In situ evaluation of the biodeteriorating action of microorganisms and the effects of biocides on carbonate rock of the Jeronimos Monastery (Lisbon)

C. Ascaso\textsuperscript{a,}\textsuperscript{*}, J. Wierzchos\textsuperscript{b}, V. Souza-Egipsy\textsuperscript{a}, A. de los Rios\textsuperscript{a}, J. Delgado Rodrigues\textsuperscript{c}

\textsuperscript{a}Centro de Ciencias Medioambientales Consejo Superior de Investigaciones Científicas (CSIC), Serrano 115 dpdo, 28006 Madrid, Spain
\textsuperscript{b}Servei de Microscopia Electrónica, Universitat de Lleida, Lleida, Spain
\textsuperscript{c}Laboratorio Nacional de Engenharia Civil, Lisbon, Portugal

Received 29 November 2000; accepted 28 June 2001

Abstract

The biodeterioration effects of microorganisms colonizing the cloister terrace wall of the Jeronimos Monastery (Lisbon) were evaluated using several microscopy techniques that allow the in situ examination of lithobiontic communities. The techniques applied were: scanning electron microscopy with back-scattered electron imaging (SEM-BSE), low temperature scanning electron microscopy (LTSEM), transmission electron microscopy (TEM) and an X-ray energy dispersive spectroscopy (EDS) microanalytical system. The stone was seen to be colonized by different lichens and microorganisms and lichen thalli of \textit{Thyrea}, \textit{Aspicilia}, \textit{Verrucaria} and \textit{Caloplaca} were identified. Cyanobacteria were frequently observed close-by, as single cells or colonies and heterotrophic bacteria were also found among these. The lithobiontic community showed biogeophysical and biogeochemical effects on the substrate. Cyanobacteria produced bowl- or pear-shaped cavities. Using SEM-BSE and TEM we were able to observe a mineral network structure adjacent to the cyanobacterial wall that might be related to calcium biomobilization processes. Neoformation of biogenic carbonate was detected in thalli of the lichen \textit{Thyrea}. This information was complemented by observing details of the response of these biological components to the biocidal agents, ALGOPHASE, METATIN and PREVENTOL R80. After treatment, \textit{Thyrea} remained on the stone, although ultrastructural alterations were observed in the photobiont. When the effects of the biocides on the ultrastructure of the cyanobacteria were analyzed, ALGOPHASE proved to be the least efficient, while PREVENTOL R80 led to the complete disorganization of the prokaryotic cyanobacterial cell. These results point to the importance of evaluating biodeterioration processes and possible treatment measures without extracting the microorganisms from their microhabitat. © 2002 Elsevier Science Ltd. All rights reserved.

1. Introduction

The stones used to construct historic buildings and monuments are frequently colonized by lithobiontic communities comprised of a great variety of microorganisms, lichens, mosses and higher plants. Understanding the complex interactions between these lithobionts and their mineral substrate is a topic of current interest, since it may shed light on the bioweathering of stone monuments. Further, detailed knowledge of bioweathering mechanisms may help to more appropriately select treatments aimed at resolving the problems associated with the presence of the lithobiontic community in the stone.

Lichens are particularly involved in a range of effects on stone buildings. Most research efforts aimed at exploring the effects of lichen thalli on monuments have been based on taxonomic methods and the presence of the lichen thallus has been related to ecological factors (Nimis et al., 1992). The ecology of the communities concerned has also been investigated through multivariate analysis for application to the conservation of monuments (Monte, 1991). The macroscopic structure of the lichens thallus is comprised of different microscopic components: fungus (the mycobiont), and alga and/or cyanobacteria (both termed the photobiont). On the stone, the microorganisms forming the lichen thallus are frequently accompanied by other microorganisms such as bacteria, cyanobacteria, free-living algae or fungi and their effects cannot be ignored. Thus, the lichen-stone interface is thought to play an important role in the biodeterioration of monuments. Results regarding the effects of lichens and other lithobiontic microorganisms on the rock are contradictory and some authors even suggest a protective role (Garg et al., 1988; Ariño et al., 1995). However, since the
application of light microscopy methods to stone weathering studies, there is evidence that lichens do cause significant alteration to the rock substrate (Bech-Andersen, 1983). This controversy stems from the difficulty in directly demonstrating the weathering effects of microorganisms on rocks. According to Nimis and Salvadori (1997), taxonomical identification must always be performed since each species could contribute in a different way to the degradation process. Traditionally, the biological elements present in the mineral substrate have been investigated by microbiological techniques, with the plate culture being the most important among these. This technique was questioned by Amann et al. (1995), on the grounds that some microorganisms are difficult to isolate and cultivate. Recently, molecular biology techniques have been used to determine the taxonomy of biofilm composing microorganisms (Rölleke et al., 1996, 1998). However, given that microbial ecology involves the study of the relationships between microorganisms and their natural environment (Brock, 1987), the researcher’s attention must be focused on the in situ examination of these epilithic and endolithic communities.

Different microscopy techniques have been used in the study of the lichen-rock interface in natural rocks and monument stones (see Ascaso and Wierzchos, 1995). Scanning electron microscopy in secondary electron mode (SEM-SE) has been widely applied to morphologically characterize the biological elements of the lichen-rock interface (Gehrmann et al., 1988, 1992; Danin and Caneva, 1990). Using SEM-SE, Dornieden et al. (2000) examined poikilotropic microcolonial fungi in cracks in marble and mortar and concluded that these are true rock pathogens. Transmission electron microscopy (TEM) has been traditionally used to observe the cytological features of lithobiontic microorganisms (Ascaso and Ollacarizqueta, 1991). The SEM technique with back-scattered electron imaging (SEM-BSE) is one of the best currently available tools for this purpose, since it permits undisturbed in situ visualization of the zone (Wierzchos and Ascaso, 1994). It also has sufficient resolution to examine ultrastructural features inside the mycobiont, photobiont and/or other biological elements (Ascaso and Wierzchos, 1994; Ascaso et al., 1995, 1998b) and also of their interaction with the mineral substrate (Sanders et al., 1994; Ascaso et al., 1998a). The simultaneous application of the EDS microanalytical system with SEM-BSE serves to chemically characterize mineral features (Wierzchos and Ascaso, 1996, 1998; Prieto et al., 1997). The LTSEM technique has several advantages over SEM, since it allows the observation of water and at times enables microbial cytological identification by providing topographic images of randomly fractured deep-frozen cells (De los Ríos et al., 1999). This technique was also used by Barker et al. (1997) who examined the complex structure of extracellular polysaccharides in bacterial biofilms coating minerals.

To develop optimal strategies for the restoration of biodeteriorated monuments, detailed knowledge of the biotic components of the lithic substrate and their actions need to be supplemented with information on the response of the components to particular treatments such as biocide application. Koestler and Salvadori (1996) discussed problems linked to the evaluation of the efficiency of biocides. So far, different methods such as changes in fluorescence or estimation of chlorophyll losses have been used to evaluate biocide efficiency (Nugari et al., 1993). However, to obtain a general picture of the effects of biocides, it is necessary to observe these effects on all the microorganisms within their microhabitat, whether they contain fluorescent photosynthetic components (algae and cyanobacteria) or not (bacteria and fungi). Also, their effects on minerals need to be simultaneously analyzed. The aim of the present study was to evaluate bioweathering processes in the stone of the Jeronimos Monastery (Lisbon, Portugal) through the use of in situ examination techniques and the direct determination of biocide effects on the lithobionthic community. Results will help define appropriate measures aimed at mitigating the bioweathering process in this Monastery.

2. Materials

The Jeronimos Monastery (Lisbon, Portugal) was built in the 15th century from “Lioz” limestone. This Cenomanian limestone is quarried in the Lisbon area. The best varieties are characterized by high mechanical resistance and low porosity (lower than 1%), while poorer varieties (porosity 1–5%) are also frequently found. The present study area was limited to a fragment of the Cloister terrace wall. Calcite is the main component of the limestone, with dolomite and a minor presence of clay minerals associated with it. Several lichen species and extensive colored areas are observed in this zone. Stereomicroscopy examination of the stone surface revealed a randomly distributed encrusted black and grayish-colored biological patina.

To evaluate the effectiveness of biocide treatment, three commercial biocides were used to treat the west-facing wall of the monastery cloister in July 1998. The bioactive products used were: ALGOPHASE® [2,3,5,6-tetrachloro-4(methylsulphonyl pyridine)]; METATIN®, a product based on tributylhexane naphthanate and an organic nitrogen compound; and PREVENTOL R80® (acyl-dimethylbenzylamine chloride and isopropanol). The biocides were tested under the following conditions: ALGOPHASE® was diluted in methylethylacetone and METATIN® and PREVENTOL R80® were diluted in water, all at 2% w/v. Four months after application, several untreated and biocide treated stone samples were collected for analysis.

3. Methods and sample preparation

3.1. LTSEM examination

Untreated stone fragments were examined using the LTSEM technique. Small fragments were mounted using
O.C.T. compound (Gurr) and mechanically fixed onto the specimen holder of the cryotransfer system (Oxford CT1500). Samples were plunge-frozen in subcooled liquid nitrogen and then transferred to the preparation unit. The frozen specimens were cryofractured and etched for 2 min at −90 °C. After ice sublimation, the etched surfaces were gold sputter coated. Samples were subsequently transferred onto the cold stage of the SEM chamber. Fractured surfaces were observed under a DSM960 Zeiss SEM mi-
croscope at −135 °C under conditions of 15 kV acceleration potential, 10 mm working distance and 5–10 nA probe current.

3.2. SEM-BSE (EDS) examination

Fragments of untreated and biocide-treated stone were processed for SEM-BSE and/or EDS analysis according to a method described elsewhere (Wierzchos and Ascaso, 1994). Stone fragments, once fixed (3.25% glutaraldehyde followed by 1% OsO4) and dehydrated in an ethanol series, were embedded in epoxy resin. After polymerization, the blocks were cut and finely polished. Transverse sections of polished surfaces of the stone were stained with uranyl acetate followed by lead citrate, carbon-coated and examined using a DSM 940 A Zeiss and a DSM 960 A Zeiss micro-
scope (both equipped with a four-diode, semiconductor BSE detector and a Link ISIS microanalytical EDS system). BSE and EDS examinations of the samples were simultaneously performed. The microscope operating conditions were as follows: 0° tilt angle, 35° take-off angle, 15 kV acceleration potential, 6 or 25 mm working distance and 1–5 nA speci-
men current.

3.3. TEM examination

Lithobiontic microorganisms were detached from the substrate by scraping the interface of the sample already examined by SEM-BSE. These microorganisms were pro-
cessed for TEM by reembedding in resin and cutting with an ultramicrotome. Some of the lithobiontic microorgan-
isms (mainly cyanobacteria) from the untreated stone were cultured for taxonomic identification and were also prepared for TEM observation according to a procedure described elsewhere (Ascaso et al., 1988).

4. Results

4.1. Direct observation and culture experiments

The samples were colonized by different lichens and microorganisms. Lichen thalli of Thyrea, Aspicilia, Ver-
rucaria and Caloplaca were observed on the surface of control samples. Cultures from the untreated stone showed the presence of several single-celled cyanobacteria of the Chroococcales order and one filamentous cyanobacterium.

4.2. In situ LTSEM and SEM-BSE examination of untreated stone

The ultrastructural details of the biological components of the substrate were observed by LTSEM. Fig. 1A is an LTSEM image of a Thyrea lichen thallus showing Gloeocapsa cyanobacteria, the photobiont of this lichen (arrows). Fig. 1B shows the ultrastructural appearance of these Gloeocapsa cells (black asterisk) together with my-

cobiont cells (white asterisk). Some areas of the Thyrea thallus were occupied by mineral particles rich in calcium, carbon and oxygen (Figs. 1C and D). EDS qualitative and quantitative data (not presented) and crystal morphology suggest that this precipitate was formed by calcium car-


bonate. These carbonate precipitates revealed two different morphological features: one characterized by a compact ag-
gregate of carbonate grains (asterisk in Fig. 1C); the other, shown in Fig. 1D, was formed by aragonite-like needles (cryo-fractured in a plane perpendicular to the aragonite needle length-axis).

The presence of non-symbiotic cyanobacteria on the stone was demonstrated using LTSEM. Figs. 2A and B are LT-
SEM images of samples from the gray-colored rock surface. Single-celled endolithic cyanobacteria (Chroococcales or-
der) were seen to be encrusted within the carbonate matrix (asterisk). Some could be seen to be complete (lower arrow Fig. 2A) while others were transversally fractured (upper arrow Fig. 2A). Fig. 2A also shows the presence of water inside the rock pores. Water, in the form of ice (upper left in the figure), appears as a network (due to eutectic struc-
tures). In other areas, colonies of endolithic cyanobacteria enveloped by sheaths may be observed (Fig. 2B). In this im-
age, all the cells were transversally fractured. On occasion, endolithic bacteria could be seen among the cyanobacterial cells (arrows in Fig. 2C and D).

All the SEM-BSE images indicate that the microorgan-
isms invading this calcareous rock showed extensive euen-
dolithic destructive activity. Fig. 3A (corresponding to the LTSEM observation in Fig. 2A) shows a general view of a zone affected by the biological activity of epilithic and endolithic microorganisms. The stone surface colonized by these microorganisms shows a sponge-like structure formed on the surface of the calcareous substrate. Single cells or colonies occupy pear-shaped cavities corresponding to the cell outlines (Fig. 3B). These pits or cavities were random-
ly distributed below the stone surface up to a depth of 0.5 mm. Channel-shaped pits were also frequently observed (arrows Fig. 3C) and, on occasion, were open to the rock surface with a single cyanobacteria cell at the base of the pit (arrow in Fig. 3D). Fig. 3E is a general view of the rock surface showing its uppermost portion (asterisk) colon-
ized by an epilithic lichen thallus and, towards the right,
Fig. 1. Low temperature scanning electron microscopy (LTSEM) observation of the *Thyrea* thallus on the surface of the monastery stone. (A) Transverse section of the thallus. Arrows indicate the photobiont (*Gloeocapsa*) cells; (B) Detail of the photobiont (black asterisk) and mycobiont (white asterisk); (C) Presence of a compact aggregate of calcium carbonate inside the thallus (asterisk); (D) Aragonite-like crystals inside the thallus.

by epilithic and euendolithic cyanobacteria (stars). Note that hyphal clusters from the epilithic lichen thallus penetrate the lithic substrate (Fig. 3F). Microbial cells from untreated stone samples showed no signs of cytological alteration; the cytoplasm was well distributed and the plasmalemma was fully adhered to the cell walls (Figs. 3B and D, 4A, and B). Fig. 4A shows the presence of bacteria near *Pseudocapsa* colonies. Cyanobacterial cells could also be seen within a fissure filled with clay material (asterisks Fig. 4B). In some zones, a dense network (black arrows in Figs. 4C and D) around the colonies of bacteria cells (white arrow Fig. 4C and D) was detected between cyanobacterial cell walls and the substrate. The EDS elemental space distribution map shown in Figs. 4E and F corresponding to the SEM-BSE images (Fig. 4C and D) demonstrated the presence of calcium in the network interior.

4.3. TEM ultrastructural characterization of lithobiontic microorganisms before biocide treatment

To gain knowledge on the specific action of each biocide at the cytological level, the ultrastructure of the lithobiontic cells living inside the substrate was established before and after biocide treatment. The typical ultrastructure of a cyanobacterium is presented in Fig. 5A and B, and represents cultivated specimens from the stone. Fig. 5A shows cultured unicellular cyanobacteria with distinct thylakoids, carboxysomes and lipids. Fig. 5B shows filamentous cyanobacteria, where thylakoids were observed within each cell.

Fig. 5C, D and E show the ultrastructural features of endolithic cyanobacteria from inside the untreated rock. Note that at the cytological level, the thylakoids (arrows in
Fig. 2. LTSEM observation of different endolithic cyanobacteria on the surface of the monastery stone. (A) Cyanobacteria (Chroococcales order) (arrows) encrusted within the calcium carbonate substrate (black asterisk). Transverse section; (B) A colony of endolithic cyanobacteria encrusted in the stone. Transverse section; (C) External appearance of a cyanobacterial cell encrusted in the stone. Arrows indicate adhered bacteria; (D) Transverse section of two cyanobacterial cells surrounded by bacteria.

Fig. 5D), carboxysomes and lipid bodies are well developed and cell walls present no alterations. Some of the euendolithic cyanobacterial cells show a characteristic dense network outlining the cell wall as shown in Fig. 5E.

4.4. In situ SEM-BSE observation and TEM ultrastructural characterization of lithobiontic microorganisms after biocide treatment

ALGOPHASE®. Following treatment with this biocide, cyanobacteria in the pores and channels show a similar appearance to those in the untreated rock. The SEM-BSE image in Fig. 6A shows a general view of the calcareous rock interface with characteristic sponge-like bioweathering patterns produced by the euendolithic microorganisms. Cell walls were well preserved and the cytoplasm was well distributed within the single cells (Fig. 6B). After ALGOPHASE® treatment, Thyrea lichen thalli and mycobiont cells showing abundant concentric bodies (small black arrows in Fig. 6C) were also observed.

METATIN®. Fig. 6D shows the appearance of free cyanobacteria after treatment with this biocide. Note that some are ultrastructurally fairly well preserved while others demonstrate a high degree of plasmolysis (arrows).

PREVENTOL R80®. After treatment with this biocide, euendolithic cyanobacteria with collapsed protoplasts (arrows) and others that had completely lost their cell structure were observed (Fig. 6E and F).

Fig. 7 presents TEM images of the cyanobacteria after ALGOPHASE® (Fig. 7A), METATIN® (Fig. 7B) and PREVENTOL R80® (Figs. 7C and D) treatment.
Fig. 3. Scanning electron microscopy with backscattered electron imaging (SEM-BSE). Transverse section of a fragment of untreated stone. (A) General view showing the alterations induced by microorganisms; (B) Detail showing bowl-shaped cavities defining the cell outline; (C) Channel-shaped pores filled with microorganisms (arrows); (D) Cyanobacterial cell within a channel-shaped pore. Transverse section; (E) Transverse section of a stone fragment. Upper micrograph, section of the *Aspicilia* thallus (black asterisk) and right-hand side, euendolithic cyanobacteria (stars). Black arrow indicates an area of endolithic fungi belonging to the lichen thallus; (F) Detail of the zone indicated by the arrow in Fig. E. Cavities containing hyphal clusters.
Fig. 4. SEM-BSE and EDS images. (A) Several cyanobacterial cells and a colony of bacteria; (B) Several cyanobacterial cells inside a fissure. Asterisk indicates microdivided minerals; (C) Cyanobacterial cell and a colony of bacteria (white arrow). Black arrow indicates a network-like structure; (D) Cyanobacterial cell (white arrow). Black arrow indicates a network-like structure; (E) Elemental distribution map (calcium) corresponding to Fig. 4C; (F) Elemental distribution map (calcium) corresponding to Fig. 4D.
Fig. 5. Transmission electron microscopy (TEM) examination of cyanobacterial cells. Bar=1 μm. (A) Detail of a cultured unicellular cyanobacterium; (B) Cultured filamentous cyanobacterium; (C) Endolithic cyanobacteria from untreated stone; (D) Detail of Fig. 5C. Arrows indicate thylakoids; (E) Network observed lining the outside of some cyanobacterial cell walls.
Fig. 6. (A) SEM-BSE image of a transverse section of a stone fragment colonized by microorganisms after Algophase treatment; (B) Detail of Fig. 
A. Cell walls are well preserved and the cytoplasm is well distributed inside the cells; (C) Thyrea mycobionts after Algophase treatment observed by 
TEM. Small black arrows indicate concentric bodies; (D) SEM-BSE image of a transverse section of a stone fragment after Metatin treatment. Some 
cells show a high degree of plasmolysis (arrow); (E) SEM-BSE image of a transverse section of a stone fragment after Preventol treatment. Arrows 
point to fully collapsed cyanobacterial cells; (F) Cyanobacteria (after Preventol treatment) showing totally destroyed protoplasts and cell walls.
Following ALGOPHASE® treatment, the cell ultrastructure undergoes minor changes and thylakoids, carboxysomes and lipid bodies can be clearly distinguished. In Fig. 7B, only remnants of cell organization, such as traces of thylakoids and lipids, can be observed after treatment with METATIN®. PREVENTOL R80® results in the total loss of cytoplasmic structures leading to the vesiculation of the cell protoplasm.

5. Discussion

The area of the lithic substrate where lichens and other lithobiotic microorganisms accumulate along with the organic compounds they produce constitutes a complex biofilm. This is where the different bioweathering processes take place on the stone. Warsheid and Braams (2000) recently described the biofilm as “microbial cells immobilized on the stone surface (substrate) and frequently embedded in an organic polymer matrix of microbial origin. The lithobiotic community present in this material is involved in the biogeophysical and biochemical processes that occur in the monastery stone.

Aspicilia and Verrucaria lichen thalli caused the disintegration of the lithic substrate beneath them. Close to these lichens (in samples taken from several areas of the cloister), cyanobacteria were also present and produced bowl- or pear-shaped cavities that sometimes constituted real channels typical of the euendolithic niche. Danin and Caneva (1990) observed similar bioweathering patterns produced by lithobiotic coccoid cyanobacteria in limestone walls in Jerusalem. Gerdes et al. (1994) also described bowl-shaped cavities produced by coccoid cyanobacteria of the Gloeocapsa type and unicellular green algae. These authors
propose that these carbonate pits are an indication that cells have regained their turgor during the biomineralization of extracellular carbonate deposits. Cavities in stone following cell outlines were also observed in samples from the Belém Tower using SEM-BSE (Ascaso et al., 1998c). The formation of larger and deeper cavities, sometimes in the form of bottle-shaped pores on limestone surfaces, has been attributed to the biocorrosive action of the fruiting bodies of *Verrucaria hochstetteris* (Gehrmann and Krumbein, 1994). According to Sanders et al. (1994) and Ascaso et al. (1998a), dissolution of the calcareous substrate could be aided by the acidity derived from respiratory carbon dioxide and possibly other organic acids produced by hyphae and rhizomorphs.

In the present study, TEM revealed a network structure in close proximity to the cyanobacterial wall in places where bacteria coexist with cyanobacteria. Albertano and Urzi (1999) also described an intimate relationship between bacterial (rod-shaped and filamentous) and cyanobacterial cells and noted the presence of mineral precipitates on some filamentous bacteria in the Roman catacombs. This biogenic network might play a relevant role in calcium biomobilization processes, leading to dissolution of the calcareous substrate and the formation of bowl-shape pores around endolithic cyanobacteria. Danin and Caneva (1990) reported that the precipitation of carbonates around cyanobacterial cells is the result of CO$_2$ molecule consumption from dissociated Ca(HCO$_3$)$_2$ in intercellular solutions during photosynthesis. It is also possible that extracellular polysaccharides, specific enzymes and/or proteins are involved in the formation of this structure. Indeed, the production of extracellular polymeric layers is a general property of microorganisms (Wingender et al., 1999). Extracellular polysaccharides (EPS) have an antidesiccant effect on cyanobacteria but at the same time can provide nucleation sites for the formation of minerals, as in the case of zones of high calcium content (Barker and Banfield, 1996; Barker et al., 1997).

The neoformation of biogenic calcium carbonate within the lichen thallus as shown in the thalli of *Thyrea* could be the result of the biological dissolution of the Ca-rich substrate and subsequent deposition within the thallus components. An example of the coexistence of different particle forms of biogenic calcium carbonate deposits within the microbial biofilm was recently reported by Gerdes et al. (1994). The authors suggest that aragonite accumulation occurs due to biologically induced physiochemical changes in the microenvironment of the interface.

Hydration–dehydration cycles might exert a clear biogeochemical effect on the stone. The antidesiccant role of EPS favors water absorption and thus the hydration–dehydration cycling of these organisms as well as that of any clay minerals present.

After the application of the biocides tested here, the lichen *Thyrea* remained on the treated stone. The most clearly observed ultrastructural change in the mycobiont was related to the concentric bodies. Concentric bodies were observed in abundance after ALGOPHASE treatment but not following the other two treatments. For cyanobacteria embedded in this lithic substrate, ALGOPHASE was the least efficient and PREVENTOL R80 the most efficient at the ultrastructural level. Following ALGOPHASE treatment it was possible to recognize thylakoids and lipid bodies, while after the application of METATIN only their traces remained. In the case of PREVENTOL R80, the cells were full of vesicles and structures observed earlier disappeared altogether. Since PREVENTOL has hydrophilic and hydrophobic chemical groups it is able to disrupt the cell membrane structure of the microorganism causing leakage of intracellular materials. A lower efficiency of ALGOPHASE with respect to METATIN has also been observed in studies based on the measurement of thallus photobiont fluorescence (Nimis and Salvadori, 1997). In previous experiments with PREVENTOL R80, moderate to high toxicity towards lichens and high toxicity towards mosses and algae was demonstrated (Frey et al., 1993). According to Bernardini (1993), this product is often used for its effects on algae, fungi, lichens and bacteria, possibly including cyanobacteria. Here PREVENTOL R80 was able to attack the lichen thallus, but greatest efficiency was shown with regard to its action on cyanobacteria. Some authors (Koestler and Salvadori, 1996) suggest that certain biocides can provoke the secretion of biopolymers. We were unable to observe this effect for the biocides tested using SEM-BSE and TEM but further investigation in the field is required.

This study shows how it is possible to analyze the organisms involved in substrate biodeterioration and evaluate the efficiency of a biocide, without extracting the microorganisms from their rock microhabitat. Using the methods described, microorganisms may be observed on the stone surface of the monument itself, and interactions among the biocide, microorganisms and stone explored. In this way, the effects of different biocidal agents may be directly compared under the same conditions of light, temperature and humidity.

**Acknowledgements**

The authors would like to thank Dr. Martin Grube of the Institut für Botanik, Karl-Franzens-Universität Graz, Austria, for identification of the lichens and Dra. Pilar Mateo of the Universidad Autonoma de Madrid, Spain, for identification of cyanobacteria. They are also indebted to Fernando Pinto for his help in developing the micrographs and to Ana Burton for reviewing the English. This study was financed by grants PB98-0679 and BOS2000-1121 from the DGICYT (Spain) and the WMF (Portugal).

**References**


