared on the basis of biological regrowth activity by ranking the cleaners from 1 to 6. The ranking of 1 was given to the cleaner that had the highest regrowth rate, and 6 was given to the cleaner with the lowest regrowth rate. Thus, lower numbers indicate more poorly performing cleaners.

Algae and cyanobacteria were not observed on any of the headstones prior to cleaning, but other bacteria and fungi were detected in almost all locations by sampling using the Liquid Amies Culture Swabs. Numbers of organisms varied greatly among cemeteries and headstones, and

FIGURE 1. Jason Church takes color measurements of a headstone after cleaning.

numbers of bacteria on the headstones (~10^5/cm^2) generally averaged one or two orders of magnitude greater than the numbers of fungi (~10^3/cm^2).

After six months, the cleaned areas showed the greatest number of changes in color measurements ($\Delta E > 5$ and 10) on test patches cleaned with Kodak Photo-Flo (Table 1). No algae or cyanobacteria were observed in samples collected after six months, but numbers of other bacteria and fungi were generally consistent with the color measurements for specific cleaners. The lowest levels of growth, for example, were often observed in samples that had been treated with D/2 and Daybreak, which performed best according to the color measurements. The numbers of bacteria (10^7–10^8/cm^2) and fungi (10^3–10^4/cm^2) were generally higher than in the initial samples, however, most likely because of seasonal effects. Samples collected in November and December could be expected to have less biological material than samples collected in the spring months of April and May.

On the basis of the data obtained for appearance change and biological activity, Kodak Photo-Flo was eliminated from further testing after six months. $H_2Orange_2$ Grout Safe cleaner performed well according to color measurements of biological activity after six months, but closer inspection of headstones indicated that biological staining was present on some test areas. On the edges of headstones at Jefferson Barracks, for example, activity is clearly visible near the edges away from areas measured for color change (Figure 2). On the basis of these observations of growth, $H_2Orange_2$ Grout Safe was also eliminated from the study.

Biological activity evaluations after 12 months did not detect algae and cyanobacteria on areas treated with the four remaining solutions (D/2, Daybreak, Marble Cleaner, and water). These organisms typically provide the most visual evidence of growth on headstones, and their absence, even from stones treated with water, suggests that a 12 month period may be too short for determination of the effectiveness of the cleaners' biocidal properties in the field. Bacterial and fungal growth varied among cemeteries.

Santa Fe National Cemetery displayed the largest amount of bacterial and fungal activity of the five cemeteries, which was five times greater than any other location. Jefferson Barracks results showed small quantities of fungal growth on all but one headstone. Fungi were found on headstones in both sunny and shady locations. Bacterial counts were limited to a few headstones in Jefferson Barracks. In Alexandria, more bacterial and fungal activity was seen on headstones in shady locations compared to sunny locations. Bacteria were not detected in many samples from San Francisco National Cemetery but, when

TABLE 1. Number of color change measurements (ΔE) greater than 5 and 10 for each cleaner applied on headstones at Alexandria, Jefferson Barracks, San Francisco, and Santa Fe National Cemeteries.

Cleaner	$\Delta E > 5$	$\Delta E > 10$
D/2	5	1
Daybreak	7	2
$H_2Orange_2$	5	2
Marble Cleaner	8	1
Photo-Flo	11	3
Water	7	2

FIGURE 2. Biological growth observed on the test stone at Jefferson Barracks after six months: area A is treated with Kodak Photo-Flo, and area B is treated with $H_2Orange_2$ Grout Safe cleaner.

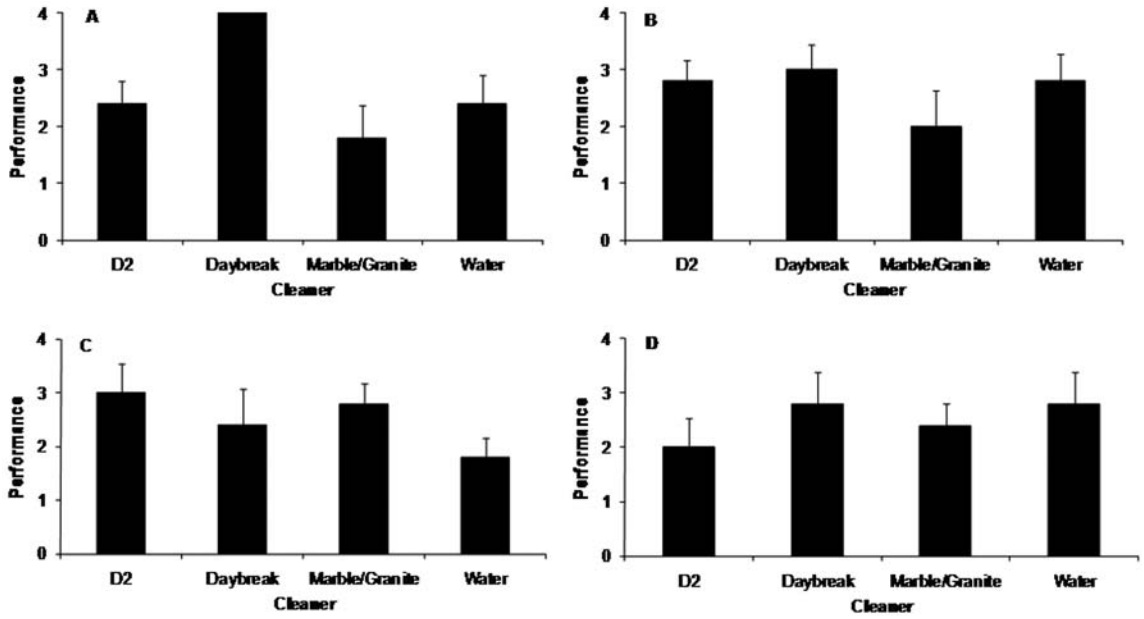

FIGURE 3. Bacterial and fungal growth on Colorado Yule marble headstones averaged for five cemeteries in two types of locations: (A) bacteria in sunny locations, (B) fungi in sunny locations, (C) bacteria in shaded locations, and (D) fungi in shaded locations. Error bars indicate the standard error of the mean.

found, were more likely to be seen in sunny locations. In contrast, bacteria and fungi were detected in few samples from Bath National Cemetery.

Initially, the presence of higher biological activity at Santa Fe National Cemetery seemed counterintuitive. Santa Fe is a drier climate, and little biological soiling had been observed in the cemetery. Locations such as Jefferson Barracks or Alexandria would be expected to have richer environments for biological growth because of their climates and higher relative humidities. It is important to note before evaluating results from initial biological analyses that each cemetery has its own regular maintenance schedule, which will influence the nature of the biological activity on headstones from that cemetery. For example, Santa Fe National Cemetery is the only one in the study where the stones have not been bleached as part of a regular maintenance schedule.

In terms of differences in location within the cemeteries, Daybreak showed better control of bacterial growth in sunny locations than the other treatments (Figure 3A); analysis of variance (ANOVA) confirmed a significant difference among the cleaners at a 95% confidence level, i.e., a probability level $p < 0.05$. No other significant differences were found, however, for fungi in sunny locations (Figure 3B), bacteria in shaded locations (Figure 3C), and fungi in shaded locations (Figure 3D).

Laboratory studies are planned to further evaluate the effectiveness of the three remaining cleaners, and accelerated studies will be carried out on stone samples in the Laboratory of Applied Microbiology at Harvard University. It must be noted, however, that none of the cleaners provide long-term protection against microbiological growth on stone.

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Case Study: Deer Stones of Mongolia after Three Millennia

Paula T. DePriest and Harriet F. Beaubien

Deer stones, Mongolia's mysterious ancient monuments, are some of the most spectacular expressions of Bronze Age megalithic art anywhere in the world. The steppe region of central Asia is home to an estimated 700 deer stones; Mongolia alone has over 550. Although they are considered to be among the most important archaeological treasures of the region, very little is understood about their stylistic development, function, and meaning within the cultural contexts that produced them, in part because of their remote and isolated locations. Renewed interest in Mongolian culture and increased ecotourism have prompted a documentation project as part of a larger archaeological and ethnobotanical research endeavor, the American-Mongolian Deer Stone Project (Fitzhugh, 2005). Since 2005, the project has documented over 100 deer stones, located in nearly 20 sites, photographically and with condition notes; over 40 have been scanned down to a submillimeter scale using 3D imaging technology (Beaubien et al., 2007; Beaubien and Karas, 2008; Wachowiak and Karas, 2009).

The deer stones, carved stone monoliths that stand 1–4 m high, are found individually, in small groups, or concentrated in larger groupings in grassy steppe environments, typically as part of stone burial complexes (Figure 1). The burial sites have been dated from the Late Bronze to Early Iron Age, between 2,000 and 3,300 years ago (Fitzhugh, 2005:17, 22–23). Deer stones bear elaborate depictions in bas relief of flying "spirit deer" with swept-back antlers and legs folded beneath their bodies, perhaps representing spirits of ancient chiefs and clan leaders (Figure 2).

Most of these monoliths are granite, varying in color from grey to pink and in quality from a very compact cohesive stone to one flawed by joints, microfractures, or foliations. The deer stones made from the more compact granites are extremely well preserved, with carved figures and symbols still sharply sculpted in the stone. Others, however, show wear from the eroding forces of wind, rain, and Mongolia's unforgiving dust storms. Rapid shifts between temperature extremes experienced on the Mongolian steppe may expand microfractures and weaken the structure as the component minerals, quartz, feldspars, and mica, have different thermal expansion coefficients. Some deer stones are almost unrecognizable, with their surface features erased or their forms broken into a trail of blocks littering the ground.

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FIGURE 1. The Ulaan Tolgoi site in Mongolia has several carved stone monoliths.

FIGURE 2. Carvings of flying "spirit deer" on the south side of a deer stone (DS4 in Beaubien et al., 2007) at the Ulaan Tolgoi site.

Whether standing, toppled and reclining on the steppe, or long buried, many deer stones are altered by biological organisms that have colonized their surfaces, ranging from microorganisms to lichens, mosses, and higher plants. Animals also leave their marks; horses, camels, and yaks use them as rubbing posts, birds use them for perching and decorate them with guano, and insect webs and cocoons can be found in nooks and crannies. Humans may be the most damaging organisms. Before the end of the Bronze Age some deer stones were reused for so-called square or slab burials where, like a four-poster bed, the stones formed the posts and the rails (Jacobson, 1993:145). Humans have pulled the deer stones down, scattered them, and moved them, including some early researchers who dug up deer stones and, after measuring, drawing, and tracing their carvings, left them scattered in the dirt (P. T. DePriest, personal observation; Fitzhugh, 2006:16). Some stones, including ones from Ulaan Tolgoi (Figures 1 and 3), have been recently reerected, sometimes set in cement, often with no information about their original orientation and location (Fitzhugh, 2005:20). The standing stones are still objects of veneration, sometimes decorated with Buddhist *khadags* (prayer scarves; see Figure 4), horsehair bundles, etc., and occasionally sprinkled with milk and smeared with oils and fats.

The focus of this case study is the gentle removal of lichens from a 3,000-year-old deer stone. Lichens, a symbiotic association of specialized fungal and algal partners, often colonize substrates such as rock outcrops and stone

FIGURE 3. Location of the Ulaan Tolgoi site. The red square shows the location of the inset map in Mongolia.

FIGURE 4. *Khadag* (prayer flag) decorated deer stone at the Ulaan Tolgoi site.

monuments. Their combination plantlike structures, called thalli, grow slowly, but in tens to hundreds of years may form extensive colonies on stone surfaces. At the Ulaan Tolgoi site (see Figure 1), the five standing deer stones have an average lichen cover of 10% or less, and this cover is concentrated on the more-humid and sheltered north and east sides. Since the colonizing lichens are tightly adhered to them, the surfaces do not collect sufficient dust or organic materials that could serve as soils for the development of higher plants. Most of the deer stones have mixed lichen colonies of different genera and species, apparent from the different colors and forms present (Figure 5).

Preliminary observations suggest that at this site lichens have not caused much obvious damage to the stones.

However, one stone (DS4) showed a fairly uniform colonization of a single type of lichen; although it is pale green (called "mineral green"), it has the structure of the crustose genus *Acarospora* (Figure 6). This deer stone is about 1.34 m high, 75 cm wide, and 23 cm thick. The colony covers over a half meter square, an area greater than that of other lichen colonies on similar stones at the site. In 2004, the lichen colony appeared healthy and thick. To determine if a simple treatment would remove lichens from deer stones, in that year a narrow strip, about 25 cm long and 10 cm wide, on the right side of this lichen cover was treated by blotting with 70% ethanol until soaked but without scrubbing or any attempt to mechanically remove the lichen thalli. Ethanol kills the lichen's food-producing algae, turning them from chlorophyll green to brown when they are dead, and without the algae, the food-dependent fungal partners starve.

After the treatment in 2004, the deer stone was examined and photographed annually, except in 2006. The first year after the ethanol application, 2005, the lichens in the treated strip turned brown, indicating that the one-time treatment had killed their algal partners. By the third year, 2007, the plantlike thalli in the treated area had dropped off the stone. Notably, during the same period the sweep of the lichen on the entire north side of this deer stone also began to turn brown and thin out, demonstrating that the colony is dynamic. By 2009, all the lichen was entirely brown and flaking, and the untreated area was almost as bare as the treated area (Figure 7). Figure 8 shows a detail of the edge of the treated area, four years after the

FIGURE 5. Another deer stone at the Ulaan Tolgoi site showing mixed lichen colonization. Note the slanted lower edge of the biocolonization, which reflects that this stone most likely was partly buried to this line.

FIGURE 6. North side of the deer stone (DS4) at the Ulaan Tolgoi site with uniform lichen colonization photographed in 2004 before treatment. The string shown serves to mark the boundary of the area that was treated with ethanol.

application of ethanol. Once this lichen drops off, it is possible that the surface will be recolonized, perhaps by the more-typical mixture of different genera and species.

Although the treatment with ethanol certainly accelerated the death of the lichens in the strip where it was applied, the ongoing decrease in the rest of the lichen coverage cannot be attributed to it. There are no other obvious changes in the stone or its environment that would account for the death of the lichen: no chemical stains, no exceptional input of toxic nitrogen compounds from bird droppings, no new shading of the area, and no ritual application of sticky materials. However, what could have changed was the position of the stone since the lichens originally colonized it. The site was discovered by Mongolian archaeologist Sanjmyatav in 1988, and in the early to mid-1990s the Mongolian-Russian archaeological team reerected some of the fallen deer stones (Fitzhugh, 2006:15), possibly including this one. It is important to note that the mineral green lichen cover originally ended rather abruptly with a slightly angled line across the stone (see Figure 6), as did the mixed lichen cover on another stone in the site (see Figure 5). The position of the lichens suggests that for many years the treated stone was lying practically flat on the ground with its upward side (now the north side) half covered with the soil up to this line. In this case, moisture in the soil's boundary layer and the sunlight's angle of insolation would have combined to support a continuous cover of the lichen species, a situation also seen in outcropped rocks in the area. Consequently, the stone may have been colonized by a single lichen rather than by the mixed lichen colonization found on most of the other standing deer stones (as in Figure 5). With a change in the stone's position to standing, the moisture content in this upper area would have dropped, and the lichen cover would have begun a steady decline over the five years it was photographed.

FIGURE 7. North side of the deer stone (DS4) from Figure 4 (a) in 2007, three years after the application of ethanol, and (b) in 2009, five years after the treatment. Note the diminishing difference between the treated area and the rest of the stone.

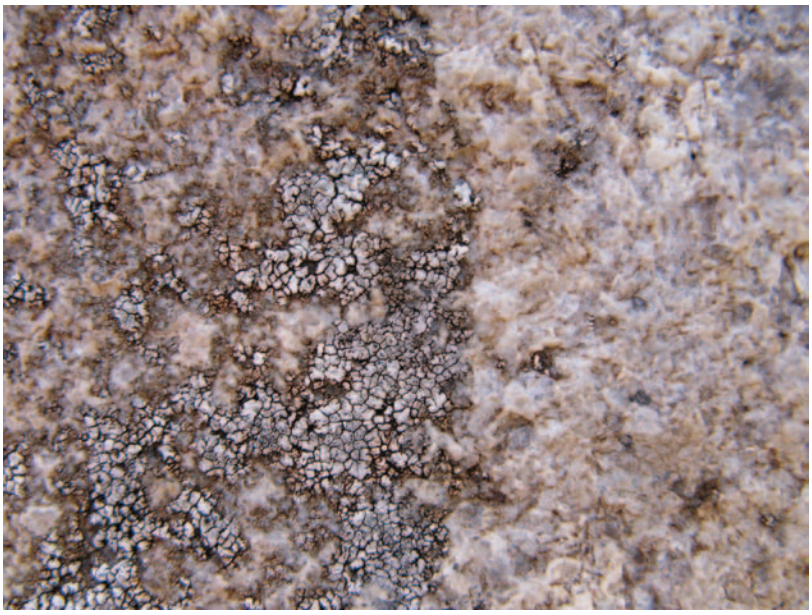

FIGURE 8. Detail of the untreated (left) and treated (right) areas in 2008, four years after the treatment. Note that the lichen colony on the left is already thinning out.

The loss of the lichen cover on this particular stone reveals 3,000-year-old carvings that are still in very good condition in spite of the years of lichen colonization they suffered, most likely because of the relatively unflawed quality of the granite surface. Although lichens will generally accelerate deterioration of the substrate, in other instances it has been shown that they also may serve to prevent deterioration resulting from the colonization of other microorganisms (Warscheid and Leisen, this volume) and perhaps from wind and rain erosion. What is important, however, is that if their removal is required for documentation purposes, then only gentle methods that do not damage the stone surface should be employed, such as the ethanol used in this instance without scrubbing or any mechanical lichen removal, and that these methods are given the required time to act on the organisms (Delgado Rodrigues et al., this volume). Biocolonization takes time to develop, and it requires time to be eliminated.

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Case Study: Field Observations on the Effectiveness of Zinc Strips to Control Biocolonization of Stone

David P. Wessel

The use of metallic strips made of copper, bronze, or zinc to control biocolonization has long been known, but the implementation of this method has not been easy. The method relies on the dissolution by rainwater of minimal amounts of metal ions that act as inhibitors for the growth of microorganisms (Ashurst and Dimes, 1999:136–137). This paper evaluates the effectiveness of zinc strips installed on the roof of the Stanford Mausoleum after 12 years.

The Stanford Mausoleum is a small neoclassical building of rectangular plan measuring approximately 8 × 12 m. In 1889 the entire building (including the roof) was constructed of gray-colored granite from Barre, Vermont (Figure 1). In 1995, Stanford University commissioned a condition survey of the building because of severe water penetration problems that had developed over the previous 106 years. Apart from those problems, the roof had developed significant lichen colonization, including foliose and crustose lichens. The former were identified as belonging to the Parmeliaceae family and probably to the *Xanthoparmelia* genus since the latter is a common lichen found in the San Francisco Bay area (Figure 2).

A conservation intervention on the building was completed in 1997. After an initial cleaning of the entire building by pressure washing, the proprietary Heavy Duty Restoration Cleaner by ProSoCo, Inc. (a mixture of glycolic, hydrofluoric, orthophosphoric, and citric acids with a nonionic surfactant) was used in a 1:5 aqueous solution to facilitate removal of the lichens. After the roof was free of the lichens, zinc strips bent to conform to the angle of the roof's ridge were installed on the ridge, and a commercial, one-part urethane sealant (Sikaflex 1a) was used to keep them in place (Figure 3) (Wessel, 2003).

Twelve years later, the roof was inspected to evaluate the effectiveness of the zinc strips. No biological colonization was evident, and only minor soiling, which could be attributed to a passing seagull, was found (Figures 4 and 5). Nevertheless, lichen colonization is slow to develop, and judging from lichen colonization of other materials, it certainly would require more than five years to develop (Henriques et al., 2007; Delgado Rodrigues et al., this volume). Only a small amount of soiling, under the roof cornice, had reappeared (Figure 6).

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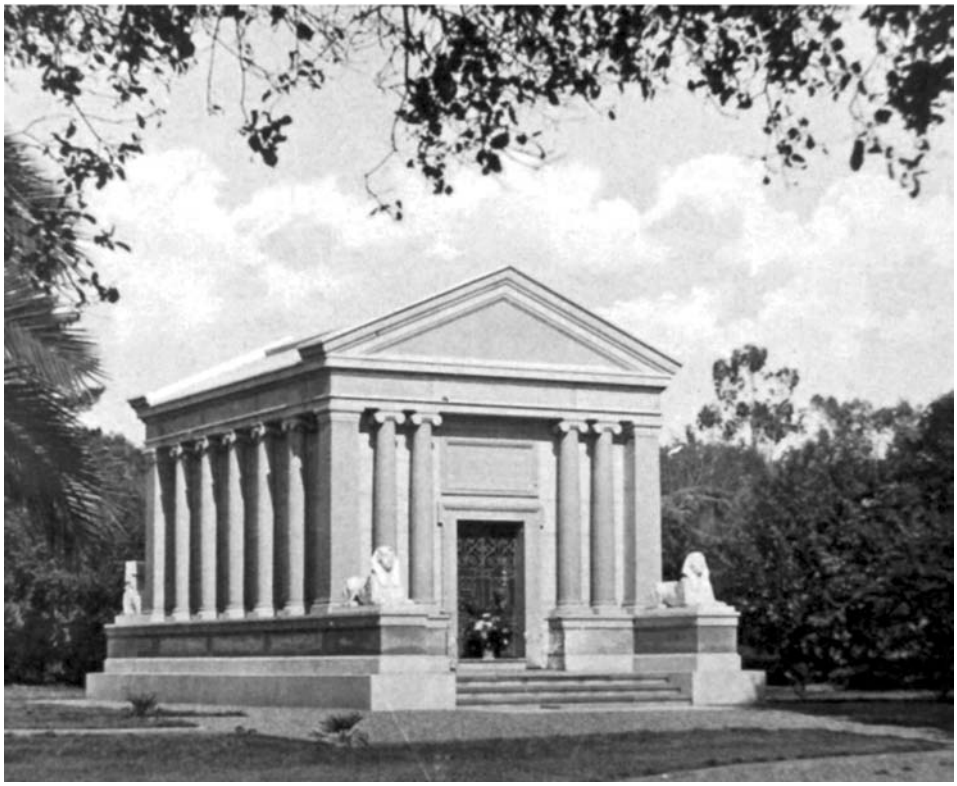

FIGURE 1. The Stanford Mausoleum (photo courtesy of Stanford Archives).

FIGURE 2. The roof of the Stanford Mausoleum in 1995, showing the heavy lichen colonization that had developed over 106 years.

FIGURE 3. The roof of the Stanford Mausoleum after cleaning in 1997, showing the zinc strips installed on the roof's ridges.

FIGURE 4. The roof of the Stanford Mausoleum in 2009, 12 years after the cleaning and the installation of the zinc strips.

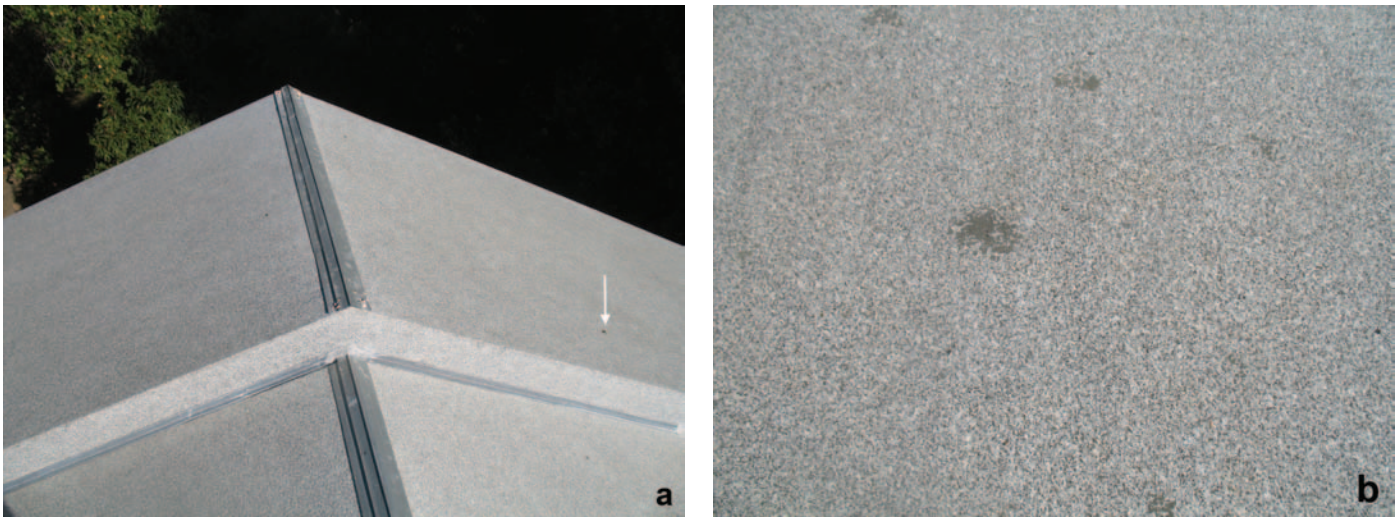

FIGURE 5. (a) Detail of the roof in 2009, showing minor soiling. (b) Close up of the area of soiling indicated by the arrow in (a).

FIGURE 6. Detail of the cornice in 2009, showing new soiling.

It is important to point out that the installation of zinc strips to prevent recolonization by lichens and other microorganisms can be most effective when the object to be protected has a regular shape and design that ensures even distribution of rain water over the surface. Furthermore, this approach should be used as part of an integral conservation intervention and not as an isolated measure. The condition of the Stanford Mausoleum 12 years after the intervention was documented to serve as reference for future monitoring. So far, the conservation intervention has been a major success. Not only has the building remained largely free of biological recolonization, but its maintenance cost has been significantly reduced, an important point in architectural conservation.

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Discussions, Conclusions, and Recommendations

A. Elena Charola, Christopher McNamara, and Robert J. Koestler

ABSTRACT. The results of the discussions and the conclusions that could be drawn from this workshop are summarized, especially with reference to the three questions that had been posed to the participants. The first question addresses the identification of criteria to determine when the colonization on a stone surface may be problematic. This requires compiling information on both the substrate and the microorganisms. The second deals with new methods for biocolonization control. This entails the development of eco-friendly compounds (e.g., biocides) methods of biocontrol, and development of long- and short-term management plans based on regular monitoring and simple maintenance practices. The final question aims to identify future research directions to achieve the two goals mentioned. Several topics were identified, ranging from rapid testing methods to interdisciplinary research methodology. Most significant was the recommendation to develop a practical database from the existing literature.

INTRODUCTION

The international workshop "Biocolonization of Stone: Control and Preventive Measures" was held at the Museum Conservation Institute in April 2009. The goal of the workshop was to provide a discussion forum for biologists, materials scientists, and conservators interested in stone biodeterioration to address the following three questions:

1. How can we develop criteria to determine when microbial colonization and growth on stone heritage materials is problematic?
2. What new environment-friendly methods are available to prevent microbial deterioration and colonization and to control recolonization of stone after a conservation intervention?
3. What future research directions will aid in achieving these goals?

The workshop featured presentations from seven experts in the field of microbial degradation of stone monuments and buildings and five case studies illustrating practical examples. A discussion period served to identify some of the key points that needed clarification, and a subsequent panel discussion with the speakers aimed to address the questions listed above. Finally, a closed-door

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half-day meeting of all the speakers focused on developing criteria that can be used to evaluate microbial colonization and growth and to determine conditions when treatment is necessary. The participants also worked on identifying future research directions for the field. For this purpose, the speakers were divided into two groups, a largely biologically oriented group and a second that could be considered the "practical approach" group. Both groups addressed the same three questions. The interesting result was that both groups came up with essentially the same answers and recommendations.

CONCLUSIONS AND RECOMMENDATIONS

The conclusions and recommendations made after the discussions can be summarized as follows.

1. How can we develop criteria to determine when microbial colonization and growth on stone heritage materials is problematic?

Microbial colonization of outdoor stone surfaces is inevitable. However, problematic microbial growth (characterized by aesthetic changes and/or biodeterioration) does not occur on all objects. Borrowing a framework developed by food microbiologists, categories of factors that could affect microbial colonization and growth were proposed, and important factors within each category were identified. These include:

1. Factors intrinsic to the substrate. Among these, the chemical and mineralogical nature of the stone, and its porosity, surface roughness, and orientation with regard to bedding planes in the building, are the most important.
2. Present state and previous history of the building or monument. These factors include the presence of soluble salts or inorganic surface crusts; the type of deterioration, e.g., powdering or flaking, that the stone has developed; contact with the soil; and previous treatments that may have been applied to the object, such as cleaning, consolidation, water repellents, and biocides.
3. Extrinsic factors defining the environment of the object. These include geographic location of the object; the environment, i.e., rural or urban; general macroclimate, e.g., average temperature and humidity, rain periodicity; exposure, e.g., whether northern or southern, and light irradiation regime; and microclimate for the object, including thermohygric changes and rate of these changes.
4. Factors describing the physiology and taxonomy of the microorganisms. Factors that should be considered include coloration (green or black pigments), the presence of biofilm or slime layers, the presence of filaments, the depth profile of colonization, the extent of colonization, comparison of the microbial community with nearby objects, and the rate of change (or seasonality) of the microbial growth.

2. What new environment-friendly methods are available to prevent microbial deterioration and colonization and to control recolonization of stone after a conservation intervention with a minimum environmental impact?

New methods are needed to prevent and control microbial colonization of stone. Particular areas of need include the development of eco-friendly compounds (e.g., biocides), methods of biocontrol, and development of long- and short-term management plans based on regular monitoring and simple maintenance practice, such as dust removal or periodic washing of the object.

3. What future research directions will aid in achieving these goals?

Five conclusions regarding directions for future research were reached:

1. Simple, quick, and accurate methods for measuring microbial colonization and growth are needed. Improved sampling techniques and a rapid biofilm sensor would be extremely useful.
2. Multidisciplinary studies of deteriorated areas that have biocolonization are needed. It is necessary to assess the contribution of biocolonization to the observed damage. The presence of microorganisms does not necessarily imply that they are responsible for the deterioration present.
3. Advances in molecular biology have improved our knowledge of the taxonomy of microorganisms found on stone. Future efforts need to make use of this information, for example, by linking taxa with function or biodeterioration. Possible methods for accomplishing this include application of genomic, proteomic, and metabolomic techniques.

4. Regarding scenario testing, weathering experiments are needed to better understand the consequences of microbial colonization and growth on historic stone.
5. Further studies and testing are needed to evaluate the interaction between two conservation products, for example, the influence of biocides on the effectiveness of water repellents and vice versa, and the long-term effect of the addition of biocides mixed directly into other conservation products, such as consolidants formulated with a biocide.

Finally, the need for the development of an interactive framework to facilitate communication and collaboration among conservators and scientists interested in the deterioration of historic stone was discussed. Scientists and conservators interested in microbial colonization and growth on historic stone are spread across the globe. In addition, studies of microbial activities on stone are found throughout the scientific and conservation literature in a wide range of journals, books, and gray literature representing many different disciplines. So there is a need for a centralized portal that can facilitate communication and access to information. Development of a Web site or wiki was proposed. The site would contain features such as a database of publications and information about biodeterioration and a discussion board.

Therefore, the immediate action recommendation is to develop a practical database from the existing literature. This would serve to identify the gaps between the various disciplines that have to collaborate in this field. For example, many of the microbiological studies reported in biological journals do not present full information on the substrate on which the microorganisms develop. On the other hand, materials scientists report the existence of biocolonization but do not identify the species in question. Therefore, to gain a better understanding of the problem from the vast literature that has already developed, it is necessary to complete the existing studies. Since these are dealing with monuments, the information is likely to be available; what is necessary is a means to bring the information together.

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