Aerobiologia **16:** 423–428, 2000.
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Evaluation of the effect of some biocides against organisms isolated from historic monuments

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Received 25 January 1999; accepted in final form 15 May 2000

Key words: biocides, monuments, organisms, weathering

Abstract

On the surfaces of monuments and buildings, organic and inorganic pollutants accumulate, as well as various microbial communities which contribute to stone decay. In order to control these organisms, we have tested some chemical products with biocide and water-repellent properties. Some of these products were tested in an agar diffusion test and on limestone slabs. Efficacy of the products and the microbial inhibition were studied with scanning electron microscopy (SEM) and confocal scanning laser microscopy (CSLM) techniques.

Introduction

It is well known that the weathering of different materials is the result of the combined actions of physical and chemical factors, and biological activity. Many monuments made of stone have been treated with a multitude of chemicals in order to: consolidate the rock materials; render them hydrophobic and, thus, less susceptible to chemical and biological attack; or kill or inhibit lower and higher organisms (Krumbein et al., 1993).

Construction of the Cathedral of Alcalá de Henares (Spain) was started in the fifteenth century, and since then it has undergone many renovations and improvements. It was built in limestone and granite. The tower was recently restored and some conservation products were applied. However, only a few months after treatment, we could see new biological growth symptoms. With the object of finding the most effective chemicals to avoid growth on this monument, there have been tested solutions containing biocides, water-repellents and consolidants often used in the restoration of weathered stones.

To determine the efficacy of biocides in killing target organisms at a minimal dose laboratory tests were carried out using Petri dishes with growth media, against bacteria, fungi and yeasts strains which were previously isolated from the weathered walls of the Cathedral.

Many factors can influence the efficiency of biocides when applied to stone substrates. These include interactions with inorganic components of rock, the presence of organic compounds on surface layers, the influence of environmental agents, etc. (Wakefield and Jones, 1996). Therefore, experiments in the laboratory using limestone and sandstone slabs impregnated with biocides and inoculated with several microbial strains were carried out to simulate outdoor conditions. These samples were treated in order to check with SEM the microbial inhibiting effects and behaviour of the biocides on the stone slabs. Finally, the application of CSLM helped to reveal the penetration depth of the organisms in the stone samples.

Materials and methods

Biocides tested

For this study we used nine commercial products supplied by different manufacturers, which have already been mentioned in the rock protection liter-

Table 1. Caracteristics of the conservation products tested.

Trade name	Chemical name	Supplier	Solvent	Activity
New Des	Benzalconium chloride 10%	C.T.S. España	Water	Bactericide
Bio Estel	Silicic acid derivatives with molecular organometalic biocides	C.T.S. España	Ethanol	Biocide, Consolidant
Mergal S97	Carbendazim octylisothiazolin-5-benzimidazol methylcarbamate	Hoechst	Ethanol	Biocide
Preventol R80	Alkylbenzyl-dimethyl-ammonium chloride	Bayer	Water	Biocide
Preventol A6	N′-(3,4-dichlorophenyl)-N,N-dimethyl urea	Bayer	Acetone	Algicide
Preventol A9D	N-dichlorofluor-methyltio-N′,N′-dimethyl-N-p-tolil sulphamide	Bayer	Acetone	Fungicide
Tegosivin HL100	Modified siloxane	Goldschmidt	Ethanol	Water-repellent
Tegovakon V	Silicic acid esther	Goldschmidt	Ethanol	Consolidant
Metatin N5810/101	Tributyl tin-naphtenate and quaternary ammonia compound	C.T.S. España	Water	Fungicide, Algicide

Table 2. Inhibitory activity of the products tested against organisms isolated from the Cathedral of Alcalá de Henares.

	New Des			Bio Estel		Mergal S97		Preventol R80		Products tested: Preventol A6			Preventol A9D			Tegosivin HL100		Tegovakon V		Metatin N5810/101		
Organism	2.5*	5	10	10	Pure	1	2	1	2	0.5	1	2	1	2	3	5	10	10	Pure	0.5	1	2
Fungi:																						
Penicillium sp.	17	16	18	27	42	−	−	11	17	+	+	+	37	35	35	+	+	+	+	10	20	17
Nectria sp.	8	10	13	17	17	29	47	27	32	+	+	+	32	28	34	+	+	+	+	15	18	22
Phoma sp.	+	+	13	12	18	−	−	22	−	+	+	+	38	40	−	+	+	+	+	22	24	22
Ulocladium sp.	14	19	29	14	25	25	39	25	26	+	+	11	41	40	−	+	+	+	+	12	18	19
Fusarium sp.	+	+	+	11	14	45	41	14	11	+	+	8	41	43	34	+	+	+	+	17	23	26
Aspergillus niger	+	+	7	17	18	33	32	8	8	+	+	+	22	19	20	+	+	+	+	21	22	24
Bacteria:																						
Micrococcus agilis	20	20	25	+	+	18	26	20	21	13	13	14	12	12	12	+	+	+	+	27	30	30
Micrococcus lylae	21	21	23	9	20	21	23	24	27	12	17	17	15	15	15	+	+	+	+	22	22	30
Micrococcus roseus	18	21	26	9	25	20	23	21	30	13	13	13	9	12	15	+	+	+	+	21	25	28
Bacillus circulans	15	17	19	+	+	21	23	18	18	9	15	15	9	12	12	+	+	+	+	20	20	20
Bacillus badius	25	25	30	12	22	21	30	20	25	15	20	20	15	18	18	+	+	+	+	25	32	32
Bacillus licheni	13	16	20	+	20	23	23	19	20	14	14	18	15	20	20	+	+	+	+	23	24	30
Yeasts:																						
Rhodotorula sp.	14	16	17	7	11	27	33	15	19	7	8	8	7	7	7	8	10	7	10	21	21	25
Cryptococcus sp.	23	24	26	8	13	30	32	26	28	7	13	13	26	29	30	10	11	+	+	13	20	28

+: growth; −: the biocide inhibition is so high than it is not possible to measure the halo size.
Numbers indicate the inhibition halo given in mm.
* Dilutions used in each product (%) (v/v).

ature (Agarossi et al., 1989; Petersen et al., 1993). The chemical compositions and effectiveness of these compounds against different organisms are given in Table 1. Seven are biocides, with Bio Estel containing a consolidant besides the biocide. Tegosivin is a water-repellent and Tegovakon is a consolidant.

Organisms tested

The organisms chosen for our tests were previously isolated from limestone and granite crusts from the Cathedral of Alcalá de Henares (Spain) (Table 2).

Agar diffusion test

Sensitivity of the organisms was determined in Petri dishes with agar medium, inoculated with a spore suspension (10^6–10^7 spores ml^{-1}). Cellulose discs (bioMerieux) of 6 mm diameter and impregnated with 10 μl of each biocide solution were put in the dishes. After an incubation period, the inhibition halo that developed around the disc was measured in duplicate plates.

Rock slab test

Limestone slabs coming from Anchuelos's quarries (Madrid, Spain) were used, with 3 cm side length and 0.3–0.4 cm thickness. First they were washed, air-dried and sterilized for 3 days in a steam autoclave. Then each slab was placed on a glass slide in a petri plate with a layer of vermiculite at the bottom (Grant et al., 1985), everything having been autoclaved. The

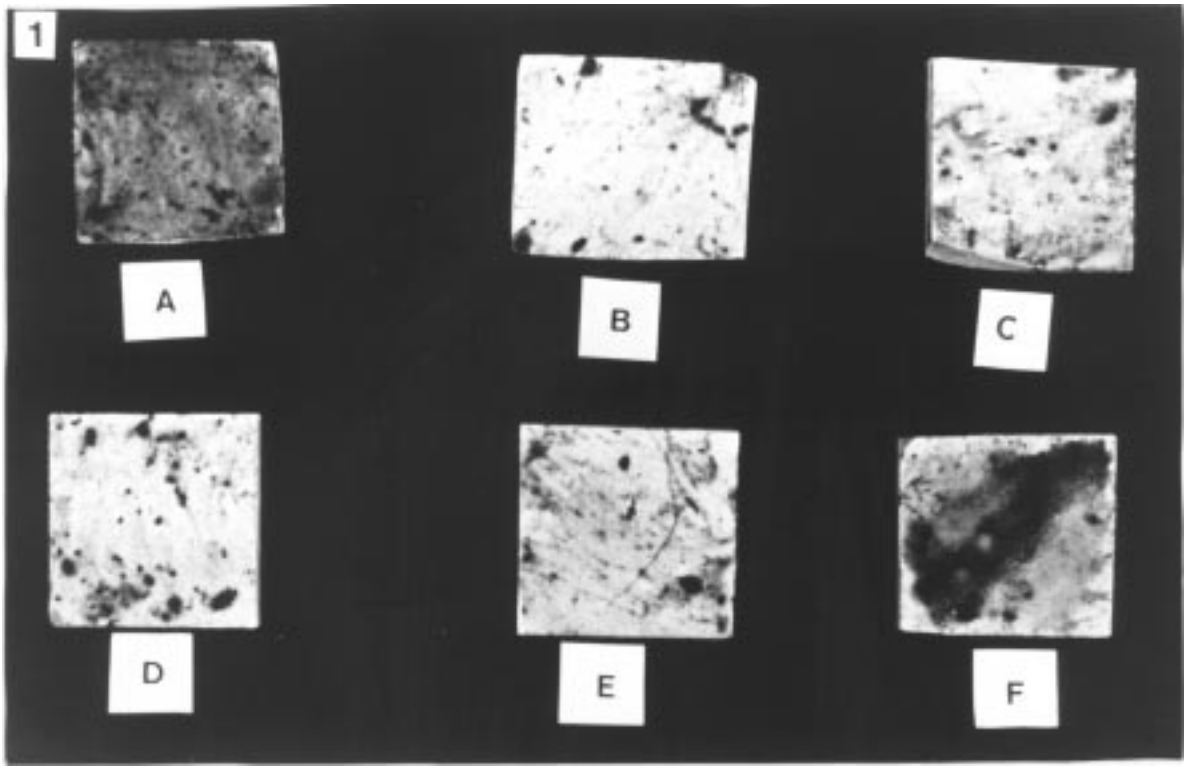

Figure 1. Slabs of quarry limestone colonized by *Ulocladium sp.* 8 weeks after inoculation, with a reinoculation at 4 weeks. Differences can be observed in growth between the control (A) and the slabs impregnated with Preventol A9D 1% (B), Preventol R80 1% (C), Metatin N5810/101 1% (D), Mergal S97 1% (E) and pure Bio Estel (F).

sterilized slabs were impregnated with biocide and then dried at laboratory temperature. Then the treated samples were inoculated with 200 μl of the microbial suspension and incubated at the optimum temperature for 4 weeks, and then reinoculated and kept for 4 weeks more. Rock slabs were then used for studying the weathering produced by organisms with the help of SEM and CSLM.

Scanning electron microscopy

Rock samples were prepared for scanning electron microscopy (SEM-EDX and SEM-BSE) according to Gomez-Alarcón et al. (1995).

Confocal scanning laser microscopy

A confocal Bio Rad MRC 1024 system and a Zeiss Axiovert 135 microscope were used. Stone material was stained with fluorochrome acridine orange (20 μg/ml) by applying the dye directly to the stone surface (Quader and Bock, 1995).

Results and discussion

Agar diffusion test

With the test in agar dishes, different results were obtained, depending on the selected organism and the respective product. The efficacy was calculated by analyzing the formation of inhibition zones with different concentrations (Table 2). Most biocides produced an inhibition halo with fungi and bacteria cultures, while water-repellents and consolidants did not. Yeasts were sensitive to almost all the products tested. In the case of Preventol A6, its low activity against fungi was evident, but this is explainable because of its mainly algicide action. Preventol A9D and Metatin N5810/101, described as fungicides, also showed bactericide activity.

The greatest efficacy against fungi was obtained with Mergal S97 and next with Preventol A9D. These results agree with those reached by Krumbein and Gross (1992). In the case of yeasts, the inhibition of these two compounds was minor. The bacteria group responded in a way similar to that of the test with

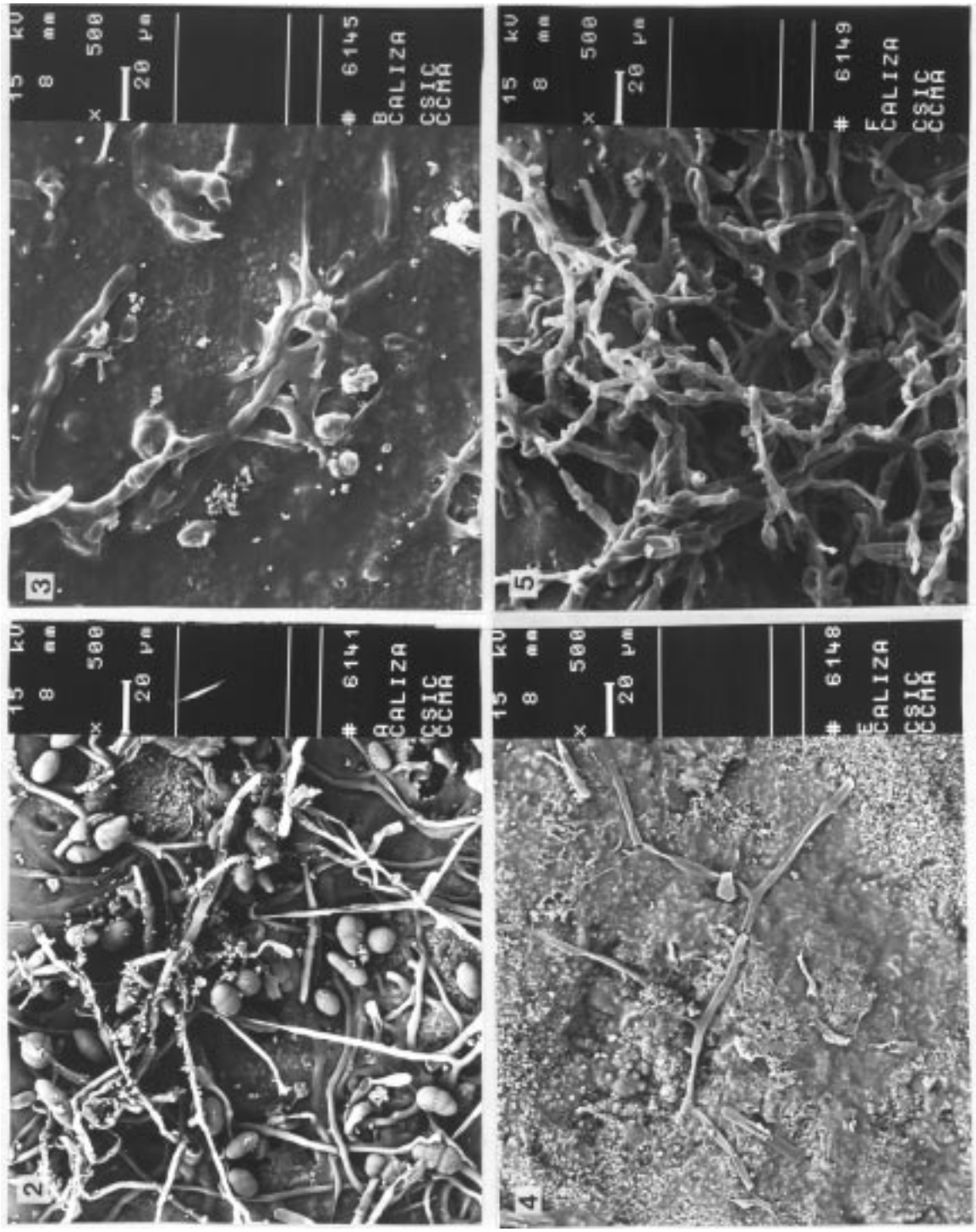

Figure 2–5. SEM images of *Ulocladium sp.* on limestone 8 weeks after inoculation (with a reinoculation at 4 weeks). Untreated sample (Figure 2); rock impregnated with Preventol A9D 1% (Figure 3), with Mergal S97 1% (Figure 4), and with pure Bio Estel (Figure 5).

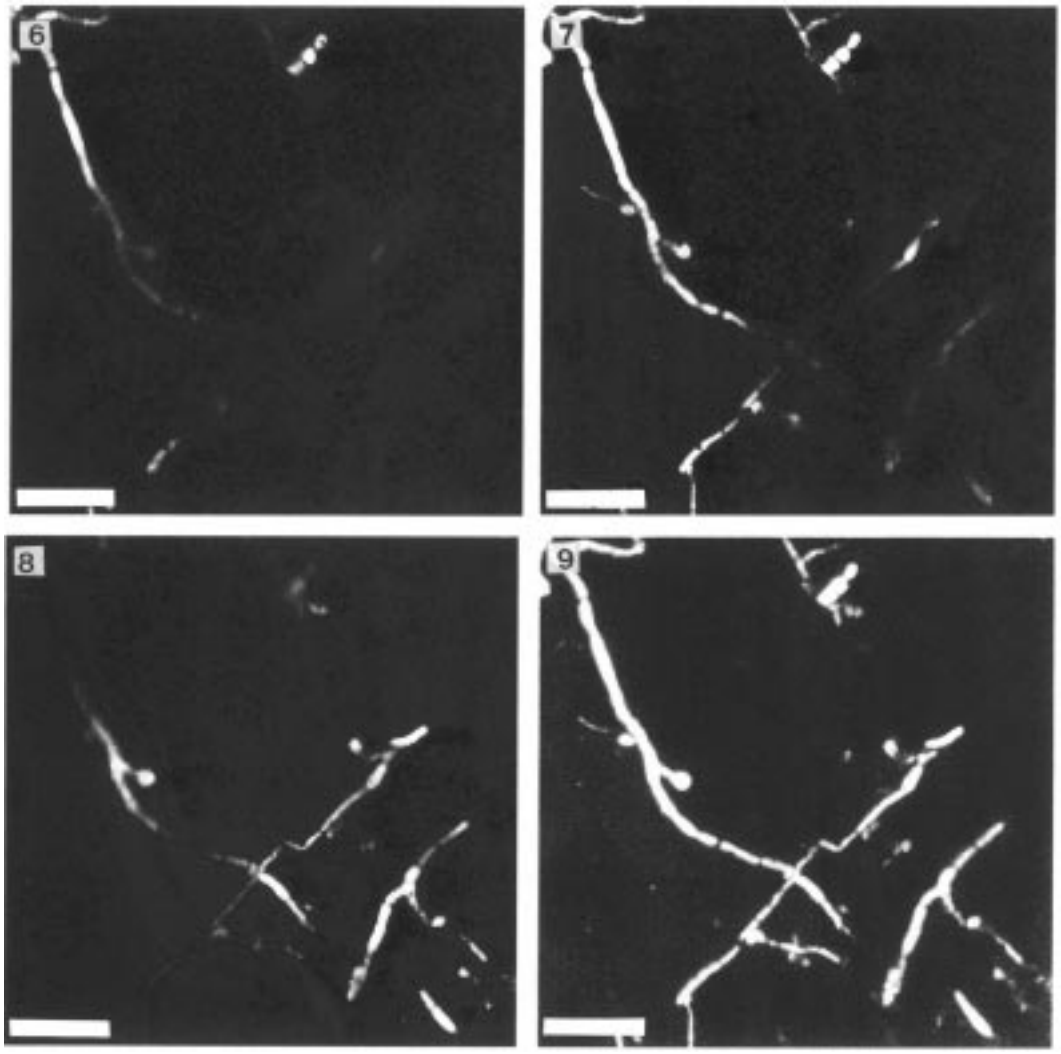

Figure 6–9. Confocal images of *Ulocladium sp.* on limestone, 8 weeks after inoculation. The fungal hiphae can be observed penetrating 3 μm into the stone (Figure 6). The hiphae penetrate at 5 μm (Figure 7) and at 10 μm (Figure 8). Superimposition of 12 images of *Ulocladium sp.* selected on one specific area of the sample along the z-axis (Figure 9). Bar = 50 μm.

Mergal S97, and were inhibited with New Des and Metatin. Finally, as was expected, Tegosivin HL100 and Tegovakon V lightly inhibited yeasts.

Growth of fungi on impregnated limestone

In experiments performed with limestone slabs inoculated with *Ulocladium sp.* and impregnated with different biocides (Figure 1), after prolonged incubation there was found an abundant growth on the non-treated sample, as well as on the Bio Estel-treated slab, with the lowest rates being on Preventol A9D-impregnated stone. Results with Bio Estel can be explained because its composition is a mixture of biocide and consolidant, and the efficiency is lower.

Activity of the products tested against *Ulocladium sp.* cellular elements was observed in detail by SEM. On non-treated limestone there was observed a good growth and sporulation of fungus (Figure 2). Nevertheless, in the samples impregnated with Preventol A9D and Mergal S97, a strong microbial inhibition appeared accompanied by swellings and cellular deformations, indicative of chemical and structural changes (Figures 3, 4). In these cases, the presence of adhered particles that must have come from constitu-

ents of the applied biocides was clear. In the Bio Estel-impregnated sample, the hiphae were disturbed although fungal development took place, as can be seen in Figure 5.

The application of CSLM has produced a considerable advance in our knowledge of the penetration and dispersion of organisms colonizing stone substrates. *Ulocladium sp.* colonized limestone slabs and micelium appeared at 3, 5 and 10 μm depths from the reference point (Figures 6–8). These observations confirm that micelial organisms spread on the surface and also penetrate to the stone interior, which helps to cause alterations in the colonized substrates. Figure 9 shows 12 images superimposed on the same area along the z-axis.

Application of biocides on the monument

After the laboratory tests, selected biocides (Metatin N5810/101, Preventol A6, Preventol A9D and Preventol R80) were applied to limestone and granite blocks, on surfaces of approximately 750 cm^2, verifying that no chromatic or chemical change was produced. The application was done with a brush, and 6 days later the treatment was repeated. Eight days after the second application, it was observed that only Preventol A6 produced a slight darkening of the limestone surface, and a less detectable change on the granite surface. Samples were taken from the blocks selected, both before and after treatment, in order to test possible changes in the microbial populations, but these observations are still in the investigative stage.

Conclusions

This work has shown that a great amount of laboratory experiments is needed before biocides and other chemicals can be applied for the protection of buildings. The use of new techniques, such as SEM or CSLM, is completely necessary and has yielded significant information for us. Thanks to SEM we can see that Preventol A9D and Mergal S97 show a good distribution on the rock surface; and with Mergal S97 we can appreciate a crystallization of the product on stone pores which could make penetration of the solutions difficult. Besides, the treatments show that sometimes microbial inhibition consists of deformations in the microbial cells. The application of CSLM allows us to know the penetration and dispersion of microorganisms in limestone slabs, which helps to evaluate microbial biodeterioration processes. Tests on the activity and longevity of biocides outdoors are necessary to understand the behaviour against microflores under environmental conditions.

Acknowledgement

This work has been supported by the Comunidad de Madrid (project 06M/024/96).

References

Agarossi G., Ferrari R. and Monte M.: 1989, Biocides in the control of biodeterioration. In: F. Zezza (ed), *Proceedings of the 1st International Symposium of the Conservation of Monuments in the Mediterranean Basin*. Bari, pp. 511–517.

Gómez-Alarcón G., Cilleros B., Flores M. and Lorenzo J.: 1995, Microbial communities and alteration processes in monuments at Alcalá de Henares (Spain). *Sci Total Environ* **167**, 231–239.

Grant C. and Bravery A.F.: 1985, A new method for assessing the resistance of stone to algal disfigurement and the efficacy of chemical inhibitors. In: G. Felix (ed), *Proceedings of the Vth International Congress on Deterioration and Conservation of Stone*. Lausanne, pp. 663–674.

Krumbein W.E. and Gross M.: 1992, Interactions of biocides with cultures of biodeteriorating microbionta in agar diffusion and rock cube tests. In: J. Delgado, F. Henriques and F. Telmo (eds), *Proceedings of the 7th International Congress on Deterioration and Conservation of Stone*, Vol. 1. Lisbon, pp. 501–509.

Krumbein W.E., Diakumaku S.E., Petersen K., Warscheid T. and Urzi C.: 1993, Interactions of microbes with consolidants and biocides used in the conservation of rocks and mural paintings. In: M.J. Thiel (ed), *Conservation of Stone and Other Materials*, Vol. 2. Paris, pp. 589–596.

Petersen K., Krumbein W.E., Haffner N., Lux E. and Mieth A.: 1993, Aspects of biocide application on wall-paintings – report on Eurocare Project EU 489, Biodecay. In: M.J. Thiel (ed), *Conservation of Stone and Other Materials*, Vol. 2. Paris, pp. 597–604.

Quader H. and Bock E.: 1995, Konfokale-Laser-Raster-Mikroskopie: Eine neue Möglichkeit gesteinbewohnende Mikroorganismen zu untersuchen. *Int J Restoration of Buildings* **1**, 295–304.

Wakefield R.D. and Jones M.S.: 1996, Some effects of masonry biocides on intact and decayed stone. In: J. Riederer (ed), *Proceedings of the 8th International Congress on Deterioration and Conservation of Stone*, Vol. 2. Berlin, pp. 703–716.