

Characterizing the Microbial Colonization of a Dolostone Quarry: Implications for Stone Biodeterioration and Response to Biocide Treatments

Beatriz Cámara · Asuncion De los Ríos · Marta Urizal ·
Mónica Álvarez de Buergo · Maria Jose Varas ·
Rafael Fort · Carmen Ascaso

Received: 7 September 2010 / Accepted: 1 February 2011 / Published online: 26 February 2011
© Springer Science+Business Media, LLC 2011

Abstract This study examines the microbial colonization of three fronts of an abandoned dolostone quarry (Redueña, Madrid, Spain) exposed to atmospheric conditions for different time periods since Roman times to the present. Through scanning electron microscopy in backscattered electron mode (SEM-BSE), endolithic colonization was predominantly detected in the most recently exposed front, while in the longer exposed quarry fronts, epilithic forms of growth were most often observed. These observations were confirmed by denaturing gradient gel electrophoresis (DGGE) analysis. Based on the distribution pattern of microbial colonization in the different quarry fronts, we then established a sequence of colonization events that took place over this long time frame. Bioalteration processes related to this sequential colonization were also identified.

Characterizing these sequential processes can be useful for interpreting biodeterioration processes in historic dolostone monuments, especially those affecting constructions in the area of the Redueña stone quarry. In a second experimental stage, different biocide treatments were tested on this quarry rock to find the best way to avoid the microbial colonization effects identified. Through combined SEM-BSE/DGGE analysis, the efficacy of several biocides against the microorganisms inhabiting the dolostones was assessed after 4 and 16 months treatment. In general, all treatments were effective at reducing around 80% of the lichen cover, although effects on endolithic lithobiontic communities were dependant on how well the rock surface had been mechanically cleaned prior to treatment and gradually disappeared over time.

B. Cámara (✉) · A. De los Ríos · C. Ascaso
Museo Nacional de Ciencias Naturales, CSIC,
C/Serrano 115 Dpdo,
28006 Madrid, Spain
e-mail: bcamara@cma.csic.es

M. Urizal
Thor Especialidades S.A.,
Barcelona, Spain

M. Álvarez de Buergo · M. J. Varas · R. Fort
Instituto de Geología Económica (CSIC-UCM), Facultad de
Ciencias Geológicas, Universidad Complutense de Madrid,
C/José Antonio Novais 2,
28040 Madrid, Spain

M. J. Varas
Dpto. de Petrología y Geoquímica, Facultad de Ciencias
Geológicas, Universidad Complutense de Madrid,
C/José Antonio Novais 2,
28040 Madrid, Spain

Introduction

The term biodeterioration refers to any decay process that affects monuments as the result of biological colonization [4, 38, 46]. However, microorganisms are not the only agents that cause the decay of monuments. Many environmental factors may synergistically or antagonistically contribute to the deteriorating actions of microorganisms [38]. For effective preventive measures to be designed, biodeterioration processes need to be accurately identified [5, 8, 10, 12]. However, owing to their artistic and historic value, monument stones cannot be extensively sampled for this diagnosis. Thus, the mechanisms of bioalteration of different types of stone in natural environments need to be determined and used to diagnosis biodeterioration and adopt appropriate control strategies [11, 31, 38]. Firstly considered, should be treatments aimed at destroying

favorable conditions for the growth of the most damaging microorganisms [10, 43]. However when this is insufficient, specific treatments have to be used to prevent as well as eliminate biological colonization. According to Ascaso et al. [5], these treatments should be ideally assessed in situ, without extracting the microorganisms from their microhabitat. The viability of cells after treatment can be examined using different methodologies. Microscopy techniques provide valuable information on the integrity of cell membranes and ultrastructural changes, but complementary techniques such as chlorophyll-a fluorescence detection can reveal changes in the physiological state of free-living or lichenized phototrophic microorganisms [44, 45].

Stone quarries are an ideal natural environment to carry out this type of study since there is a non-limited supply of sample material and the information obtained is directly transferable to any nearby monuments built out of the same rock [10, 15, 19]. A further benefit of this type of sampling site is that the mineral surfaces of exploited natural outcrops are of known age, such that it is easy to calculate time frames for the development of biofilms and their weathering rates [6, 10].

Several studies have been performed in old quarries of different rock types (see references in [15]). For example, some of the most recently reported applications of such studies have been the induction of the growth of biofilms in a quartz quarry to correct the visual impacts of past mining activities [36] and bacterially induced mineralization of quarry stone to protect and consolidate new stone pieces to replace the damaged stone of a historic building [29].

The Madrid region has several ancient stone quarries and much of the region's architectural and monumental heritage was built from stone materials extracted from these sites [24]. The Redueña site is a Cretaceous dolostone quarry in the north of Madrid, whose characteristics of workability, availability, and proximity to urban areas made its stone one of the most used materials for the construction of historic monuments in the region from Roman times until the mid-twentieth century [32]. Although many of these dolostone monuments still stand today, signs of deterioration are visually apparent.

The aim of this study was to characterize the types of lichen and microbial colonization of different fronts of the Redueña quarry exploited at different times in history using an integrated microscopy and molecular biology approach. The information obtained will be used to identify biodeterioration processes related to these types of colonization in historic monuments built out of this stone. In addition, we evaluated the in situ effects of different biocide treatments on the lithobiotic communities inhabiting the microhabitats of the Redueña quarry to design an experimental assay for future use on monument stones.

Methods

Sampling

Pieces of dolostone were taken from the Redueña quarry. This natural outcrop forms part of a discontinuous narrow band of Upper Cretaceous dolostone running NE-SE in the Spanish Central System (917 m above sea level; N 40° 47.66'; O 3° 33.25'). From Roman times to the mid-twentieth century, dolostone from the Redueña quarry has been intermittently used to construct monuments of different architectural styles in the region. Today, the quarry has three different fronts that were exploited in different time periods. The increasing dimensions of these fronts and ashlar over the centuries reflect improvements in the tools used to extract the stone facings typical of the time and new forms of transport [32].

- Quarry front I (QF-I). This is the largest front, which was mainly exploited to reconstruct most of the existing dolostone historic monuments in the 1940s. Materials were extracted from three sites ordered in time. The base of this front was the most recently abandoned.
- Quarry front II (QF-II). The rocks of this front were intermittently extracted in the fourteenth to sixteenth centuries.
- Quarry front III (QF-III). This is the oldest and smallest front (Fig. 1a), which could correspond to the start of dolostone extraction activities in the area. The earliest mining activities have been dated as Roman based on evidence of the rudimentary methods used to extract the stone [32].

To examine microbial colonization, representative samples were collected from each of the fronts (I, II, III) of the Redueña quarry in October 2006. In all cases, rock specimens were taken from a vertical south-facing wall, since throughout history it was mainly this southern aspect of the three quarry fronts that was exploited. The same samples were used for scanning electron microscopy-backscattered electron mode (SEM-BSE), isolating fungi and denaturing gradient gel electrophoresis (DGGE) analysis.

In addition, samples were collected from historic monuments constructed using Redueña dolostones as dimension stone to compare with the quarry stone including the Roman Bridge (Talamanca del Jarama, second century), and the Inmaculada Concepción church (Soto del Real, sixteenth to eighteenth centuries).

Biocide Experiments

The effectiveness of different biocide treatments was tested in the oldest quarry front (Q-III) since this one was the most

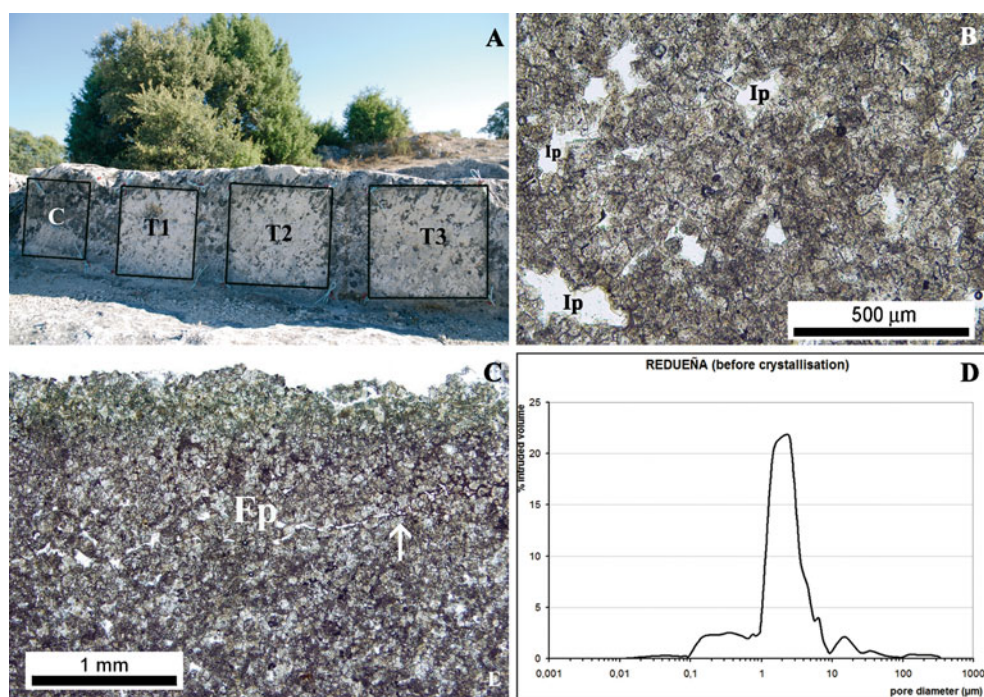


Figure 1 **a** General view of the quarry front in which the last mining activity dates back to Roman times (QF-III) showing the different experimental plots used to assess the efficacy of the biocide treatments (*C* control; *T1* disinfectant+fungicide a; *T2* T1+(fungicide a+algicide); *T3* T1+(fungicide a+fungicide b)). **b** POM micrograph under parallel polars of the Redueña dolostone revealing it is mainly composed of rhomboedric dolomite crystals and shows high inter-

crystalline porosity (*Ip*). Intercrystalline pores are sometimes filled with calcite cement. **c** POM micrograph under parallel polars of fissure porosity (*Fp*) associated with the exposed surface. Note that this type porosity only affects the first 1.5 mm of the rock. **d** Curve of the pore size distribution of the Redueña dolostone estimated by mercury intrusion porosimetry: 17.9% of the total porosity is in the range 400–0.01 µm

extensively colonized. The experiment was conducted in four 25×25 cm grid plots on a vertical surface (*C*, control; *T1*, treatment 1; *T2*, treatment 2; and *T3*, treatment 3; Fig. 1a). The biocide treatment was administered according to the manufacturer's instructions. This consisted of mechanically removing the biological cover using a sponge followed by application of the chemical compounds. Three commercial biocides provided by THOR were applied in different combinations and concentrations using a paintbrush: ACTICIDE® LV706 (10% in distilled water), ACTICIDE® CF (1.5% in water-repellent resin), and ACTICIDE® IOG (0.5% in water-repellent resin). The experiment was performed on three consecutive days in July 2007. The treatment regimens and compositions are shown in Table 1. Samples were collected 4 and 16 months after applying the biocides and their efficacy assessed in terms of the reduction in lichen cover (%), changes in chromatic variables (L^* , a^* , b^* , C^* , IB, IA) and analysis of the lithobiontic communities by SEM-BSE and DGGE.

Mercury Intrusion Porosimetry

The porosity and pore size distribution of the Redueña dolostone were determined using a Micromeritics Autopore IV 9520 mercury intrusion porosimeter. The limit between

micro- and macroporosity was set at a pore diameter of 5 µm.

Polarized Optical Microscopy

Thin sections of samples from the three quarry fronts were petrographically characterized using an Olympus BX 51 microscope connected to an Olympus DP 12 (6 V/2.5 Å) digital camera. The software used was Olympus DP-Soft (version 3.2).

Epilithic Colonization Cover

Epilithic colonization was estimated in each experimental plot by measuring percentage cover in 12 randomly selected zones of smaller size (4×4 cm) through digital image analysis using the UTHSCSA Image Tool 2.0 (University of Texas Health Science Center in San Antonio, USA). Cover values for each treatment at the two time points (4 and 16 months after application) were compared by two-way analysis of variance, including biocide treatment and time as categorical predictors. Tukey's HSD post hoc test was used to identify differences among treatments. All statistical tests were performed using STATISTICA 6.0 (Tulsa, OK, USA).

Table 1 Biocides and treatment regimens (T1, T2, and T3) applied to 25×25 cm vertical plots on the quarry rock surface

Biocide	Active ingredients	Concentration and solvent used	Treatment regimen	Day 1	Day 2	Day 3
			Control	–	–	–
A: ACTICIDE® LV706	2-n-octyl-3(2 H)-isothiazolone (f)+ benzalkonium chloride (d)	10% in distilled H2O	T 1	Mechanical cleaning A	A	–
B: ACTICIDE® CF	2-n-octyl-3(2 H)-isothiazolone (f)+ terbutryn (a)	1.5% in water-repellent resin	T2	Mechanical cleaning A	A	B
C: ACTICIDE® IOG	2-n-octyl-3(2 H)-isothiazolone (f)+3-iodopropynyl butylcarbamate (f)	0.5% in water-repellent resin	T3	Mechanical cleaning A	A	C

Chromatic Characterization of the Experimental Plots

Colonization cover was also determined colorimetrically at the 16-month time point. The color of each experimental plot was measured by reflectance using a MINOLTA CM 700d spectrophotometer and analyzed by COLOR DATA SPEC-TRAMAGIC™ NX CM-S100W (Minolta) software. Results are expressed as the mean of 20 direct readings following two diagonals in each of the experimental plots. The chromatic variables determined defining the color of a material were: L* (lightness), a* and b* (chromaticity coordinates indicating red (+a*), green (−a*), yellow (+b*) and blue (−b*) directions), C* (chroma or saturation defined by the equation $C^* = (a^*)^2 + (b^*)^2 / 1/2$), IB (whiteness index) and IA (yellowish index) [16]. The L*/C* diagram represented here was the most appropriate to determine differences in color between the untreated and treated plots.

Scanning Electron Microscopy with Back-Scattered Electron Imaging

Fragments of dolostone from the different quarry fronts, historic monuments, and biocide–assay plots were prepared for SEM-BSE according to a technique [48] described elsewhere with some modifications. Briefly, hand-cut pieces of rock were fixed in glutaraldehyde (3.25% in 0.05 M cacodilate buffer) and then in osmium tetroxide solution (1% in 0.025 M cacodilate buffer), dehydrated in a graded series of ethanol solutions, and embedded in LR-White resin. Samples finely polished and carbon-coated were observed in a Zeiss DMS 960 SEM equipped with a four-diode, semiconductor BSE detector, and an ISIS Link EDS microanalytical system.

Isolation of Fungal Strains

Free-living fungi were isolated from deep areas of recently fractured rock samples. Small fragments of each sample

were incubated on 1.5% malt extract agar (MEA) at room temperature for 3–4 weeks. Isolates were purified in two or three steps by transfer to fresh medium.

DNA Extraction from Rock Samples and Fungal Cultures

To characterize microbial colonization, DNA was extracted from epilithic and endolithic areas (~0.5–1 cm below the rock surface) of the rock samples. For DNA extraction, the Power Soil DNA Isolation kit (MoBio Laboratories Inc, Solana Beach, CA, USA) was used according to the manufacturer's instructions. The extraction process was started by resuspending 250 mg of sample material in lysis solution.

To identify the fungal strains isolated, total DNA was extracted from different mycelia according to a modified CTAB method [9].

PCR Amplification and Sequencing

The ITS regions of the rRNA gene were used for polymerase chain reaction (PCR) amplification. For the DGGE analysis of environmental samples, the primer pair used was ITS4GC/ITS3 [47] and to obtain the sequences of the isolated fungal strains the ITS1F/ITS4 pair [22, 47].

PCRs were carried out in a 25 µl final volume using Illustra™ puReTaq Ready To-Go PCR Beads (GE Healthcare) containing 0.1 U/µ of puReTaq DNA polymerase in Tris–HCl (pH 9.0), 50 mM KCl, and 1.5 mM MgCl₂, 200 µM of each dNTP, 0.5 µM of each primer and ca 10–50 ng of genomic DNA. The thermocycling program was: 5 min denaturation at 94°C, followed by 30 cycles at 95°C for 30 s, 52°C for 1 min, and 72°C for 1 min 30 s. The final extension step was 10 min at 72°C or 30 min at 72°C for the DGGE samples.

The PCR products from the fungal cultures and DGGE bands were cleaned on a QIAGEN quick spin column (QIAGEN, Valencia, CA, USA). Both complementary

strands were sequenced separately at the SECUGEN sequencing company (S.L. Madrid, Spain).

Denaturing Gradient Gel Electrophoresis

DGGE was performed using a D-Code Universal Mutation Detection System (Bio-rad). Samples (22 µl) were loaded on 6% acrylamide/bisacrylamide (37.5:1) gels with a denaturing gradient of 30–60% (where 100% denaturant is 7 M urea and 40% formamide). The gels were run at 70 V for 16 h at 60°C. After staining with ethidium bromide 10 mg/ml for 10 min, the gels were rinsed in distilled water prior to viewing and capture in a UV transilluminator.

Well-defined DGGE bands were excised using a scalpel blade and incubated at 4°C overnight in sterile distilled water and then reamplified using the same primers as for PCR but without the GC clamp. The resultant PCR products were purified using a QIAGEN quick spin column (Qiagen) and sequenced at the SECUGEN company (S.L. Madrid, Spain). Sequences have been deposited in GenBank under accession numbers JF330171–173.

Results

Mineralogical Study

The samples from the three quarry fronts showed the same petrographic features confirming they all belonged to the same Cretaceous dolostone outcrop. The Redueña stone is a dolostone with a massive internal structure comprised mainly of rhombic, idiomorphic dolomite crystals (MgCa_2CO_3) [18]. These clean and unaltered crystals form dense crystalline masses of fairly uniform size (70–80 µm). The presence of high porosity (≈20%) should be highlighted. Main porosity is intercrystalline of regular morphology and small size (100–150 µm; Fig. 1b), although a larger and more irregular intercrystalline porosity (0.5–1 mm) may occasionally appear infilled with crystals of calcite cement. A further type of porosity appears close to the surface and is attributable to surface weathering. These cracks (fissure porosity; Fig. 1c) run parallel to the surface and are only a few millimeters deep. The pore size distribution plot for the Redueña dolostones determined by mercury intrusion porosimetry indicates that 17.9% of the total porosimetry is in the range of 400–0.01 µm of pore size (Fig. 1d).

Microbial Colonization

Microbial colonization in the three quarry fronts was macroscopically and microscopically assessed to compare epilithic and endolithic communities in terms of their cover, spatial occurrence, and weathering actions.

Microbial Colonization of the Quarry Front Exploited in the 1940s (QF-I)

This quarry front was the most recently exposed of the three examined. Because of its large size, we sampled three sites following an exposure time gradient. At the macroscopic scale, epilithic lichen thalli were practically absent on the rock surface at the base of the front (Fig. 2a) but increased in frequency towards the top (Fig. 2b). These observations were confirmed by SEM-BSE. Thus, at the base of this front, extensive endolithic fungal colonization was predominantly observed (Fig. 2c). The scarce epilithic growth was associated with fragmentation and detachment of dolomite crystals (arrowheads in Fig. 2c). In contrast, at the top of this front or site exploited longest ago, the rock surface was colonized by small lichen thalli, some of which showed mineral particles in their interior (arrowheads in Fig. 2d). At this site, it was also possible to detect fungal hyphae inside the rock (empty arrow in Fig. 2d), approximately 200 µm below the surface. Occasionally, besides colonizing the intercrystalline porosity of the dolomite crystals, these fungi also penetrated the crystals.

Microbial Colonization of the Quarry Front Exploited Between the Fourteenth to Sixteenth Centuries (QF-II)

In this area, epilithic colonization was more extensive than in QF-I (Fig. 3a), with an increase in lichen thallus size observed (Fig. 3b). Microdivided mineral crystals of different size were trapped among the mycobiont hyphae, which showed a capacity for intense mechanical weathering. In the attachment area, numerous fungal hyphae emitting an intense BSE signal were closely associated with the lithic substrate (black arrow in Fig. 3b). These hyphae generated an extensive network of colonized fissures and cracks inside the rock (arrowheads in Fig. 3b). Clusters of algal cells and fungal hyphae were also frequently observed in deep areas of the dolostone, also forming an extensive network inside the rock through the spaces between the dolostone crystals (arrows in Fig. 3c).

Microbial Colonization of the Quarry Front Exploited in Roman Times (QF-III)

At this sampling site, abundant epilithic lichen colonization was both macroscopically and microscopically observed (Fig. 4a–b). *Verrucaria nigrescens* Pers. was found to colonize extensive areas of the rock. The weathering capacity of this lichen was reflected by the presence of several mineral fragments completely embedded in its medulla (asterisk in Fig. 4b), possibly as the result of its mechanical actions. Hyphae were also seen to colonize

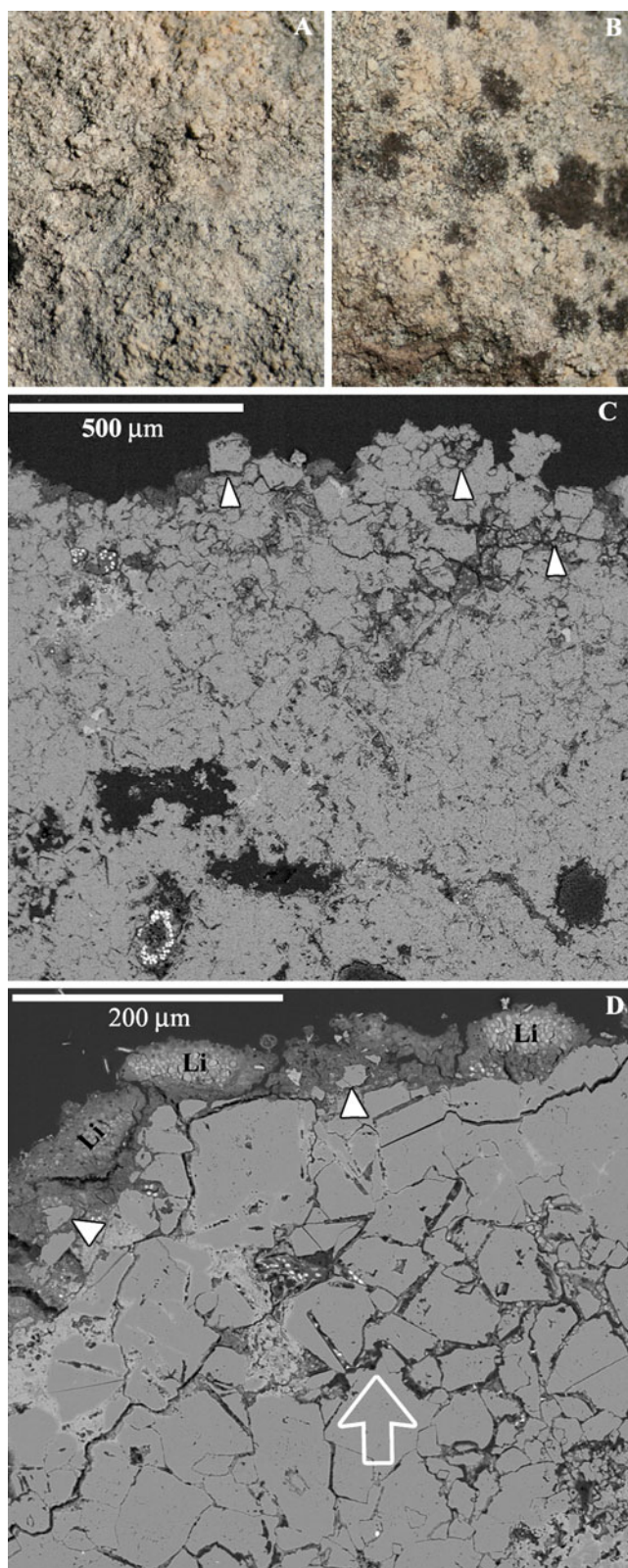


Figure 2 Time-dependent succession of endolithic and epilithic fungal growth in QF-I. **a–b** Images showing a macroscopic view of the different microbial colonization stages detected in QF-I. **c** First stage of colonization detected in QF-I. SEM-BSE image of endolithic fungal colonization randomly distributed throughout the rock. Note the surface growth of these endolithic forms promotes disgregation of the dolomite crystals (*arrowheads*). **d** SEM-BSE image of small lichens (*Li*) colonizing the lithic surface in an advanced stage of colonization corresponding to the images in Fig. 2b. Note the mineral fragments embedded in some of the lichen thalli (*arrowheads*). Endolithic fungal colonization (*empty arrow*) appears beneath the lichen thalli, occupying pores between the dolomite crystals, but also penetrating the crystals

deep areas of the dolostone (arrows in Fig. 4b), mainly through its intercrystalline porosity (arrows Fig. 4c) and intracrystalline pores (arrowheads).

Fungal Isolation

Ten fungal strains were isolated in culture medium (MEA 1.5%) and identified using the ITS rRNA region as a genetic marker (Table 2). Most of the isolates were identified as ascomycetes and basidiomycetes. All isolates came from quarry fronts QF-I and QF-II. No fungal strains could be isolated from the oldest quarry front (QF-III).

DGGE Profiles

The structure of the epilithic and endolithic free-living and lichenized fungal communities was analyzed by DGGE (Fig. 5). The DGGE profiles of rRNA isolated from epilithic zones tended to show a number of bands that increased with time of exposure to the atmospheric conditions. Some bands of the same electrophoretic mobility were shared by epilithic growths from the three different fronts (1 in Fig. 5) while others only appeared in one of the fronts. In addition, some of the bands were detected in both epilithic and endolithic locations (2 in Fig. 5). The number of bands in all the endolithic profiles was low and decreased with quarry front exposure time. Notably, some of the endolithic forms appeared only in the fronts recently exploited. In Ic, II, and III, endolithic profiles were restricted to a single phylotype, not detected at epilithic locations (3 in Fig. 5).

Analysis of Monument Rock

Patterns of microbial colonization and weathering detected in monument stones (Fig. 6), were similar to those observed in the rock quarry. In areas mainly colonized by lichen thalli, intense alteration of the rock surface was observed and this was mainly due to the mechanical actions exerted (Fig. 6a). This mechanical action was especially aggressive in the attachment area, where numerous fragments of

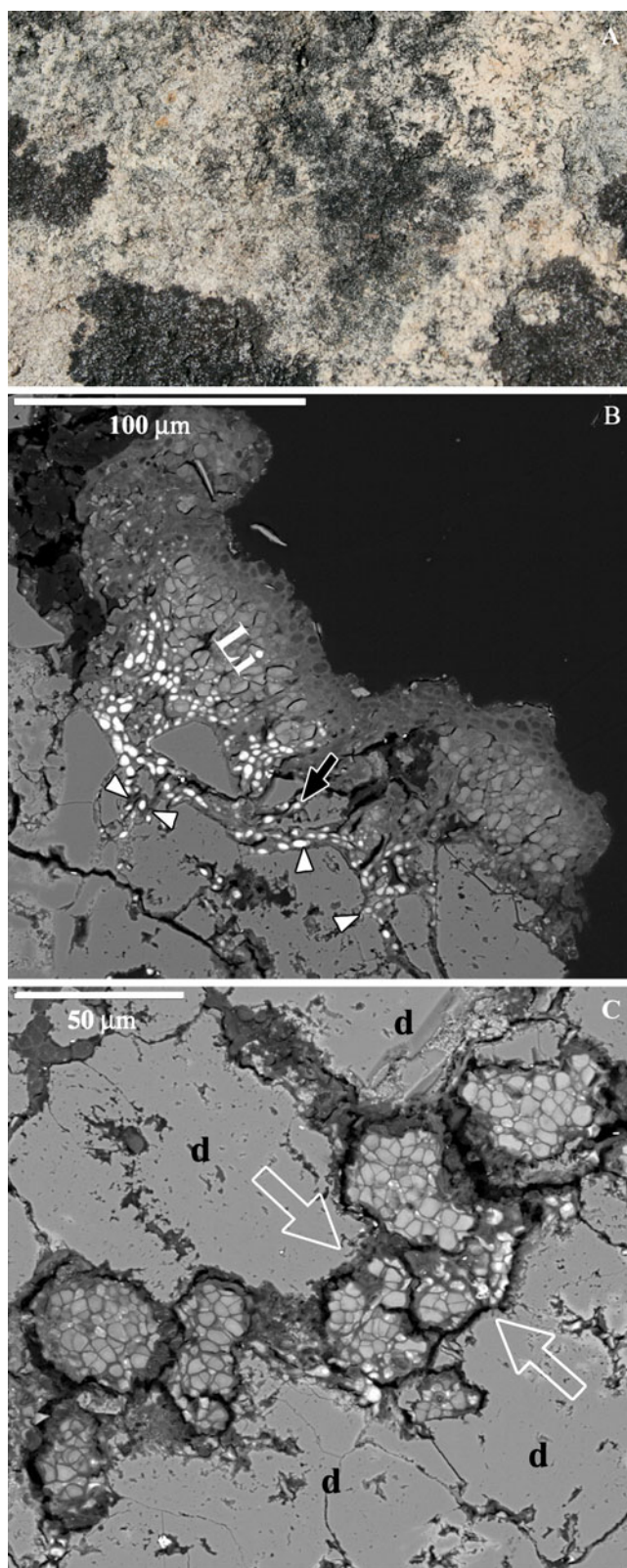


Figure 3 a–c Microbial colonization in QF-II. **a** Image showing a macroscopic view of the epilithic cover. **b** SEM-BSE image of intermediate-size epilithic lichen (*Li*). The mycobiont's hyphae (arrowheads) are strongly attached to the lithic substrate provoking fragmentation of the dolomite crystals (arrows). **c** Endolithic colonization occurring as algal and fungal associations in the intercrystalline porosity of the dolostone (arrows)

dolomite crystals appeared between the mycobiont hyphae (Fig. 6b). As observed in the natural dolostone, these bioalteration processes were not only associated with epilithic lichens but also with deep extensive colonization by endolithic mycobiont hyphae (empty arrow in Fig. 6c). In addition to lichen colonization, it was possible to observe the microbial colonization of both epilithic and endolithic locations. Thus, the hyphae of free-living fungi were frequently detected in areas below lichen thalli (arrows in Fig. 6d), but were also commonly observed in areas where epilithic lichens were absent (Fig. 6e), resembling the early stages of microbial colonization of the Redueña dolostones described above. Bacteria were also observed in the proximity of mycobiont hyphae penetrating the substrate (black arrows in Fig. 6c). Finally, algae sometimes appeared both on the rock surface and deep within some of these monument dolostones (Fig. 6f).

In situ Biocide Efficacy Against the Lithobiontic Community

The efficacy of the different biocide treatments (T1, T2, and T3) applied to the oldest quarry front (QF-III) was assessed in the short- (4 months) and long-term (16 months).

After 4 months of biocide treatment, a highly significant reduction ($F=373$; $p<0.0001$) in lichen cover approximating 80% of the total cover was recorded in the three experimental plots compared to the control (Fig. 7a–i). However, no significant ($F=1.60$; $p=0.194$) differences between biocide treatments were detected. After 16 months of treatment, these effects persisted though a marginally significant ($F=8.20$; $p=0.06$) increase in epilithic cover was detected for all three biocide treatments.

The chromatic parameters (L^* , lightness and C^* , chroma) recorded at 16 months confirmed these observations. Hence, L^*/C^* values indicated lightness ($L^*>65$) increases induced by all three biocide treatments compared to the control plot which showed values close to those for a gray color ($L^*<10$ and $C^*\sim 55$) (Fig. 7a–II). Both techniques provided similar information and we plan to conduct further color measurements on experimental plots in the future because of benefits such as speed, repetitive sampling, and easy monitoring of the effectiveness of biocide treatments [36].

Biocide effects on the lithobiontic community were determined at the cellular level through SEM-BSE. This

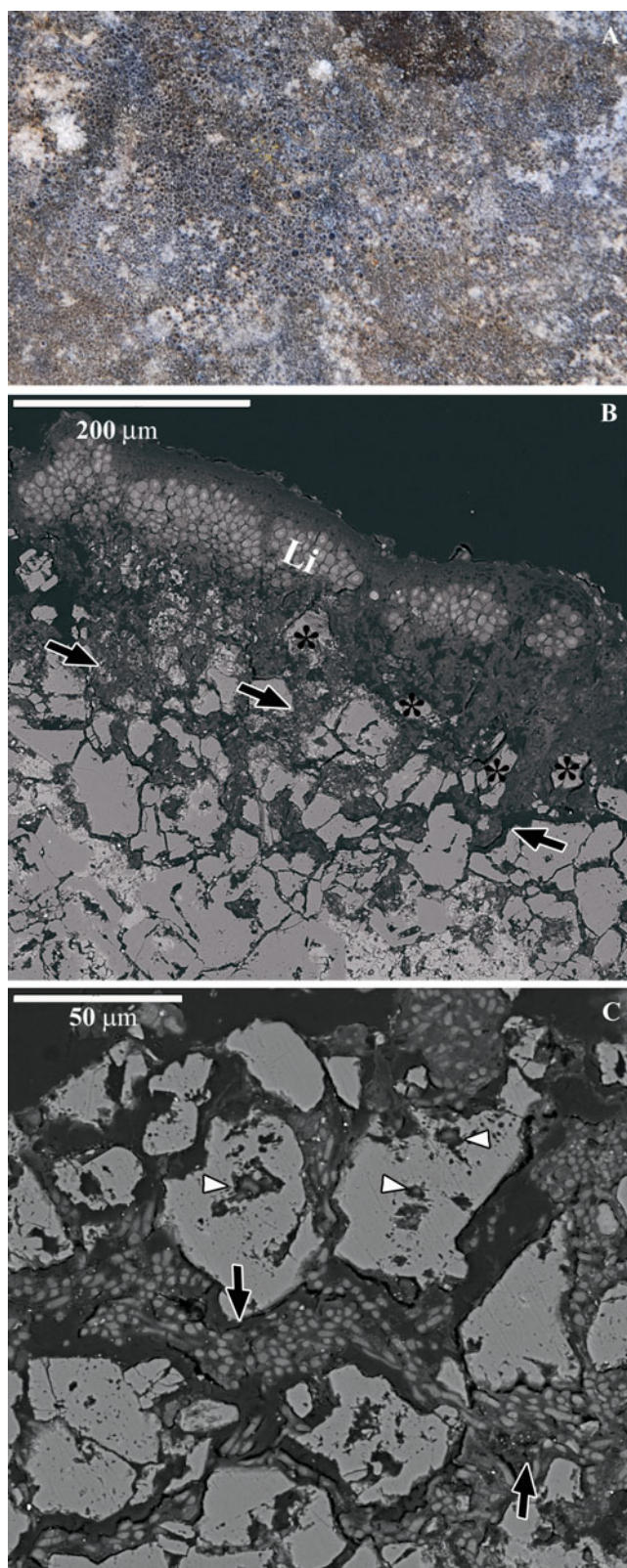


Figure 4 a–c Microbial colonization in QF-III. **a** Macroscopic view of the epilithic colonization in QFIII. **b** SEM-BSE of large epilithic thalli (*Li*). Mineral fragments (*asterisk*) of variable size appear embedded in the medulla of a lichen between mycobiont hyphae, indicating the lichen’s weathering capacity. **c** Mycobiont hyphae detected in deep areas of rock substrate colonizing intercrystalline (*arrows*) and intracrystalline (*arrowheads*) pores of dolostone

quarry front showed abundant epilithic lichen colonization (Fig. 4), which was not observed after the treatments (Fig. 7b–f). However, treatment effects on all microbial colonizers were not always the same. In areas where there were no remains of lichen thalli on the rock surface after biocide treatment endolithic fungal hyphae and algal cells lost their typical morphology, especially in places close to the rock surface (Fig. 7b and inset). This effect disappeared at greater depths where we observed unharmed endolithic microbial cells. When there were large amounts of lichen thalli remains, it was possible to observe an interface of living and non-living cells (see above and below—white arrow and black arrow—the line in Fig. 7c). After 4 months of treatment, no differences among samples subjected to the different biocides were observed.

In the longer term (16 months), most of the biocide effects observed at 4 months were also detected (Fig. 7d, e, f). However, of note did the detection in some endolithic areas of extensive fungal colonization inhabit spaces between the dolomite crystals (Fig. 7d and inset), absent in the untreated samples. It should also be noted that in this area, most of the calcite cement in intercrystalline spaces had disappeared. In other areas, abundant fungal hyphae were detected not only at endolithic locations (Fig. 7e), but also at specific points on the rock surface (Fig. 7f). Our DGGE analysis of the fungal endolithic community after 16 months of treatment revealed some differences in profiles between the three biocide treatments and control (Fig. 8). First, the control profile contained one band not present in the other profiles (arrowhead), indicating an endolithic form sensitive to the biocide treatments. Second, there were bands common to all profiles (empty arrow), meaning the resistance of some phylotypes to treatment. And finally, the recolonization of endolithic positions by new fungal forms was revealed by the appearance of new bands after the T2 and T3 treatments (black arrows). This observation was complemented by sequencing some of these DGGE bands, which indicated the presence of some lichenized fungal forms after 16 months of biocide treatment.

Discussion

Through in situ electron microscopy examination of the microbial colonization of an abandoned dolostone quarry

Table 2 Fungal strains identified in dolostones taken from the different fronts of the Redueña quarry by culturing and sequencing the ITS region of the rRNA gene

Code	Best BLAST match [GenBank accession number]	Similarity (%)	Length (bp)	Quarry front
Ascomycota				
Re-H1 [JF330174]	<i>Foliar endophyte</i> [AY566890]	92	447	QF-Ia
Re-H2 [JF330175]	<i>Aspergillus fumigatus</i> [HQ026746]	96.7	626	QF-Ib
Re-H3 [JF330176]	<i>Chaetomium funicola</i> [EU552109]	99.8	611	QF-Ib
Re-H4 [JF330177]	<i>Phialophora sessilis</i> [AB190381]	88	652	QF-Ib
Re-H5 ^a [JF330178]	<i>Coniosporium</i> sp. [AM279682]	99.8	641	QF-Ic
Re-H6 [JF330179]	<i>Cladosporium</i> sp. [HQ631003]	96.7	602	QF-II
Re-H7 ^a [JF330180]	<i>Phaeococcomyces</i> sp. [AJ972801]	98.6	665	QF-II
Re-H8 [JF330181]	<i>Phoma herbarum</i> [FN868459]	98.7	526	QF-II
Basidiomycota				
Re-H9 [JF330182]	<i>Bjerkandera adusta</i> [EF441742]	99.8	612	QF-II
Re-H10 [JF330183]	<i>Oxyporus latemarginatus</i> [FN252852]	99.5	631	QF-II

^a Slow-growing fungi

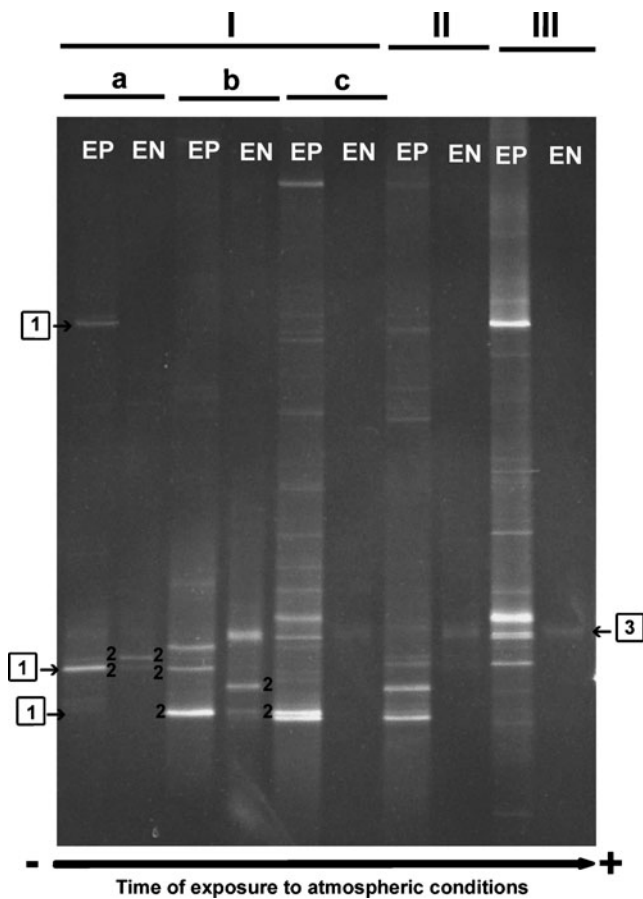


Figure 5 DGGE profile of the fungal diversity of epilithic (EP) and endolithic (EN) locations in the different quarry fronts (I, II, III). DGGE banding patterns correspond to the ITS regions of the rRNA gene (~400 pb): 1 bands common to epilithic positions in the different quarry fronts; 2 bands appearing both in epilithic positions and endolithic positions; 3 bands common to all the endolithic locations in the different quarry fronts not present in the epilithic profiles

(Redueña), we were able to assess the extent of colonization of the different quarry fronts (QF I, II, III) corresponding to different time periods. Free-living and lichenized fungi were the main inhabitants of these dolostones, and these occupied different positions and showed different types of colonization. Each type seemed to correspond to different stages of colonization of the dolostones. Thus, it was possible to establish a sequence of fungal colonization. Colonization begins at endolithic sites and epilithic locations are colonized at a later stage. Thus, endolithic fungi were pioneers and epilithic lichens appeared as the predominant forms later on. It is also worth mentioning that endolithic communities showed differences in composition when the rock surface was colonized by lichen thalli, a finding supported by DGGE and culture techniques. Accordingly, when lichens were present on the rock surface, a lower diversity of free-living fungi was detected, which could indicate some degree of competition.

The fungal pioneer endolithic colonization observed here could have arisen from spores or vegetative propagules transported by wind or water, but we cannot rule out the possible spread of endolithic growth from nearby areas [7]. The development of lithobiontic communities is influenced by abiotic factors, such as the physico-chemical properties of the lithic substrate, by interrelations between different community members and the substrate itself [13], but also by the specific mode of reproduction and growth rate of each type of microorganism. In effect, ascospores of lichenized fungi in the natural environment need to invest more time to find a compatible photosynthetic partner and undertake morphogenesis processes relative to the time taken for the reproduction

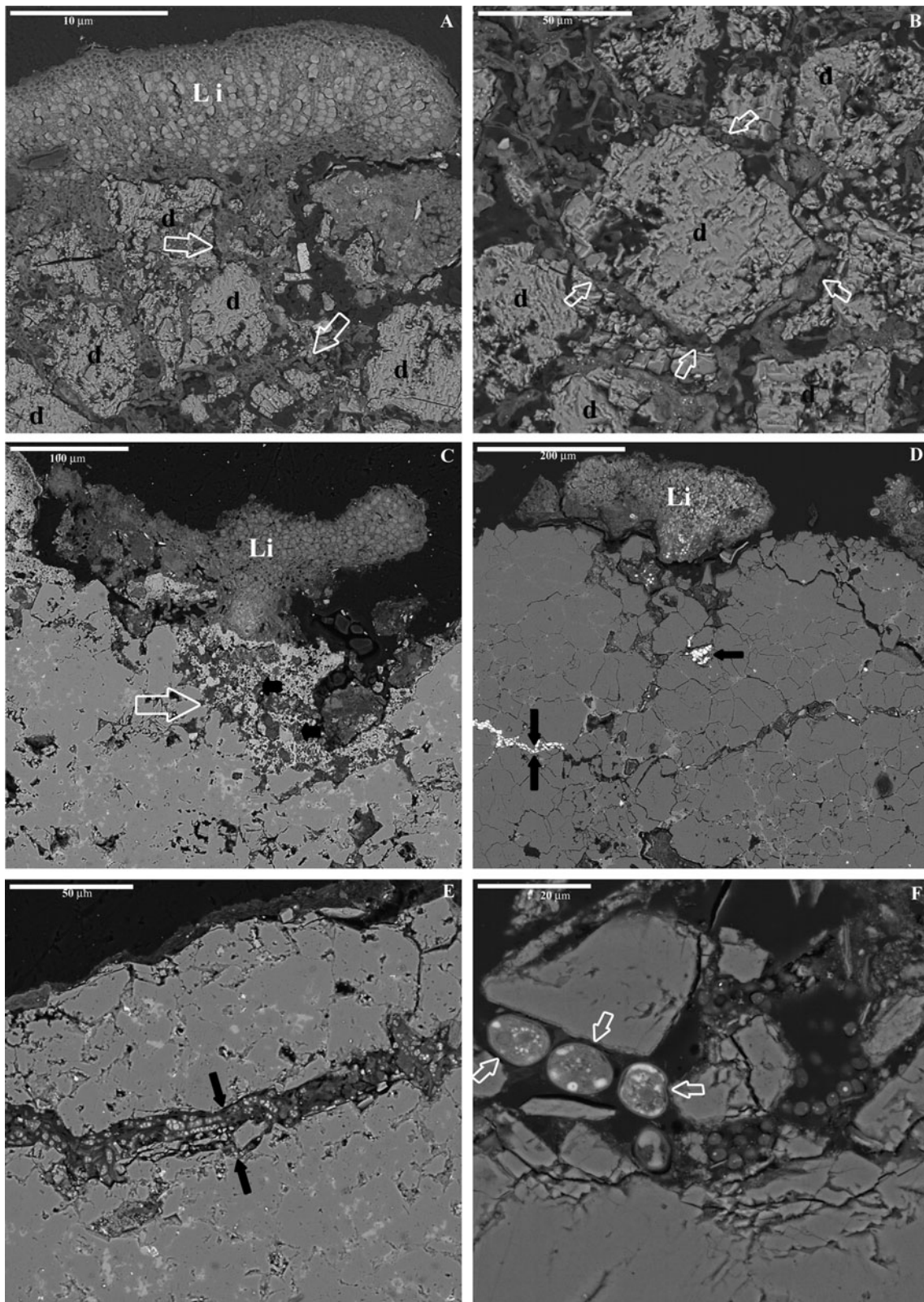


Figure 6 SEM-BSE images of lichen and microbial colonized areas in historic monuments built using Redueña dolostones. **a** Epilithic lichen thallus (*Li*) provoking intense alteration of the dolostone (arrows). **b** Fungal hyphae (arrows) surrounding dolomite crystals (d) in endolithic positions beneath a lichen thallus. **c** Epilithic lichen thallus (*Li*) with extensive endolithic colonization (empty arrow) where mycobiont hyphae coexist with other microorganisms (black arrows). **d** Lichen thallus (*Li*) colonizing the rock surface of dolostones from the church La Inmaculada Concepción (Soto del Real, Madrid). Note fungal hyphae emitting an intense BSE signal occurring several micrometers beneath the lichen thallus (arrows). **e** Endolithic colonization by free-living fungi inhabiting preexisting fissures in the dolostone (arrows) in the absence of epilithic lichens. **f** Epilithic colonization by algae (arrows) in dolostones from the Roman bridge at Talamanca del Jarama (Madrid)

and growth of non-lichenized fungi [28, 34]. The prior presence and activity of endolithic microorganisms could help the lichen symbiosis to settle in later stages of colonization [13, 27].

Changes in the endolithic community, such as the loss of some endolithic fungal forms in more developed communities, could also be related to the effects of the lichen thalli on the substrate. Indeed, it is well-known that lichens interact geochemically and geophysically with the lithic substrate leading to the formation of different microhabitats and chemical microenvironments beneath them [3, 6, 7, 13]. Other biotic interactions involved in the succession of saxicolous lichen communities have been described [1, 30, 35, 49] including competition for limiting resources such as space, nutrients, and light [26]. The fungal communities examined here may have also been shaped by competition between lichens and free-living endolithic fungi [7].

The chemical composition and intrinsic properties of the lithic substrate could also affect the intensity and type of microbial colonization present [23, 25]. Indeed, the textural features of Redueña dolostone could be one of the main determinants of the type of colonization observed in this study. The extent and type of porosity of dolostones have been reported to condition fungal penetration pathways along their intrinsic discontinuities [8]. Thus, the lack of intracrystalline porosity in the Redueña dolostone could favor the intercrystalline penetration of fungi. This has been recently described by Favero-Longo et al. [15], who stressed that differences in the modality of mycobiont hyphae penetration (depth of maximum penetration and penetration pattern) are basically dependent on the mineral composition and structure of the different lithotypes rather than on species differences.

The dynamics of microbial colonization has been scarcely explored. However, a microbial colonization sequence similar to that observed here was described by Hoppert et al. [27] in limestone across an area of receding glaciers in the Austrian Alps. The first microbial settlers

were endoliths encompassing a wide variety of microorganism groups. However, epilithic lichen colonization was not detected in these limestones exposed to the environmental conditions for 100 years. Ascaso et al. [2] also described a process of sequential colonization of carbonate rocks (dolostones and biocalcarenes) in the Mola quarry (Novelda, Alicante), but the sequence differs to that observed in the present study. Thus, in the abandoned area of the Novelda quarry epiliths appeared, while in the limiting zone of the quarry, an non-exploited area, lichens and cyanobacteria occupied both endolithic and epilithic positions. This difference in the sequence of colonization could be attributed to differences in mineralogical composition, textural features, and petrophysical characteristics [17, 18], but also to climate differences (Mediterranean in Alicante versus continental in Madrid). Indeed, seasonal and environmental variations have often been incriminated in changes in the dynamics of colonization and composition of microbial communities, as described in different marble stones from both quarries and monuments [19–21].

We detected similar bioalteration processes in dolostone from the quarry and from monuments built out of the same stone. Long-standing colonization by lichen communities has meant intense alteration of the lithic substrate beneath these communities. These effects appear mainly due to mechanical actions of the lichens although we cannot rule out the possibility of chemical alterations. Several stages of dolostone alteration were observed, similar to those described by De los Ríos et al. [11]. Thus, areas colonized by epilithic lichens were the most weathered and as we moved away from the lichen thalli, alteration of the rock substrate diminished with depth and it was possible to distinguish the other stages of alteration described by these authors. However, we should not underestimate the decay of the lithic substrate in deeper areas by endolithic forms under these lichens [37]. In effect, slow-growing fungi showing a high similarity to *Phaeococcomyces* sp. (98.6%) and *Coniosporium* sp. (99.8%) were isolated from deeper areas of the dolostones. These microcolonial fungi are common inhabitants of rocks and considered very aggressive biodeterioration agents in stone monuments [39–41], since their melanized pigmentation is thought to confer extra-mechanical strength to the hyphae [14, 42]. All our results indicate that observations in quarry stones may be translated to the situation in the historic monuments in which Redueña dolostone was used as dimension stone. All this detailed information of microbial colonization strategies obtained in this study, especially the presence of different endolithic forms, helped us select treatments to combat the problems caused by the entire lithobiontic community.

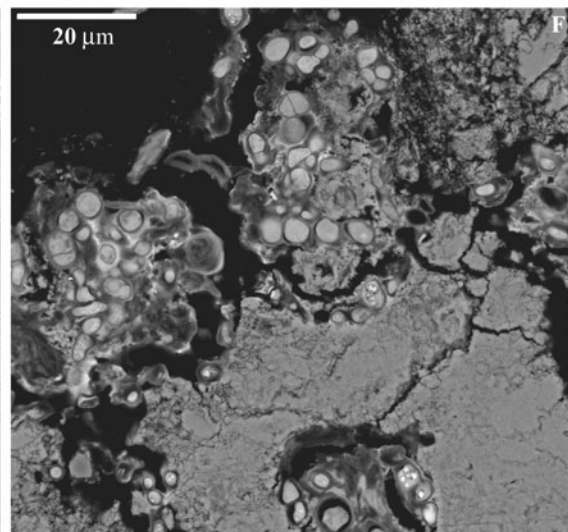
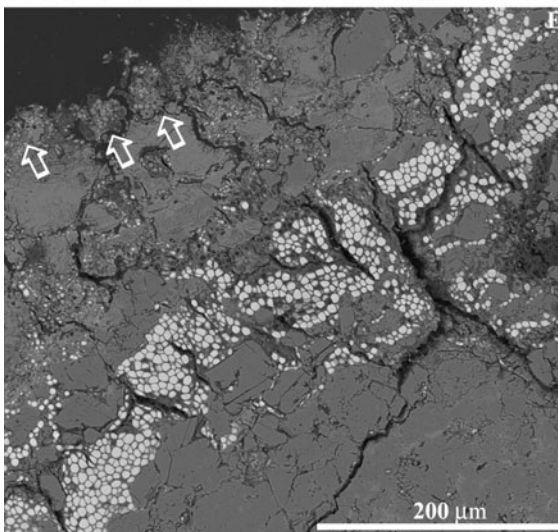
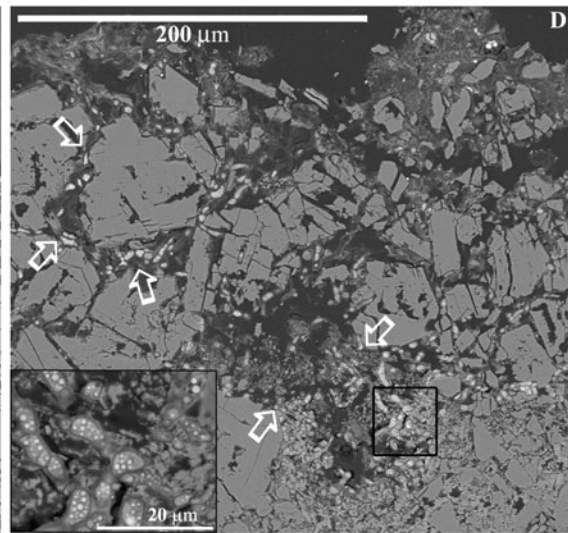
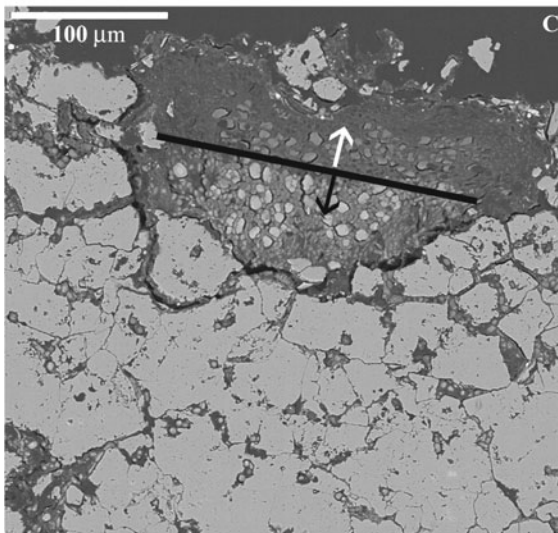
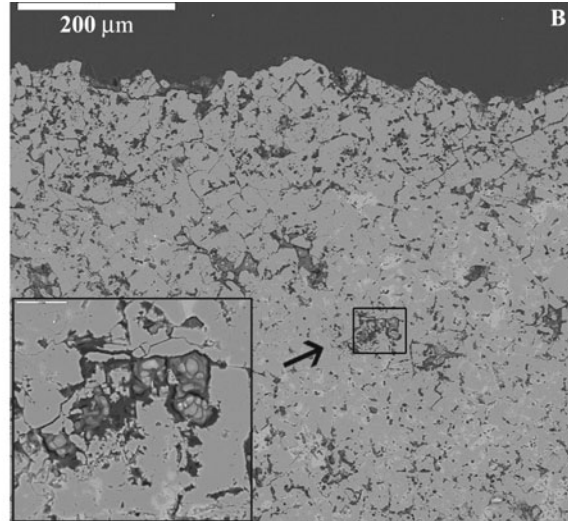
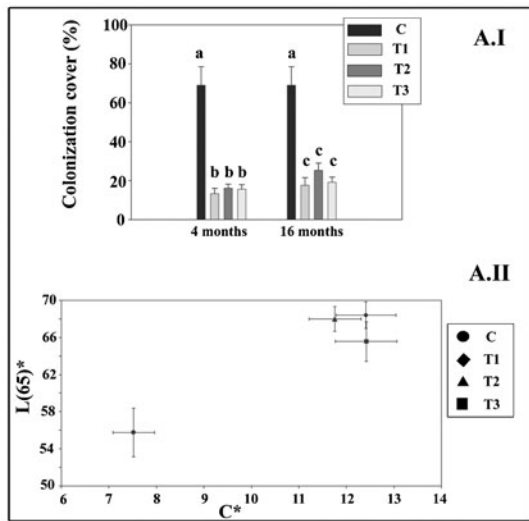


Figure 7 Assessment of the efficacy of the biocide treatments on the Redueña quarry dolostone; **a-I** Epilithic lichen cover determined in the experimental plots at 4 and 16 months post treatment. After 4 months of treatment, epilithic cover was reduced to 1/5 and no differences between treatments were observed. Different letters indicate significant differences; **a-II** Diagram of L* (lightness)/C*(chroma) of the experimental plots measured by reflectance using a spectrophotometer. The treated plots show similar lightness values which were higher than the value recorded for the untreated plot. **b–c** SEM-BSE images of the effects at 4 months on the lithobiotic community commonly observed in the three treatment plots. **b** Area with no lichen remains on the rock surface. Detailed view of the endolithic alga cells and fungal hyphae showing loss of their typical morphology; **c** Remains of a lichen thallus. Note the interface (*line*) between the zone of biocide action (*white arrow*) and the zone of no biocide action (*black arrow*). **d–f** SEM-BSE images of samples from plots after 16 months of treatment. **d** Abundant endolithic colonization of Redueña dolostone in areas lacking lichen remains. Note that associated with this fungal endolithic colonization there is intense alteration of the internal structure of the dolostone, especially in areas with calcite cement (*arrows*). Detailed view of the area showing fungal hyphae with numerous lipid bodies. **e** Area extensively colonized by endolithic fungi showing incipient epilithic recolonization (*arrows*). **f** Detailed view of the newly colonized area indicated with *arrows* in Fig. 7e

By in situ assessing the biocide treatments on quarry stone, our aim was to simulate one of the strategies commonly used in the conservation of historic monuments while avoiding any risks of damaging this cultural heritage. The biocide treatments per se were efficient in that they all managed to reduce lichen cover by over 80%. However, the

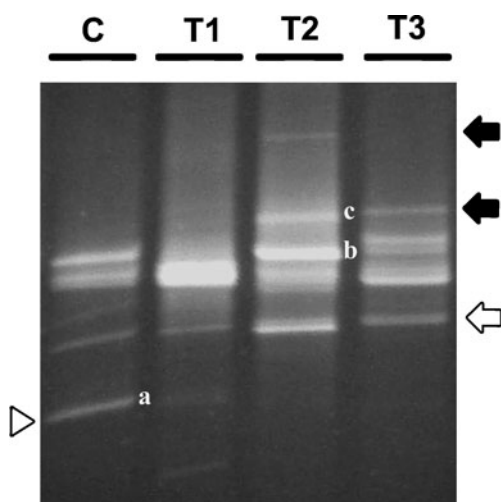


Figure 8 DGGE analysis of the fungal endolithic colonization of the Redueña dolostone 16 months after biocide treatment. The control (C) is included for comparison. Note that some bands are common to all the profiles (*empty arrow*), new bands appear in some of the profiles for the different treatments (*black arrow*) and one of the bands only appears in the control profiles (*arrowhead*). **a–c** DGGE bands and percentage Genbank matches obtained for the control and T2 profiles: **a** *Verrucaria csernaensis* [FJ664723] 94%; **b** *Caloplaca concreticola* [EU192154] 98%; **c** *Aspicilia contorta* [EF332108] 95%

lack of significant differences between treatments ($p=0.194$) indicates that the most resolving strategies were mechanical cleaning and treatment with ACTICIDE® LV706 (disinfectant plus fungicide) since these were common to all the experimental plots. In addition, the effectiveness of treatment was dependent on the persistence of lichen remains after cleaning the rock surface indicating that our cleaning method was not totally effective and should be modified in future experiments. These lichen remains act as a protective layer, avoiding the penetration of the agents into deeper areas of the lithic substrate and their actions on endolithic microorganisms. Irregularities in the rock surface could be the main reason for the lack of effectiveness of the treatments in some areas. The recolonization of dolostones observed after 16 months of biocide treatment reveals a need for retreatments. Effectively, the presence of remains of dead free-living and lichenized fungi and the absence of competitors could have promoted the growth of free-living fungi [10, 33]. Future experiments should be aimed at mitigating the effects of the factors that could have interfered with the success of the biocide treatments tested here before these biocides are used on these monuments. Other measures that could be tested in future trials are, for example, wetting the rock surface and applying a higher biocide concentration as pretreatment before mechanical elimination of the epilithic biomass [33, 44, 45].

Our findings also indicate that the Redueña quarry is an ideal natural laboratory for analyzing lithobiotic communities and the efficacy of biocide treatments designed to eliminate them. Indeed, this use of quarry stone is recommended to optimize the different protocols and conditions in the in situ evaluation of biocide treatments [29]. Once the most effective method has been established, this information could be extrapolated to monuments built out of the same rock. The sequence of microbial colonization of natural dolostones inferred here and the in situ assessment of biocide treatments contribute to our present understanding of the microbial ecology of lithobiotic communities and also have implications for conserving our cultural heritage.

Acknowledgments The authors would like to thank the following persons for their contributions to this study: Fernando Pinto from the Electron Microscopy Service of the Institute of Agricultural Sciences (CSIC) and Teresa Carnota and Maria Jose Malo from the National Museum of Natural Sciences (CSIC) for their technical assistance; Manolo Castillejo and José Manuel Hontoria for polishing the samples for microscopy (MNCN, CSIC); Dr. Silvia Matesanz for help with the statistical analysis; Dr. Sergio Perez-Ortega for help with identifying the lichens; Ana Burton for editorial assistance; and reviewers for their constructive comments. This study was supported by grants GEOMATERIALES (S2009/MAT-1629) from the CAM and CTM2009-122838-C04-03 from the Spanish Ministry of Science and Innovation, and by a predoctoral fellowship (FPI program, BES-2007-15145) awarded by the Spanish Ministry of Science and Education.

References

- Armstrong RA (1982) Competition between three saxicolous species of *Parmelia* (lichens). *New Phytol* 90:67–72
- Ascaso C, García del Cura MA, De Los RA (2004) Microbial biofilms on carbonate rocks from a Quarry and Monuments in Novelda (Alicante, Spain). In: Clair LS, Seaward M (eds) *Biodeterioration of stone surfaces*, vol. 6, vol 6. Kluwer Academic Publishers, Netherlands, pp 79–98
- Ascaso C, Wierzychos J (1995) Estudio de la interfase talo líquénico-sustrato lítico con Microscopía Electrónica de Barrido en Modo de Electrones Retrodispersados. In: Daniels FJA, Schulz M, Peine J (eds) *Contributions to lichenology in honour of Gerhard Follmann Flechten Follmann*. The Geobotanical and Phytotaxonomical Study Group. Botanical Institute, University of Cologne, Cologne, pp 43–54
- Ascaso C, Wierzychos J, Castello R (1998) Study of the biogenic weathering of calcareous litharenite stones caused by lichen and endolithic microorganisms. *Int Biodeterior Biodegrad* 42:29–38
- Ascaso C, Wierzychos J, Souza-Egipsy V, De Los RA, Rodrigues JD (2002) In situ evaluation of the biodeteriorating action of microorganisms and the effects of biocides on carbonate rock of the Jeronimos Monastery (Lisbon). *Int Biodeterior Biodegrad* 49:1–12
- Banfield JF, Barker WW, Welch SA, Tauton A (1999) Biological impact on mineral dissolution: application of the lichen model to understanding mineral weathering in the rhizosphere. *P Natl Acad Sci Usa* 96:3404–3411
- Bjelland T, Ekman S (2005) Fungal diversity in rock beneath a crustose lichen as revealed by molecular markers. *Microb Ecol* 49:598–603
- Cámara B, De Los RA, García del Cura MA, Galvan V, Ascaso C (2008) Dolostone bioreceptivity to fungal colonization. *Mater Constr* 58:113–124
- Cubero OF, Crespo A, Fatehi J, Bridge PD (1999) DNA extraction and PCR amplification method suitable for fresh, herbarium-stored, lichenized, and other fungi. *Plant Syst Evol* 216:243–249
- De Los RA, Ascaso C (2005) Contributions of in situ microscopy to the current understanding of stone biodeterioration. *Int Microbiol* 8:181–188
- De los Ríos A, Cámara B, Cura del García MA, Rico VJ, Galván V, Ascaso C (2009) Deteriorating effects of lichen and microbial colonization of carbonate building rocks in the Romanesque churches of Segovia (Spain). *Sci Total Environ* 407:1123–1134
- De Los RA, Galvan V, Ascaso C (2004) In situ microscopical diagnosis of biodeterioration processes at the convent of Santa Cruz la Real, Segovia, Spain. *Int Biodeterior Biodegrad* 54:113–120
- De Los RA, Wierzychos J, Ascaso C (2002) Microhabitats and chemical microenvironments under saxicolous lichens growing on granite. *Microb Ecol* 43:181–188
- Dornieden T, Gorbushina AA, Krumbein WE (2000) Biodecay of cultural heritage as a space/time-related ecological situation—an evaluation of a series of studies. *Int Biodeterior Biodegrad* 46:261–270
- Favero-Longo SE, Borghi A, Tretiach M, Piervittori R (2009) In vitro receptivity of carbonate rocks to endolithic lichen-forming aposymbionts. *Mycol Res* 113:1216–1227
- Fort R (1996) Características cromáticas de los materiales en construcción. In: Mingarro F (ed) *Degradación y conservación del Patrimonio Arquitectónico*. Complutense, Madrid, pp 213–226
- Fort R, Bernabéu A, García del Cura MA (2002) Novelda stone: a stone widely used within the Spanish architectural heritage. *Mater Constr* 266:19–32
- Fort R, Fernández-Revuelta B, Varas MJ, Álvarez de Buergo M, Taborda-Duarte M (2008) Influence of anisotropy on the durability of Madrid-region cretaceous dolostone exposed to salt crystallization processes. *Mater Constr* 58:161–178
- García-Vallés M, Urzú C, Leo FD, Salamone P, Vendrell-Saz M (2000) Biological weathering and mineral deposits of the Belevi marble quarry (Ephesus, Turkey). *Int Biodeterior Biodegrad* 46:221–227
- García-Vallés M, Urzú C, Vendrell-Saz M (2002) Weathering processes on the rock surface in natural outcrops: the case of an ancient marble quarry (Belevi, Turkey). *Environ Geol* 41:889–897
- García-Vallés M, Vendrell-Saz M, Molera J, Blazquez F (1998) Interaction of rock and atmosphere: patinas on Mediterranean monuments. *Environ Geol* 36:137–149
- Gardes M, Bruns D (1992) ITS primers with enhanced specificity for basidiomycetes—application to the identification of mycorrhizae and ruts. *Mol Ecol* 2:113–118
- Gleeson DB, Clipson N, Melville K, Gadd GM, McDermott FP (2005) Characterization of fungal community structure on a weathered pegmatitic granite. *Microb Ecol* 0:1–9
- Gómez-Heras M, Fort R (2004) Location of quarries of non traditional stony materials in the architecture of Madrid: the Crypt of Cathedral of Santa María la Real de la Almudena. *Mater Constr* 54:33–48
- Guillitte O (1995) Bioreceptivity: a new concept for building ecology studies. *Sci Total Environ* 167:215–220
- Harris PM (1996) Competitive equivalence in a community of lichens on rock. *Oecologia* 108:663–668
- Hoppert M, Flies C, Pohl W, Günzl B, Schneider J (2004) Colonization strategies of lithobiotic microorganisms on carbonate rocks. *Environ Geol* 46:421–428
- Hoppert M, König S (2006) The succession of biofilms on building stone and its possible impact on biogenic weathering. In: Fort, Alvarez de Buergo, Gomez-Heras, Vazquez-Calvo (eds) *Heritage, weathering and conservation*. Taylor and Francis Group, London, pp 311–315
- Jimenez-Lopez C, Jroundi F, Pascolini C, Rodriguez-Navarro C, Piñar-Larrubia G, Rodriguez-Gallego M, González-Muñoz MT (2008) Consolidation of quarry calcarenite by calcium carbonate precipitation induced by bacteria activated among the microbiota inhabiting the stone. *Int Biodeterior Biodegrad* 62:352
- Lawrey JD (1991) Biotic interactions in lichen community development: a review. *Lichenol* 23:205–214
- Macedo MF, Miller AZ, Dionisio A, Saiz-Jimenez C (2009) Biodiversity of cyanobacteria and green algae on monuments in the Mediterranean Basin: an overview. *Microbiology* 155:3476–3490
- Mendiúña J, Fort R, García del Cura MA, Galán L, Pérez-Soba C, Perez-Monserrat EM, Fernández-Revuelta B, Bernabéu A, Varas MJ, (2005) Las piedras utilizadas en la construcción de los bienes de interés cultural de la Comunidad de Madrid anteriores al siglo XIX. Instituto Geológico y Minero de España
- Nascimbene J, Salvadori O, Nimis PL (2009) Monitoring lichen recolonization on a restored calcareous statue. *Sci Total Environ* 407:2420–2426
- Nash TH III (1996) *Lichen biology*. Cambridge University Press, Cambridge
- Pentecost A (1980) Aspects of competition in saxicolous lichen communities. *Lichenol* 12:135–144
- Prieto B, Silva B, Aira N, Laiz L (2005) Induction of biofilms on quartz surfaces as a means of reducing the visual impact of quartz quarries. *Biofouling* 21:237
- Salvadori O (2000) Characterisation of endolithic communities of stone monuments and natural outcrops. In: Ciferri O, Tiano P, Mastromei G (eds) *Of microbes and art: the role of microbial*

- communities on the degradation and protection of cultural heritage. Kluwer, Florence, pp 89–102
38. Scheerer S, OrtegaMorales O, Gaylarde C, Allen I, Laskin SS, Geoffrey MG (2009) Microbial deterioration of stone monuments—an updated overview advances in applied microbiology, vol. 66. Academic Press, pp. 97–139
 39. Sert H, Sterflinger K (2009) A new coniosporium species from historical marble monuments. *Mycol Prog*. doi:10.1007/s11557-009-0643-z
 40. Sert H, Sümbül H, Sterflinger K (2007) Microcolonial fungi from antique marbles in Perge/Side/Termessos (Antalya/Turkey). *Anton Leeuw* 91:217–227
 41. Sterflinger K, De Baere R, de Hoog GS, De Wachter R, Krumbein WE, Haase G (1997) *Coniosporium perforans* and *C. apollinis*, two new rock-inhabiting fungi isolated from marble in the sanctuary of Delos (Cyclades, Greece). *Anton Leeuw* 72:349–363
 42. Sterflinger K, Krumbein WE (1997) Dematiaceous fungi as a major agent for biopitting on Mediterranean marbles and limestones. *Geomicrobiol J* 14:219–230
 43. Sterflinger K, Sert H (2006) Biodeterioration of buildings and works of art—practical implications on restoration practice. In: Fort, Buergo Ad, Vazquez-Calvo, Gomez-Heras (eds) Heritage, weathering and conservation, vol. 1. Taylor & Francis Group, London, pp 299–304
 44. Tretiach M, Crisafulli P, Imai N, Kashiwadani H, Hee Moon K, Wada H, Salvadori O (2007) Efficacy of a biocide tested on selected lichens and its effects on their substrata. *Int Biodeterior Biodegrad* 59:44–54
 45. Tretiach M, Bertuzzi S, Salvadori O (2010) Chlorophyll a fluorescence as a practical tool for checking the effects of biocide treatments on endolithic lichens. *Int Biodeterior Biodegrad* 64:452–460
 46. Warscheid T, Braams J (2000) Biodeterioration of stone: a review. *Int Biodeterior Biodegrad* 46:343–368
 47. White TJ, Bruns TD, Lee SB, Taylor JW (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JH, White TJ (eds) PCR protocols—a guide to methods and applications. Academic, New York, pp 315–322
 48. Wierzos J, Ascaso C (1994) Application of back-scattered electron imaging to the study of the lichen–rock interface. *J Microsc* 175:54–59
 49. Woolhouse MEJ, Harmsen R, Fahrig L (1985) On succession in a saxicolous lichen community. *Lichenol* 17:167–172