their respective reaction to mechanic and chemical treatments and their role in the biodeterioration processes can only be understood as a whole and needs to be done with very little manipulation of the precious works of art. ESEM and CSLM allows for each of these conditions and has the additional advantage of avoiding pre-treatments of the samples that could give false impressions of the whole living community.

References

Introduction
The deterioration of ornamental stone, as well as other materials used both in sculpture as well as in the construction of historical buildings or in new works, has in recent times accentuated due to environmental pollution and acid rain, especially in urban and/or industrialized areas. In the last few decades, awareness has grown concerning the importance of these types of problems because, in a great number of cases, key architectonic monuments have been degraded and even lost.

During this time, conventional treatments, both inorganic was well as organic, used for the consolidation and/or impermeabilization of these deteriorated materials have yielded poor results because of the incompatibility between the stone or material treated and the nature of the material applied in the treatment. In many cases, treatments have in fact proved harmful by accelerating the alteration (Clifton 1980, Torraca 1976), (Figure 1).

An attempt to use treatments more in line with the nature of these materials has, in the last few decades, directed attention to biomaterials, generally carbonates, produced.

In the case of bacterial biomineralization, the most common process is induced biomineralization (Lowenstam and Weiner 1989), with certain exceptions (such as the production of magnetite by magnetotactic bacteria) (Bäuerlein 2003), and this process is commonly extracellular, occurring in the area surrounding the cell, often leading to the mineralization of the bacterial cells (i.e., Ehrlich 1999, Rodriguez-Navarro et al. 2007).

Bacteria can change the chemistry in their surroundings by the release of metabolites. This, together with the contribution of structures (i.e., cells, cell membranes or cell debris; González-Muñoz et al. 1996, Schultze-Lam et al. 1996) that would act as nuclei for heterogeneous crystallization, lead to mineral precipitation. Therefore, the precipitation of minerals induced by bacteria can take place under conditions in which precipitation would not occur in their absence. In addition, the types of minerals that the bacteria can produce are very diverse (i.e., calcium oxalate, silicates, calcite, apatite; Ben Omar et al. 1997) and consequently so are the materials that can be cemented (consolidated) by the deposition of a broad range of compatible minerals. Nevertheless, applied research on cementing agents has to date focussed mainly on consolidation by calcite deposition, partly because bacterial carbonatogenesis is a widespread phenomenon among bacteria, occurring through many metabolic pathways. However, for the problem at hand, it is generally accepted that bacteria suited for stone-consolidation treatments are aerobic heterotrophs or microaerophiles (Castanier et al. 2000). In general, metabolic pathways able to increase the environmental pH toward alkalinity can, in the presence of calcium ions, foster calcium carbonate precipitation.

Some authors consider that these processes represent a method to produce just a sacrificial layer, being the part most exposed to the deteriorating action of physical, chemical, and biological agents. However, other authors think that these biomineralization processes also create a calcium-carbonate cement that consolidates and protects the material being treated.

Consequently, the newly formed carbonate: 1) reduces the water permeability of the stone while maintaining its transpiration to permit gas exchange; and 2) promotes a greater cementing of the mineral particles that make up the stone, conferring it greater resistance. These processes should occur, moreover, without altering the colour of the stone and without plugging its natural pores. In this sense, noteworthy results have been achieved by Rodriguez-Navarro et al. (2003) treating calcarenite with cultures of Myxococcus xanthus, in which case the original porosity of the stone was 28% and that of the treated stone 26%, with almost no alteration of the mean pore-size distribution. With regard to stone consolidation, after the use of different culture media, treated and untreated samples exposed to five 5-min cycles of ultrasonic treatment showed that the untreated stone lost somewhat more than 0.9% of its weight while the samples treated with M. xanthus in one of the media did not reach 0.4% loss, and another remained below 0.6%. Figure 2 shows two samples of Macael marble (Almería, Spain), one untreated and the other treated, following the protocol described for calcarenite in the work of Rodríguez-Navarro et al. (2003). The newly formed calcite crystals are visibly very compact, and grow epitaxially on the substrate.
Despite of the many investigation carried out on this problem and the goodness of the results obtained, some researchers call for attention to the drawbacks that these types of treatments can have. For example, the supply of nutrient media to the stone or to the construction materials with the end of encouraging the bacterial growth of those able to produce the precipitation of CaCO₃ could promote the development of undesirable microbiota that could later harm the treated material (Perito et al. 2000).

Researchers and European projects
A great number of researchers, from one standpoint or another, work on these types of processes some of them working within the framework of a series of European projects. As representatives of different lines under development, the following ones have been chosen, from which a brief commentary will be provided:

- Research team of Adolphe, Castanier, Loubière, Le Métayer-Levrel and collaborators (French group).
- Research team of González-Muñoz and Rodríguez-Gallego (group of the Universidad de Granada, Spain).
- Group of Verstraete (Ghent University, Belgium).
- Group of Bang (South Dakota School of Mines and Technology, USA).
- Project BIOBRUSH (http://www.heritage.xtd.pl/pdf/Bio_3_May.pdf), coordinator Eric May (University of Portsmouth, UK). Within the framework of this project, the group of Claudia Sorlini (Università degli Studi di Milano, Italy).
- Project BIOREINFORCE (http://www ub.es/rpat/bioreinforce/bioreinforce.htm), coordinator Piero Tirano (Istituto por la Conservazione e la Valorizzazione dei Beni Culturali, Sesto Fiorentino, Italy). Within the framework of this project, the group of Brunella Perito and Giorgio Mastromei (Università degli Studi di Firenze, Italy).

French group. The large group of French researchers, including, among others, Adolphe, Castanier, Loubière, Le Métayer-Levrel, have been working on applying bacterial biominerilization to the consolidation of stone since the 1980s. These works have led them to develop two patents (European Patent n° 90400G97.0, Adolphe et al. 1990; French Patent n° 9505861, Castanier et al. 1995) and, based on these, have developed the process CALCITE. With these patents, they support the use of any microorganism for the restoration of stone materials, though in the CALCITE process the bacterium used is Bacillus cereus. The data offered by these authors indicate that the treatments made (e.g. in the tower of the church Saint Mèdard and in the castle walls of Champs-sur-Marne, both structures from the 12th century) have the following effects: do not notably alter colour; slightly reduce the original roughness of the stone; provide a calcite film for a few micrometers; reduce water permeability; the general biotope is not changed; the bacteria provided by the treatment are well calcified; the undesirable microbial populations decreased; the number of fungal spores are augmented (data taken from the Spanish version of the CALCITE procedure).

The works of the research group of the Universidad de Granada present treatments that improve the results shown by the French group. In this sense, a coherent carbonate cement of 10–50 µm coated the treated stones and this cement was rooted down to a depth of 1 mm while, at the same time, stone porosity remained unaltered (Rodriguez-Navarro et al. 2003) as commented above. In addition, this group has investigated the role of microbiota in stone when the treatment is applied. The culture media used by these authors activate, among the microbiota inhabiting the stone, those bacteria able to induce the precipitation of calcium carbonate. Such a precipitation notably contributes to the consolidation of the stone (Jimenez-Lopez et al. 2007). In some of these treatments, the new calcium carbonate produced was rooted down to a depth of 5 mm (unpublished data).

Group of Verstraete proposes: (a) the use of diverse species of Bacillus selected on the basis of their capacity to produce urease, of the bacterial membrane potential ζ at pH 9.0, and of the production of a biofilm (Dick et al. 2006); and (b) a treatment method that proceeds sequentially and in alternating cycles (1) production of the biofilm and (2) CaCO₃ precipitation. The application of the treatment was made on Euvile limestone (from quarries of the “Département de la Meuse” in France). These researchers conclude that “B. sphaericus strains with a very negative ζ -
potential, a high initial urea degradation, and a continuous formation of dense calcium carbonate crystals are most suitable for coherent calcite production on degraded limestone. These strains also significantly decreased capillary water absorption of the treated limestone.”

Along another line, the Group of Bang has investigated the microbiological remediation of concrete cracks and demonstrated the improvement of compressive strengths of cement mortar cubes in the presence of microorganisms and in particular reported that the “physicochemically versatile Poly Urethane has shown to be an effective enhancement tool in microbiologically induced calcite precipitation in concrete cracks” (Bang et al. 2001). In this work, they utilized B. pasteurii and suggest that the role of urease in CaCO3 precipitation is paramount.

European project BIOBRUSH (2002-2005). Some of the objectives proposed were:

1: Collection, cataloguing and analysis of stone samples from historic buildings and monuments across Europe, showing evidence of salt deposits and incrustations.
2: Selection, screening and identification of bacterial cultures for use in bioremediation.
3: Evaluation of delivery systems to carry biological agents onto the stone.
4: Assessment of mineral changes in stones during bioremediation treatment in laboratory studies.
5: Assessment of the effect of the bioremediation process on bulk-stone properties in laboratory studies with stone cubes and blocks.
6: Field trials of bioremediation on buildings and monuments in Europe.”

The work of Cappitelli et al. (2007) argues for this double use of the treatment, precisely to avoid applying a culture medium that has a complex composition in order to overcome the problems mentioned above. Nevertheless, although these authors state that they do not apply organic matter, the solution that they use is a phosphate buffer containing sodium lactate, which can be used by diverse microorganisms as a carbon source, and therefore the problem that the researchers wish to avoid is not completely eliminated. In addition, although the idea of the treatment with a double aim is a priori worthwhile, before proposing it as a practical alternative, the consolidation effect of the calcite formed in this process should be evaluated. At the moment, there is no information on how the new calcite crystals produced are attached to the surface of the pores in the weathered stone or on the degree of the stone reinforcement.

European project BIOREINFORCE (2001-2004).

“The objective of the project is to develop and validate a new methodology for monumental stones conservation based on biomineralization processes. This could satisfy the request for more durable and safer products in order to reduce the costs, delay the maintenance interventions and pose no risk both for the personnel and the environment, conciliating the end-users and stakeholders with the application of innovative treatments. The molecular biology and the bacterial genetic engineering are the innovative technologies chosen to improve the bio-mediated calcite precipitation method. These tools will be applied for finding the genetic expression of crystal formation in bacteria. This will be cloned and the bio-inducing proteins will be overproduced by an appropriate expression vector (host cell). With
these bio-derived low cost renewable macromolecules, a Bio-Mediated calcite Treatment (BMT) will be developed for the stone reinforcement, due to new calcite precipitation inside its pores. The BMT will be finally validated, by end-users, in monumental test-sites applications.”

As stated above, some authors such as Perito et al. (2000) suggest that negative processes may occur as a consequence of the application of bacterially-inoculated culture media. Therefore, in the execution of this European project, in accord with its objectives, alternative methods have been proposed and developed in what could be considered two research lines. One of these is the one followed by the group of PieroTiano. These researchers have investigated, in particular, the growth of new calcite crystals inside stone pore space with a biomineralization process induced by the “Organic Matrix Macromolecules (OMMs)” extracted from marine shells and by Polyaspartic acid (Poly A) (Tiano et al. 2006), in the absence of living cells. In their study, these authors present excellent results when the treatments are applied under laboratory conditions, but monumental test-sites have not rendered the results expected. Also, the drawbacks of applying organic matter do not appear to be avoided, since the treatments used (OMMs or Poly A) could foment the development of heterotrophic microorganisms that may use these macromolecules as a carbon, nitrogen, and energy source.

The other line, developed by the group of Brunella Perito, has sought to identify the genes involved in bacterial carbonatogenesis with the aim of large-scale production of the corresponding macromolecules for use in free applications of bacterial cultures. These authors, in a recent publication (Barabesi et al. 2007), show that Bacillus subtilis has an operon, lcfA, which is involved in calcite precipitation, and that the gene etfA is essential for this precipitation. On the other hand, as a consequence of the research in this work, they suggest the possibility of a link between calcite precipitation and fatty-acid metabolism. The extraordinary genetic work done by these authors does not, however, allow the identification of the macromolecules implied in the carbonatogenesis process. Consequently, the development of a large-scale method based on low-cost renewable macromolecules for the reinforcement of the stone is not possible at the present.

**Conclusions and new perspectives**

In consideration of the above in relation to the use of microorganisms for bioremediation, it can be stated, in agreement with Alison Webster and Eric May (2006), that “although the technology is still in its infancy and, therefore, not readily available, the results so far indicate that it promises to offer a viable alternative to those working to preserve our cultural heritage”. In addition, the research performed to date will indicate paths that are useful to follow and those that are not. The data indicate that bacterial carbonatogenesis processes are appropriate and promising for treating and reinforcing altered stone and it is advisable, in order to proceed in a suitable manner and to avoid undesirable secondary effects, to investigate in depth the following aspects: 1) nutritive solutions used as bacterial culture media, so as to help activate carbonatogenic microbiota while inhibiting the development of microbiota harmful to the stone; 2) the role of the formation of biofilm, to study the cases in which its presence might be positive and in which cases it could provoke the inappropriate loss of porosity and/or provoke undesired supply organic matter.

In addition, a consensus should be sought among researchers working in this field in order to establish both the type of characteristics or parameters that should be evaluated as well as the techniques to undertake such an evaluation. This would enable the effective comparison of the treatments applied by the different researchers. I suggest that evaluations should be made on measurement of the porosity and water uptake, surface hardness, stone cohesion, and chromatic coordinates of the stone surface. In terms of the techniques to be used, a distinction should be made between those exclusively for laboratory treatment (testing laboratory samples) and those that could be used also in the field (validation of treatments on monumental test-sites). To date, the most adequate appear to be those used by Rodriguez-Navarro et al. (2003) and Tiano et al. (2006):
Changes in stone porosity and pore-size distribution, using mercury intrusion porosimetry (Rodriguez-Navarro et al. 2003) for laboratory samples.

Water uptake, by contact sponges (Tiano et al. 2006), for laboratory and monumental site-tests.

Stone cohesion, using ultrasonic treatment (Rodriguez-Navarro et al. 2003) and by drilling resistance measuring system (DRMS) (Tiano 2006) for laboratory and monumental site-tests, respectively.

Superficial hardness using the DRMS and the peeling tape test for monumental site-tests (Tiano 2006).

Chromatic coordinates of stone surface by using Minolta Chromameter (Tiano et al. 2006), for both tests.

Finally, I would also like to mention the works that in recent years have questioned the paradigm of controlled biomineralization: i.e., DiMasi et al. (2003), Nassif et al. (2005), Yu et al. (2005). In this regard, the question of DiMasi et al. (2003) “When is template directed mineralization really template directed?” is truly provocative. I think that it is interesting to go further in this research, since it could provide new insights regarding consolidation treatments based on biomineralization processes.

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References


Until now viable cells of bacteria can be applied to remove the following alterations and undesired materials from the surfaces: (i) “sulfatation” of calcareous stones, resulting in black, brown and grey crusts; (ii) “nitratation” resulting in disgregation and pulverization; both alterations are caused by atmospheric pollutants on the stones and, in the case of “nitratation”, also by the capillary rise of water rich in nitrates in the walls; (iii) layers of organic patinas used in particular techniques of frescoes detachment from the walls; (iv) residues of synthetic polymers during conservation treatments in order to protect the works of art. (Figure 1).

The procedure used for this application consists on: (1) the selection of appropriate bacterial strains that must be effective in the alteration materials removal and safe for the works of art, operators and environment; (2) the selection of the delivery system effective to immobilise the bacteria and adapt for an easy application also to vertical surfaces and vaults; (3) to set up the procedure of delivery system application and to define the duration of the treatment; monitoring of environmental parameters during the application; (4) removal of the bacteria after the treatment and long-term monitoring of the treatment effects.

Selection of the bacteria
The bacteria used in these cases are, respectively: (a) sulfate-reducers, able to convert sulfates to hydrogen sulfide; initially, Desulfovibrio desulfuricans has been used, but subsequently it has been substituted with Desulfovibrio vulgaris subsp. vulgaris, because the latter showed a better tolerance to the oxygen; (b) nitrate-reducers, able to convert nitrates to molecular nitrogen; among the bacteria of this functional group a Pseudomonas stutzeri has been selected among the several bacteria tested; (d) aerobic heterotrophic bacteria able to attack and degrade synthetic undesired polymers. In this last case the applications are not yet performed on the real works of art.

The APPLICATION OF VIABLE BACTERIA FOR THE BIOCLEANING OF CULTURAL HERITAGE SURFACES
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Microorganisms generally are considered biodeterioration agents. Indeed they are often responsible of alteration of works of art, but they can be also used as means of conservation. In fact during the last ten years a new technology, based on the use of microorganisms to clean the surfaces of altered works of art, has been set up.

The application of this new technology to stone monuments and frescoes gave very interesting results.

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