International Biodeterioration & Biodegradation 62 (2008) 352–363

Contents lists available at ScienceDirect

International Biodeterioration & Biodegradation

journal homepage: www.elsevier.com/locate/ibiod

Consolidation of quarry calcarenite by calcium carbonate precipitation induced by bacteria activated among the microbiota inhabiting the stone

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ARTICLE INFO

Article history:
Received 29 July 2007
Received in revised form 6 March 2008
Accepted 11 March 2008
Available online 20 June 2008

Keywords:
Bacterial biomineralization
Stone consolidation
Myxococcus xanthus
Calcite
Vaterite

ABSTRACT

A new method is described to protect and to increase the consolidation of new pieces of stone that will replace damaged ones in restoration procedures, in order to make the new piece more resistant to deterioration. Culture media were applied to quarry porous limestone whose natural microbial community was not eliminated. The culture media activated bacteria belonging to the microbial community that are able to induce the precipitation of calcium carbonate. The newly produced calcium carbonate was compatible with the substrate and consolidated the stone with no pore plugging. This is the first study to propose the use of a bacterial non-inoculated culture medium in the consolidation of newly quarried stone. Application of the culture media specified in this study is proposed as a procedure for effective protection of ornamental limestone.

1. Introduction

Progressive deterioration of our built and sculptural heritage is a problem that considerable resources have attempted to address and manage (Price, 1996). In Western countries, the rationale behind cultural heritage restoration is promulgated through several charters. The most well-known, the 1964 Charter of Venice (Conti, 2002), established a three-step procedure to protect/restore a deteriorated ornamental stone: (a) prevention of damage, (b) in situ restoration/consolidation of damaged parts, and (c) replacement of the altered piece by a new one in those cases where the piece is seriously damaged and/or represents a danger to people or for the structure. The consolidation of damaged pieces has been studied much more than protection of new pieces used to substitute for seriously damaged ones. Within the context of degraded ornamental pieces of stone, there are numerous treatments based on the application of a consolidating agent to the substrate that result in the organic and/or inorganic precipitation of new cement within the porous system of the deteriorated ornamental stone (Lazzarini and Tabasso, 1980). However, the beneficial effects of such treatments have not been as extensive as expected, because of compositional and textural complexities encountered. Among these complexities are the long-term incompatibilities of the substrate and the new cement used for consolidation (Clifton, 1980), and the plugging of pores in the treated material induced by the new cement (Lazzarini and Tabasso, 1980). The chemistry, crystallography, and porosity of the new cement are often so different from those of the original stone (substrate) that, after an apparently successful restoration, within a time frame of decades or even years, the restoring cement, typically an organic resin, separates from the substrate and falls, causing damage to the stone much more intense than what would have occurred without restoration (Clifton, 1980; Lazzarini and Tabasso, 1980; Price, 1996). The observed incompatibility of most organic consolidants has prompted the search for more compatible (inorganic) and effective conservation treatments in recent years (Hansen et al., 2003).

Among such new conservation treatments, there is a promising procedure to consolidate ornamental stone that makes use of bacterially induced mineralization (Castanier et al., 2000; Rodriguez-Navarro et al., 2003; Jimenez-Lopez et al., 2007). Orial et al. (1993) and Castanier et al. (2000) tested the effectiveness of *Bacillus cereus* to protect stone and found that this bacterium was able to induce extracellular precipitation of calcium carbonate on decayed limestones. This bacterially induced calcium carbonate was compatible with the substrate and significantly reduced the water sorptivity of the treated stone (Le Métayer-Levrel et al., 1999). However, the layer of the new cement induced by *B. cereus* was very

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doi:10.1016/j.ibiod.2008.03.002

thin – only a few microns-thick. Another treatment was proposed by Rodriguez-Navarro et al. (2003), who tested a bacterial conservation method based on the use of *Myxococcus xanthus*. This method appears to be more effective than that proposed by Castanier et al. (2000). The Gram-negative, non-pathogenic, common soil bacterium *M. xanthus* belongs to the δ-subdivision of the Proteobacteria. Depending on the chemistry of the culture media, *M. xanthus* is able to induce the formation of phosphates (struvite, schertelite, newberyite: González-Muñoz et al., 1993, 1994; Ben Omar et al., 1994, 1998), carbonates (calcite, Mg-calcite, vaterite: González-Muñoz et al., 2000; Rodriguez-Navarro et al., 2003, 2007; Ben Chekroun et al., 2004) and sulfates (barite, taylorite: González-Muñoz et al., 2003). This capacity for biomineralization is of particular importance because, by creating a new cement that is compatible with the substrate, *M. xanthus* may thus be used to consolidate a wide spectrum of materials. Furthermore, *M. xanthus* displays gliding motility (McBride et al., 1993) and, being that such a motility is linked to interfaces, *M. xanthus* could then be able to colonize not only the surface of the stone, but also more deeply into the stone pores, enabling the newly formed carbonate to root in the porous system, thus increasing its consolidation efficiency. Rodriguez-Navarro et al. (2003) tested the ability of *M. xanthus* to induce calcium carbonate precipitation on sterilized porous limestone, finding that: (1) a coherent carbonate cement of 10–50 μm in thickness coated the treated stones; (2) the new cement was compatible with the substrate; and (3) this cement was rooted down to a depth of ∼1 mm, at the same time maintaining the porosity of the stone. The newly formed bacterial cement was more resistant to mechanical stress, i.e., more consolidated, than the substrate.

Despite the success of the restoration procedures, which involved the application of a bacterial inoculated culture medium, these procedures were technically problematic since they required specialized personnel/equipment to manipulate the product to optimize conditions for bacterial growth. Because the stones being treated are not sterile and may contain microorganisms that can induce the precipitation of calcium carbonate (Urzi et al., 1999), a simpler, more user-friendly restoration procedure would be to apply a sterile culture medium that could activate, from within the microbial community of the stone, only those bacteria able to induce the extracellular formation of calcium carbonate (González-Muñoz et al., 2008). Such a study was carried out by Jimenez-Lopez et al. (2007) on altered calcarenite exposed for centuries to the environment and urban contamination. In particular, the samples were pieces of a deteriorated calcarenite pinnacle of the Granada Cathedral (Spain) that was substituted during a conservation treatment. In this study, three culture media were applied to the altered calcarenite in which the microbial community inhabiting the stone was not eliminated prior to the treatment. These authors found that the application of the culture media to the stone resulted in the activation of carbonatogenic bacteria among the microbial community of the stone. As a consequence of the activation of those bacteria, a new calcium carbonate precipitation was induced that rooted into the porous system and effectively consolidated the treated stone without pore plugging.

The present paper is based on the results of Rodriguez-Navarro et al. (2003) and Jimenez-Lopez et al. (2007) and takes into account the following ideas: (1) The number of species of bacteria capable of producing calcium carbonate is high [i.e.: sulfate-reducing bacteria and cyanobacteria (Wright, 1999), *Bacillus* (Castanier et al., 2000), Myxobacteria (Ben Chekroun et al., 2004), *Halobacillus* (Rivadeneyra et al., 2004) and *Pseudomonas* (Baskar et al., 2006)]; (2) carbonatogenic bacteria are commonly found in stones collected from different locations (Urzi et al., 1999); and (3) despite the fact that the protection of quarry calcarenite used to substitute for seriously damaged stone is crucial to ensure the durability of the new piece, there are no studies on how to protect such a new quarry stone. Specifically, this study determines the effects on the consolidation of quarry and non-degraded porous limestone following the application of a culture medium to the stone whose natural microbial community has not been eliminated.

2. Materials and methods

Biomineralization experiments were conducted by immersing sterilized and non-sterilized stone slabs in a sterile culture medium that activates, among the microbiota inhabiting the stone, those microorganisms with a potential to precipitate calcium carbonate and, thus, to increase the consolidation of the stone being treated. Identical experiments were conducted immersing another set of slabs in a culture medium inoculated with *M. xanthus*. Variations in the chemistry of the culture media (pH and total aqueous calcium concentration) as well as in other parameters such as mineralogy, weight, mechanical resistance, and porosity of the treated stones, were used to evaluate the potential for calcium carbonate precipitation of the bacteria activated from the microbial community and the effects on stone consolidation of the *M. xanthus*-inoculated culture media. The ability to form calcium carbonate from individual isolates was also analyzed. Although it is being checked in experiments that go beyond the focus of the present manuscript, the procedure proposed in the present study also opens a new field for in situ protection of new pieces, where restoring solution may be applied by means of conventional restoration methods (i.e., spray).

2.1. Stone slabs, inoculum, and culture media

Quarry porous calcarenite stone from La Escribanía (Escúzar, Spain) was used for biomineralization tests. Tortonian (Tertiary) calcarenite, a porous bioclastic limestone, was used in our experiments because it is a widespread ornamental stone used since Roman times along the Mediterranean Basin (Spain, Portugal, France, Greece, Italy, etc.) and it is highly susceptible to decay once used for building and/or sculptural purposes (Rodriguez-Navarro, 1998). There are a number of quarries in Europe from which this porous limestone is extracted. All these outcrops show very similar characteristics (biofacies, depositional environment, and degree of cementation), resulting from nearly identical geological processes that culminate in the formation of a calcarenite with the compositional and textural properties that define this stone type. Collateral circumstances, such as the presence of organic matter due to heterotrophic activity in the surroundings, may vary from one outcrop to the other. Calcarenite from a representative quarry, Escúzar, located in southern Spain and important since, at least, the Roman era, was used in the present study. Southern Spain, more specifically, the area of Granada and its surroundings, is well known for its architectural and sculptural legacy, with the predominant building material, calcarenite, extracted from the Escúzar quarry. The oldest ornamental building in which the use of calcarenite from the Escúzar quarry is documented comes from the eighth century. Due to the importance of this quarry and the likelihood that new stones would come from it to replace damaged stones, this study chose to test stone from here.

Thirty-six stone slabs, $2 \times 5 \times 0.5$ cm in size, were cut out of Escúzar quarry stone using a diamond saw. Samples were weighed with a Mettler AJ 100 scale with an error of ±0.1 mg. Eighteen stone slabs were sterilized and the other 18 were not. To minimize alteration of the original stone, stone slabs were sterilized by tyndallization (heating the slabs in flowing steam, at 100 °C for 1 h, 4 days in a row, with intermediate storage at room temperature). Tyndallization is essentially a fractional method of sterilization, the theory being that vegetative cells and some spores are killed at the first heating and that the most resistant spores subsequently germinate and are killed at either the second or third heating (Sykes, 1969).

The microorganism used was *M. xanthus* (strain 422 provided by the Spanish Type Culture Collection, Burjasot, Valencia, Spain). For inoculum preparation, *M. xanthus* was cultured in liquid medium CT (Rodriguez-Navarro et al., 2003). The culture was incubated on a rotary shaker (18.85 rad s^{-1}) for 48 h at 28 °C to reach a late exponential growth phase with a cell density of $\sim 3 \times 10^8$ cells ml^{-1}.

Biomineralization tests were conducted in three liquid media: M-3 [1 wt% Bacto Casitone, 1 wt% $Ca(CH_2COO)_2 \cdot 4H_2O$, 0.2 wt% $K_2CO_3 \cdot 1/2H_2O$ in distilled water, pH 8; Rodriguez-Navarro et al., 2003]; M-3P [1wt% Bacto Casitone, 1 wt% $Ca(CH_2COO)_2 \cdot 4H_2O$, 0.2 wt% $K_2CO_3 \cdot 1/2H_2O$, 10 mM phosphate buffer in distilled water, pH 8; Rodriguez-Navarro et al., 2003]; and CC [0.3 wt% Bacto Casitone, 0.4 wt% $Ca(CH_2COO)_2 \cdot 4H_2O$, 0.1 wt% $CaCl_2$ 0.3% $NaHCO_3$, 0.1 wt% yeast extract in distilled water, pH 8; Ben Chekroun, 2000]. Solid M-3, M-3P, and CC culture media were prepared by adding 1.8 wt% agar–agar to the liquid culture media.

2.2. Experimental procedures

Fifteen 250-ml Erlenmeyer flasks were filled with 100 ml of filtered M-3 liquid culture medium. Culture medium was then sterilized by autoclaving for 20 min at 120 °C. Once sterilized, six of the Erlenmeyer flasks containing sterile M-3 were inoculated with 2 ml of *M. xanthus* inoculum culture (described in the previous section) and the remaining nine Erlenmeyer flasks were not inoculated with *M. xanthus*. The six *M. xanthus*-inoculated flasks were treated as follows. Three slabs of

sterile calcarenite were introduced into three of them (one per flask), while three slabs of non-sterile calcarenite were introduced into the remaining three. Concerning the nine Erlenmeyer flasks, three received no slabs (control), three received sterile slabs, and three received non-sterile slabs.

The same procedure was followed in all other sets of experiments with M-3P and CC culture media. All Erlenmeyer flasks were incubated in the dark at 28 °C for 30 days in a rotary shaker at 5.97 rad s^{-1}. Shaking was performed to ensure enough homogenization and aeration of the flask. Such aeration is achieved naturally when treatment is performed in situ. Evaporation rate was measured by weighing each flask every 24 h. All the slabs (both sterilized and non-sterilized) immersed in any culture media are here referred to as "treated slabs" versus "non-treated" slabs, which are raw slabs to which no treatment was given (i.e., they were not immersed in any culture media and/or sterilized). Although immersion of the damaged part is, obviously, not the procedure to follow when restoration procedures are used in situ, such a method can be applicable to protect small pieces before they are installed to substitute for damaged ones.

At predetermined time intervals (0, 1, 3, 5, 7, 10, 15, and 30 days) an aliquot of 7 ml of culture media was withdrawn from the Erlenmeyer flasks under aseptic conditions. A series of decimal dilutions were prepared from 500 μl of the sample and then a volume of 100 μl of each dilution was platted on the corresponding solid M-3, M-3P, and CC culture media by spreading the volume homogenously on the surface of the solid media. Petri dishes containing inoculated culture media were incubated at 28 °C for 4 days. Plating on solid culture media was performed to determine the size of the bacterial population. The rest of the aliquot was filtered through a 0.2-μm Millipore membrane and kept under refrigeration in sealed vials for chemical analysis. At the end of the experiment (30 days), 2 ml of the culture media were withdrawn from the flasks under aseptic conditions and centrifuged at 1571.05 rad s^{-1} for 5 min, and the pellet was stored at −80 °C for further molecular analyses of the microbiota growing in the culture media. Stone slabs were collected, rinsed twice using distilled water, and dried in an oven at 40 °C for 48 h. Samples were weighed on a Mettler AJ 100 balance with an error of ±0.1 mg.

2.3. Analyses

Solution pH was measured with a combination pH electrode (Crison micro-pHmeter 2001). Total calcium concentration in solution, $Ca_{T(aq)}$, was determined by atomic absorption spectrophotometry (AAS, Perkin–Elmer 1100B) using an air–acetylene flame atomizer. In order to prevent further precipitation of solid carbonate, samples were acidified using HCl. Based on repeated measurements, experimental error for pH measurements was ±0.05 (1σ) and for $Ca_{T(aq)}$ it was ±0.05 mM (1σ).

The weight the stone gained after treatment was calculated by comparing the weight value measured before and after the treatment for each stone slab. Error was ±0.2 mg. These measurements, along with $Ca_{T(aq)}$ data, were used to determine the existence of newly precipitated calcium carbonate on the treated stone. Moreover, the amount of newly precipitated calcium carbonate was quantified based on the measurements of the weight gained during the treatment.

The mineralogy of the new calcium carbonate precipitated on treated stone slabs was analyzed by X-ray diffraction (Philips PW1547 diffractometer, equipped with graphite monochromator and using Cu Kα radiation, $\lambda = 1.5418$ Å). Six XRD analyses were performed per slab at different locations over the surface of the slabs.

Scanning electron microscopy analyses (SEM, Leo Gemini LV 1530) were performed forboth the surfaces and cross-sections of the non-treated and treated stones. To perform SEM analyses of the surface, small fractions of the stone slabs were separated and gold-coated prior to observation by SEM. Scanning electron microscopy observations enabled the identification of bacterial carbonate cement formed on the treated stone blocks. Cross-sections of the stones were also observed by back-scattered electron imaging (BSE) with the goal of determining the thickness of the overgrowths and the depth at which the new cement was rooted in the original stone. In addition, SEM analyses were performed to observe the existence of special features that could potentially affect the consolidation and protection of the stone (i.e., presence of biofilm, massive precipitation that could plug the pores, accumulations of crystals at specific sites). The mineralogy of the newly precipitated carbonate, the degree of consolidation of the stone and/or its porosity variations were not determined based on SEM observations. Nonetheless, SEM analyses were instrumental in confirming the results of XRD, mercury intrusion porosimetry, and consolidation tests (sonication).

Consolidation tests were carried out on the remaining fractions of the treated stone slabs. Considering that there is no standard test for determining the consolidation efficiency of a bacterial conservation treatment, we chose to use the same sonication test that was successfully applied by Rodriguez-Navarro et al. (2003). These slabs were sonicated in deionized water at 5-min intervals, five times in succession (50 kHz ultrasonic bath, Ultrasons model, 200 W; J. P. Selecta). Sonication estimates both the adhesion force and the consolidation efficacy of the newly formed carbonates (Rodriguez-Navarro et al., 2003), being a laboratory analogue to wind erosion and alteration due to vibrations. Sonication removes loose carbonate grains in order to measure the real consolidation achieved in the treatment compared to that of the non-treated stone. Unlike other physical–mechanical tests, such as drilling-resistance, sonication in water also tests the resistance toward dissolution of the treated stone, an important issue considering that chemical weathering strongly contributes to the decay of carbonate stones. Note that Rodriguez-Navarro et al. (2003) have reported that bacterial carbonates are typically less soluble than carbonates in limestone due to the incorporation of organics within the former. The number of cycles and the duration of each cycle were selected with an eye to facilitating differentiation between treated and non-treated slabs. Samples were collected, dried for 24 h in an 80 °C oven, and weighed after each 5-min sonication cycle. Based on repeated measurements, analytical error wa ±7 wt%.

Changes in stone porosity and pore size distribution were studied using mercury intrusion porosimetry (MIP) (with a Micromeritics Autopore 5510 device). Samples (weighing about 2 g) were dried overnight in an oven at 80 °C prior to MIP analysis.

At the end of the experiment (30 days), 10 samples were taken from the M-3 and M-3P culture media and plated on solid M-3 and M-3P, respectively. Those samples correspond to runs containing both non-sterile stone and non-sterile stone immersed in culture medium inoculated with *M. xanthus*. Plates were incubated at 28 °C for 3 days. After that, colonies were observed with an optical microscope and all the colonies with different appearance were isolated and plated again in M-3 and M-3P culture media. Isolates were incubated at 28 °C. The production of crystals in the colonies was observed every day for up to 6 days. Once crystals were detected, the colonies were harvested and placed on an XRD sample-holder to determine the mineralogy of the precipitate.

The microbial community associated with quarry stone, as well as the bacteria activated by the application of the culture media, was studied using a culture-independent approach. The purpose of this phylogenetic study was to determine whether the activation of the microorganisms by the culture media could be potentially dangerous for stone consolidation (i.e., could acid-producing-bacteria be activated). Such analyses were performed on M-3P runs, since they induced the best consolidations on the treated stones, as described below. A more detailed study concerning the molecular characterization of the natural microbiota (identifying and comparing the natural microbiota inhabiting the stone as well as quantifying *M. xanthus* in inoculated samples) is being prepared for submission. In this subsequent paper, DGGE fingerprints, sequence analysis, and results of real-time PCR (RT-PCR) using *M. xanthus* specific primers will be reported on.

DNA extraction was performed as previously described by Schabereiter-Gurtner et al. (2001). Purified DNA was further amplified by PCR using the 16S rDNA specific bacterial primers 341f (Muyzer et al., 1993) and 907r (Teske et al., 1996). PCR reactions were carried out in 25-μl volumes containing 12.5 pmol of each primer, 200 μM of each deoxyribonucleoside triphosphate (MBI Fermentas), 2.5 μl of 10× PCR buffer (100 mM Tris–HCl, 15 mM $MgCl_2$, 500 mM KCl; pH 8.3), 400 μg ml^{-1} of bovine serum albumin (BSA) (Roche Diagnostics, Mannheim, Germany), 5% dimethylsulphoxide (DMSO), 0.5 U of Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany), made up to 25 μl with sterile water. A 1.5–2 μl aliquot of the DNA extraction was used as template DNA.

PCR was performed in a Robocycler (Stratagene, La Jolla, CA) with the following thermocycling program: 5 min denaturation at 95 °C, followed by 30 cycles of 1 min denaturation at 95 °C, 1 min annealing at 55 °C, and 1 min extension at 72 °C. A final extension step of 5 min at 72 °C was added at the end.

For denaturing gradient gel electrophoresis (DGGE) analysis a semi-nested PCR was performed by using the primer 341f GC as forward primer to which, at its end, a 40-base GC clamp was added to stabilize the melting behavior of the DNA fragments (Muyzer et al., 1993). As reverse primer the universal consensus primer 518r (Neefs et al., 1990) was used. The PCR reaction was carried out in a volume of 100 μl (2 × 50 μl) each with 3 μl of PCR product of the first round as template DNA. Cycling conditions were as described above. Eight microliters of PCR products were visualized by electrophoresis in 2% (wt vol^{-1}) agarose gels and ethidium bromide (0.5 μg ml^{-1}) staining.

For genetic fingerprinting of bacterial communities present in the stone samples, the 100 μl of PCR products, obtained by the semi-nested PCR, were precipitated with 96% EtOH, resuspended in 20 μl ddH_2O, and separated by DGGE. Gel electrophoresis was performed as described in Muyzer et al. (1993). Gels were run at a constant temperature of 60 °C, with 200 V for 3.5 h. Linear denaturant gradient of urea and formamide [100% denaturant contains 7 M urea and 40% (vol vol^{-1}) formamide] used ranged between 30 and 55%. After completion of electrophoresis gels were stained in an ethidium bromide solution and documented with a UVP documentation system.

To obtain sequence information on individual members of the bacterial communities, clone libraries were constructed by cloning 4 μl of the purified PCR product amplified with primers 341f and 907r. PCR conditions and cloning were performed as described by Schabereiter-Gurtner et al. (2001). The pTZ57R/T vector was used for cloning (InsT/Aclone™ PCR product cloning kit, MBI Fermentas). The clone libraries were screened by PCR using the standard M13 primers. Screening for different clones was carried out by comparing the migration of reamplified inserts by DGGE analyses (Schabereiter-Gurtner et al., 2001). Clones showing different positions in DGGE were sequenced as previously described by Schabereiter-Gurtner et al. (2001). The sequences were compared with known sequences using the FASTA search option (Pearson, 1994) for the European Molecular Biology Laboratory (EMBL) database to search for close evolutionary relatives.

2.4. Calculations

To calculate the generation time (g) for different bacteria grown in the experiments, *M. xanthus* and four different isolates that grew in the experiments (three of

them corresponding to *Bacillus fusiformis* and one to *Bacillus* sp.) were cultured in the liquid culture media used in the experiments. Measurements of optical density (OD) of the cultures were performed over time with a spectrophotometer (Spectronic 20D). The optical density of *M. xanthus* was measured from 0 to 180 h. Data were taken every 3 h within the first 48 h and then every 7 h until the end of the experiment. The optical density of the other four isolates was measured from 0 to 32 h every 2 h. The purpose of these analyses was to compare the growth rate of *M. xanthus* with that of the activated bacteria.

The parameter μ was calculated for each bacterium growing in each culture medium, as the slope of the curves representing optical density (as ln OD) versus time during the exponential growth phase. Such a parameter is defined as the specific growth rate or the instantaneous growth rate and determines the growth rate of the bacterial mass as a function of time. The parameter μ is inversely related to the generation time (g) through a constant (ln 2), as indicated by the following equation: $\mu = \ln 2/g$. Therefore, the longer the generation time, the smaller the growth rate of the bacterial mass (Willet et al., 1989).

3. Results and discussion

3.1. Microbial growth and identification

The culture media used in the experiments had a particular composition that had to comply with two important goals: (1) to promote the growth of those bacteria, among the microbial community inhabiting the stone, capable of inducing precipitation of calcium carbonate; and (2) to prevent the production of acids that can form due to the nature of the metabolic activity of the activated bacteria, since the production of acids would arrest the formation of calcium carbonate and/or lead to the dissolution of previously formed calcium carbonate coating, or even to the stone's dissolution. With these two objectives in mind, carbohydrates were excluded from the culture media composition, while a pancreatic digest of casein (and yeast extract in CC medium) was introduced as a source of carbon and nitrogen. Removal of carbohydrates as a source of carbon excluded the growth of microorganisms that produce organic acids as the result of the metabolism of those carbohydrates, while the growth of bacteria that use amino acids (from Bacto Casitone and/or yeast extract) as a source of carbon and nitrogen was enhanced. The latter is of particular importance, because the oxidative deamination of the amino acids results in a release of ammonia, which alkalinizes the culture media, and it is this alkalinization that then favours the formation of calcium carbonate. In addition, alkalinization limits the growth of fungi, which preferentially grow in more acidic conditions. This is important, since fungi must be avoided because they too can produce undesirable acids due to their metabolic activity (Strzelczyk, 1981). Furthermore, they could colonize the stone being treated and seriously interfere with the conservation treatment. It is worth noting that most of the methods proposed so far to consolidate ornamental stone based on the application of a bacterially inoculated culture medium use culture media containing a considerable amount of carbohydrates (Castanier et al., 2000). Interestingly, as will be discussed below, our results demonstrated that carbohydrates could be excluded from the culture media without limiting the growth of bacteria able to induce precipitation of calcium carbonate. There are other potential problems that have to be considered in the choice of culture medium. One of these problems is possible pH decreases during treatment. Such decreases may under-saturate the system with respect to calcium carbonate and result in the dissolution of the newly formed calcium carbonate or even the treated stone. Therefore, such pH decreases were prevented by the use of calcium acetate as the source of calcium in the culture media, since the acetate ion forms the acetic/acetate ion pair that acts as a buffer.

No growth was detected in the sterile culture media containing sterile stone and only the growth of *M. xanthus* was detected in *M. xanthus*-inoculated culture media containing sterile stone. This holds true for all culture media used in our experiments and indicates that tyndallization was an effective method of sterilizing the stone. No growth of fungi was detected in any culture media. In contrast, microbial growth was detected in the solid culture media inoculated with samples collected from runs containing non-sterile slabs immersed in both sterile and *M. xanthus*-inoculated culture media, thus indicating that those culture media were able to activate the bacteria belonging to the microbial community of the stone. The microbial community had a size of $\sim 10^8$–10^9 UFC ml^{-1} and no significant variation in the number of the different colonies was observed. In agreement with this observation, it is worth noting that the liquid culture media in those runs containing non-sterile stone became very dense as a consequence of the intense microbial growth and also of the homogeneous nucleation of calcium carbonate in the bulk liquid.

Phylogenetic identification of the activated bacteria was performed in order to assess whether the application of the culture media had enhanced the growth of any microorganism that could be potentially dangerous for the consolidation of the stone. The microbiota inhabiting the non-treated porous limestone had the highest score similarities phylogenetically with cultivated genera of Sphingomonadaceae (*Sphingomonas* sp. and *Novosphingobium* sp.; >97% similarities) and of Comamonadacea (*Acidovorax* sp., *Diaphorobacter* sp., and *Comamonas* sp.; >99% similarities). Highest score similarities were also affiliated with uncultured bacterium clones found in contaminated aquifer waters and with an uncultured bacterium clone inhabiting a dolomite aquifer. Another sequence was phylogenetically affiliated with the genus *Imtechium* (*Imtechium assamiensis*, 95.7% similarity), as well as with different uncultured bacteria clones related with equine faecal contamination and activating sludge.

Once the stone was treated, the activated bacteria detected in the culture media containing non-sterile stone were phylogenetically affiliated with: (1) members of the low G + C gram-positive bacterium [strain D-N(1)-3C] from sediments of the Southern Basin of lake Biwa (>99% similarity); (2) members of the Bacillales, such as Paenibacillaceae (*Brevibacillus brevis* and *Brevibacillus formosus*; similarity >99%), and Bacillaceae (halophilic microorganisms such as *Halobacillus* sp. SA-Hb3 and *Bacillus* sp. SA-B1; >99% similarities); and (3) *Oceanobacillus iheyensis* HTE831 (>99% similarity), which is an alkalophilic and halotolerant *Bacillus*-related species isolated from deep-sea sediments.

These activated bacteria are aerobic chemo-organotrophs and can grow under the physical–chemical conditions (i.e., temperature, pH, salinity) and in the culture media used in our experiments, since these bacteria can use amino acids as a source of carbon and nitrogen: for instance, PY (*O. iheyensis*; Lu et al., 2001) and nutrient agar (*B. brevis* Shida et al., 1996; *Bacillus*; Sneath, 1986). These bacteria are spore-forming, as has been described in the relevant literature: *B. brevis* (Shida et al., 1996), *Bacillus* sp. (Sneath, 1986), *O. iheyensis* (Lu et al., 2001), *Halobacillus* sp. (Piñar et al., 2001). Such an ability to form spores would allow them to resist dry conditions, thus being able to germinate upon the application of the culture media used in our experiments. These same strains were not detected in non-treated quarry porous limestone; this is probably due to the kind of potential pitfalls that molecular techniques suffer from, e.g., the possibility of missing DNA from spores during DNA extraction (Kuske et al., 1998; Laiz et al., 2003). In contrast, some strains detected in the non-treated stone were not detected when the stone was treated. This probably occurs because those strains, which are non-spore-forming, went into dormant stages and were not reactivated by the culture media (Roszak and Colwell, 1987).

It is worth noting that the bacteria activated in our experiments are commonly found in natural environments and have also been found in ornamental rocks from different locations (Urzi et al., 1999). In particular, Urzi et al. (1999) report that bacterial isolates from calcarenite samples collected at Mont Blanc Church, Noto

quarry (Siracusa, Italy), and Malta, as well as from other calcareous stones (limestone and marble), show the presence of bacteria belonging to the following genus: *Geodermatophylus*, *Pseudomonas*, *Micrococcus*, *Actinomycetes*, *Coryne*, *Micromonospora*, and *Bacillus*. Most of the bacteria isolated by Urzi et al. (1999) were able to precipitate calcium carbonate. Besides, some of the species of the *Bacillus* genus have been reported to be highly effective in the production of calcium carbonate (*B. cereus*; Castanier et al., 2000; *Bacillus thuringiensis* and *Bacillus pumilis*; Baskar et al., 2006). Other species of the genus *Halobacillus* (e.g., *H. trueperi*) play an active role in the precipitation of carbonates, such as calcite, magnesium calcite, and monohydrocalcite (Rivadeneyra et al., 2004). Therefore, none of the activated bacteria seem to be potentially dangerous for the consolidation of the stone; instead, those bacteria have the potential to produce calcium carbonate, thus contributing to that consolidation.

Comparing the microbial community identified in the non-treated quarry stone (analyzed in the present study) with that identified in the non-treated altered stone analyzed in the work of Jimenez-Lopez et al. (2007), it is worth noting that, in the altered stone, it was possible to identify microorganisms related to the quarry from which the stone was once extracted (similar to those identified in the present study) plus other microorganisms related to the environment and contamination to which the altered stone was exposed for centuries. Regarding treated stone, although the diversity of the microbial community identified in the culture medium in which the altered stone was being treated (Jimenez-Lopez et al., 2007) is greater than that identified in the culture medium in which the quarry stone was being treated (present study), all of these microorganisms are able to induce the precipitation of calcium carbonate.

The study of calcium carbonate production by the different bacteria isolated from the culture media in which the non-sterile stones were treated shows that all the isolates were able to induce the precipitation of calcium carbonate in M-3 and M-3P culture media. The only difference was in the time at which each isolate produced the crystals and the relative mass of carbonate production (Tables 1 and 2). Similar colony types were observed in the two culture media. Differences between them were related to the relative amount of crystals produced, which was higher in M-3 than in M-3P. Crystals began to show up inside the colony. During the first stages they appeared mainly at the borders, but with time, the production of calcium carbonate crystals extended to the whole colony and even to the culture media surrounding the colony (Fig. 1). Since the production of calcium carbonate crystals occurs as the result of bacterial metabolism, it will occur where metabolites are most concentrated (inside the colony) and then at any place – in the colony or even in the culture medium – where supersaturation with respect to calcium carbonate is reached due to the diffusion of those metabolites (González-Muñoz et al., 2000). The fact that all the bacteria isolated from the culture media (M-3 and M-3P) in which the non-sterile stones were immersed were able to induce the precipitation of calcium carbonate in those media is very important, since it demonstrates that (1) the application of the culture media specified in our study only activates the growth of calcium carbonate producing-bacteria among the microbial community that inhabits the stone; and (2) such an activation has a favourable role in the consolidation of the stone, as will be discussed below.

Table 1
Calcium carbonate crystal production over time by isolates from runs containing non-sterile calcarenite immersed in sterile M-3 and M-3P culture media

Isolate	Calcium carbonate production over time (days)		
	1	4	6
Nst-a	++	++	+++
Nst-b	+++	+++	+++
Nst-c	+	++	+++
Nst-e	+	++	+++
Nst-d[a]	–	–	–

–: No crystals detected.
+: Low production of crystals.
++: Moderate production.
+++: Intensive production.
[a] The isolate Nst-d produced crystals of calcium carbonate at 10 days.

Table 2
Calcium carbonate crystal production over time by isolates from runs containing non-sterile calcarenite immersed in *M. xanthus*-inoculated M-3 and M-3P culture media

Isolate	Calcium carbonate production over time (days)		
	1	4	6
Nst + Mx-a	+++	+++	+++
Nst + Mx-b	+++	+++	+++
Nst + Mx-c	+	+	+
Nst + Mx-d	+++	+++	+++
Nst + Mx-e	+	+++	+++

–: No crystals detected.
+: Low production of crystals.
++: Moderate production.
+++: Intensive production.

3.2. Evolution of the chemistry of the culture media

The pH values of sterile culture media containing no stone slabs (controls) were almost constant throughout the time course experiment. Variations were in the range of ±0.1 pH units (Fig. 2a–c). The pH values of sterile culture media containing sterile slabs varied within ±0.3 pH units and were lower than those of the controls. This was not, however, the case for runs containing sterile slabs in which culture media were inoculated with *M. xanthus*. In the latter case, pH values oscillated within the first 10–15 days and drastically rose thereafter to final values of 9.62, 9.00, and 9.69 at 30 days for M-3, M-3P, and CC runs, respectively (Fig. 2a–c). In contrast, runs containing non-sterile stone usually displayed higher pH values. The maximum pH values were observed in *M. xanthus*-inoculated culture media containing non-sterile stone, reaching final values of 9.85, 10.37, and 10.28 for M-3, M-3P, and CC culture media, respectively (Fig. 2a–c). Evaporation rates of ~0.5 ml day^{-1} were measured over the entire course of the experiments.

Only a slight decrease in $Ca_{T(aq)}$ was detected in control experiments (with the exception of M-3 culture medium), showing limited calcium carbonate precipitation. In sterile M-3 containing sterile slabs there was a decrease in $Ca_{T(aq)}$ of ~43% with respect to the initial value, while in sterile M-3P and CC containing sterile slabs, reductions in $Ca_{T(aq)}$ were below 5%. This is probably due to the higher initial calcium concentration to supersaturation in M-3 culture medium that induced heterogeneous calcium carbonate precipitation on the carbonate support, even in the absence of microorganisms. Conversely, a decrease of ~98% of the initial values was observed in runs where *M. xanthus* and/or the microbial community were present, demonstrating a highly noticeable calcium carbonate precipitation. This decrease occurred mainly within the first 15 days of the experiment, the values remaining almost constant thereafter. In summary, the decrease of $Ca_{T(aq)}$ (related to calcium carbonate precipitation) was more important in the following sequence (from higher to lower): (a) *M. xanthus*-inoculated media containing non-sterile stone; (b) sterile culture media containing non-sterile stone; (c) *M. xanthus*-inoculated media containing sterile stone; and, finally, (d) sterile culture media containing sterile stone (Fig. 2d–f). Final values for (a), (b), and (c) are nevertheless highly similar. Therefore, the presence of *M.*

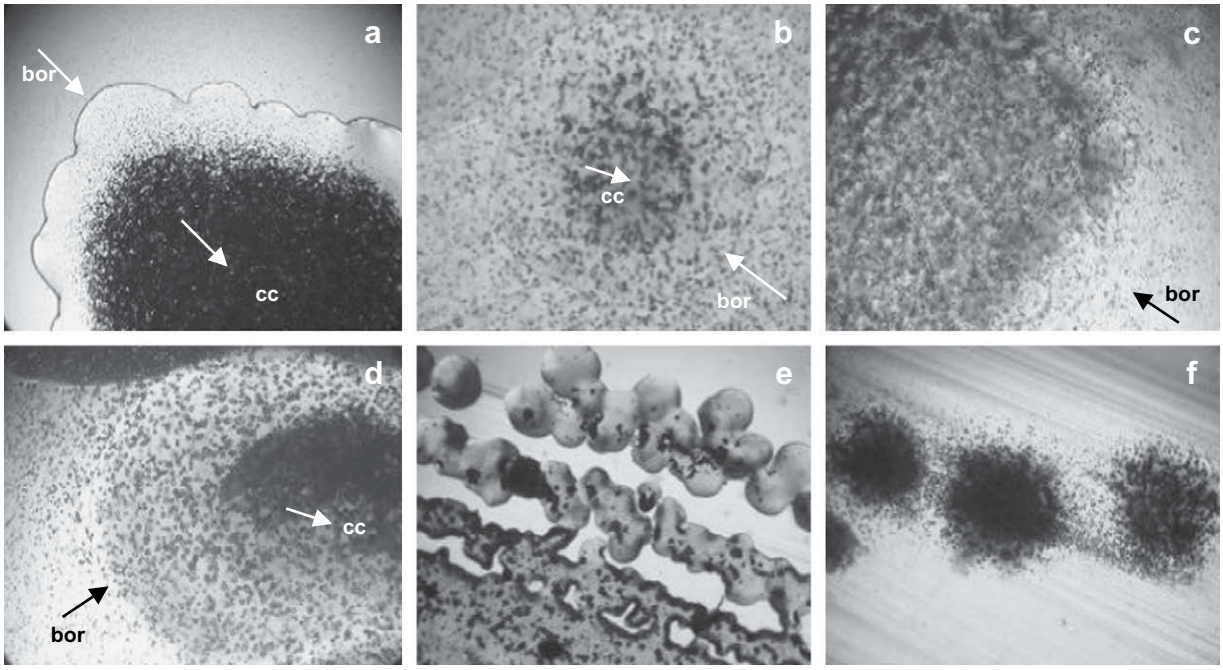

Fig. 1. - Optical microscopic images of colonies from different isolates showing calcium carbonate production. (a) Isolate Nst-a: detail of a colony with considerable calcium carbonate production; (b) isolate Nst-b: remarkable calcium carbonate precipitation within the colony and also in the surrounding culture medium; (c) isolate Nst-d: border of a colony showing an intense calcium carbonate precipitation; (d) isolate Nst + Mx-a: intense calcium carbonate precipitation in the inner and borders of a colony and even in the adjacent culture medium; (e) isolate Nst + Mx-c: colonies showing few crystals of calcium carbonate; (f) isolate Nst + Mx-d: colonies showing a remarkable production of calcium carbonate. cc = Center of the colony; bor = border of the colony. The mineralized areas within the colonies appear dark (Fig. 1a, d and f).

xanthus and/or activated bacteria from the microbial community of the stone seems to be crucial to induce a noticeable precipitation of calcium carbonate.

$Ca_{T(aq)}$ and pH data demonstrate that the activated bacteria from the microbial community induced an alkalinization (pH rise) that increased the supersaturation of the system with respect to a particular phase of calcium carbonate, resulting in the precipitation of such a phase. Although increases in pH values were observed when either *M. xanthus* or the microbial community were present, it was in their combined presence that such increases were most

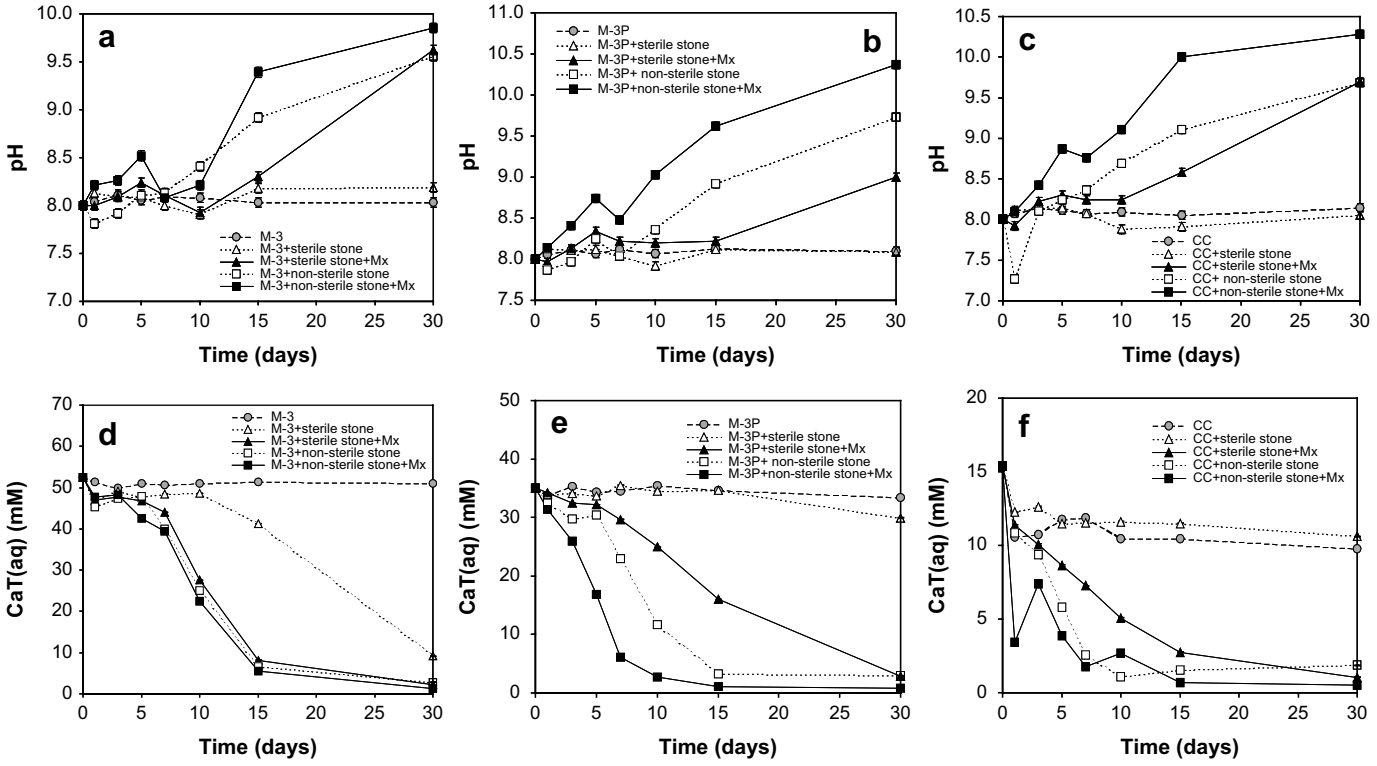

Fig. 2. - Measured pH and calcium concentration in the media during the time course of the experiments for M-3: (a) and (d), for M-3P: (b) and (e) and for CC: (c) and (f) CC culture media.

significant (Fig. 2a–c). These results suggest that between *M. xanthus* and the stone microbial community there is a cooperative effort regarding alkalinization that results, according to $Ca_{T(aq)}$ data, in the enhancement of the precipitation of calcium carbonate.

M. xanthus increases alkalinity because its metabolic activity produces CO_2 and NH_3. Extracellular ammonia release raises pH values and therefore $CO^{2-}_{3(aq)}$ concentration, according to these equilibria

$$NH_{3(g)} + H_2O \Leftrightarrow NH^{+}_{4(aq)} + OH^{-}_{(aq)} \quad (1)$$

$$CO_{2(g)} + H_2O_{(l)} \Leftrightarrow H_2CO_{3(aq)} \Leftrightarrow HCO^{-}_{3(aq)} + H^{+}_{(aq)} \Leftrightarrow CO^{2-}_{3(aq)} + 2H^{+}_{(aq)} \quad (2)$$

Precipitation of a calcium carbonate phase occurs when a sufficient supersaturation is reached with respect to this phase (Rodriguez-Navarro et al., 2007):

$$Ca^{2+}_{(aq)} + CO^{2-}_{3(aq)} \Leftrightarrow CaCO_{3(s)} \quad (3)$$

The metabolism of the activated bacteria reinforces this effect. The bacteria activated from the stone microbial community are capable of using the amino acids that are contained in the culture media, producing an oxidative deamination that results, as in the case of *M. xanthus*, in the release of NH_3. The pH of the solution may also increase due to physical factors such as evaporation (constant through the experiment) and dissolution of a solid carbonate (not observed). Therefore, the rises and decreases of pH values are due to bacterial metabolic activity and calcium carbonate precipitation, respectively.

The precipitation of calcium carbonate can be determined by the decrease in $Ca_{T(aq)}$ values over time. Such decrease would account for both heterogeneous and homogeneous calcium carbonate precipitation and for adsorption of calcium by the cells. In this context, intracellular calcium uptake by cells was shown to be almost negligible by Rosen (1987). On the contrary, it is well known that calcium may be effectively adsorbed in charged areas of the cell walls and/or extracellular polymeric substances, being that considered as a first step for heterogeneous nucleation of biologically induced calcium carbonate (Dittrich and Sibler, 2006). Such bioadsorption and the homogenous precipitation in the culture media may account for the differences observed between the mass of new precipitate calculated from the consumption of $Ca_{T(aq)}$ compared to the mass of overgrowth determined from the measurements of the weight gained for the slab during the treatment.

Interestingly, and, as occurred in the case of pH, although calcium carbonate precipitation occurred when *M. xanthus* and/or the microbial community were present in the solution, such precipitation was enhanced in the presence of both (Fig. 2–f). In all cases, the microbial community inhabiting the stone appears to favour calcium carbonate precipitation in a more efficient way than *M. xanthus*, which is probably related to the higher size of bacterial population of the former. The bigger the size of the bacterial population of activated carbonatogenic bacteria has implications in the (1) faster the supersaturation with respect to calcium carbonate is reached, and (2) the number of nuclei available for heterogeneous nucleation of calcium carbonate. Therefore, the bigger the size of the activated bacteria, the faster the optimal conditions for calcium

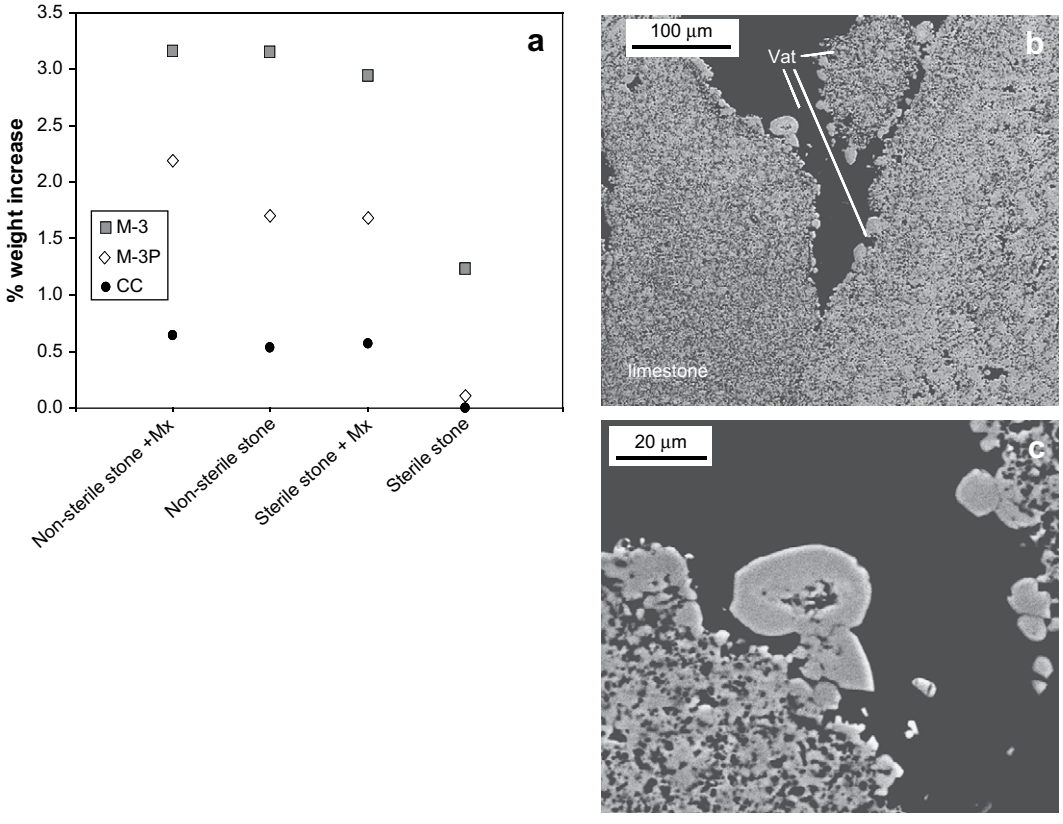

Fig. 3. (a) Weight increase of treated stone in the M-3, M-3P and CC runs. Error bars are smaller than the symbols. (b) BSE of a cross-section of the stone slab, showing the substrate (original carbonate) and an overgrowth formed by a layer of newly precipitated vaterite (vat) with a thickness ranging from 10 to 30 microns. (c) Details of Fig. 2b showing a BSE image of a newly formed vaterite globule inside a pore at a depth of 3 mm.

carbonate precipitation are reached in the media. The size of the population is related to the generation time (g). Our results indicate that *M. xanthus* has a μ ranging from 0.03 to 0.06 ($g = 11.5$–24 h), while that for the other isolates ranges between 0.19 and 0.26 ($g = 2.5$–3.6 h). Therefore, although the initial cell density of *M. xanthus* was high (10^8 cells ml^{-1}), the shorter generation time of the other bacteria growing in the culture media results in the overlap of the population size of *M. xanthus* over the course of the experiment. This result confirms the hypothesis that, at a given time, bacteria activated among the microbial community of the stone reach a greater bacterial population than that reached by only *M. xanthus*. Such a larger population could, thus, explain the higher production of calcium carbonate in the experiments where activated bacteria were present versus those in which only *M. xanthus* was present.

Regarding the effect of *M. xanthus* on sterile porous limestone, our results are in agreement with those observed by Rodriguez-Navarro et al. (2003) when working with quarry sterile stone immersed in M-3 and M-3P culture media inoculated and not inoculated with *M. xanthus*. As in Rodriguez-Navarro et al. (2003), very little calcium carbonate precipitated in M-3 and M-3P culture media not inoculated with *M. xanthus* and containing no stone slab. According to our experiments, the highest amount of precipitate was detected in M-3 runs, while the lowest occurred in CC runs. This is probably related to the initial calcium concentration in the culture media, which is maximum in M-3 (52.5 mM) and minimum in CC (15.4 mM; see Fig. 2d–f).

3.3. Analysis of the treated stone

According to XRD analyses, non-treated calcarenite slabs were more than 95% calcite with <5% quartz. Regarding treated stone slabs, XRD analyses show that vaterite precipitated on slabs immersed in M-3 and CC while only calcite precipitated in M-3P runs, irrespective of the presence of *M. xanthus*. Quantification of the amount of the precipitated vaterite (present in the overgrowth in CC and M-3 runs) was not possible, given the relatively small amount of overgrowth compared to that of the substrate and the small XRD reflectance of the vaterite.

Both the culture media and bacterial activity determine the mineral phase that precipitates (Rodriguez-Navarro et al., 2007). The culture medium influences the selection of the polymorph by setting the initial supersaturation. Calcite, the most stable of the three polymorphs of calcium carbonate, usually forms at relatively low supersaturation while vaterite precipitates at higher supersaturation, forming spherulites (Lippmann, 1973; Ogino et al., 1987; Jimenez-Lopez et al., 2001). Also, bacteria contribute to polymorph selection by increasing supersaturation as a result of their metabolic activity, thus enabling the precipitation of the polymorphic phases which require higher supersaturation to form (i.e., vaterite versus calcite), and by stabilizing vaterite. In this regard, Rodriguez-Navarro et al. (2007) observed that the incorporation of organics (cell structures, cell debris, and/or byproducts of bacterial activity) within vaterite crystals stabilizes this metastable phase, preventing its transformation into calcite during the time period studied. Nevertheless, since it has not been determined whether this vaterite may potentially transform into the most stable polymorph (calcite) at longer time periods, and since it is yet unclear if such transformation may represent a long-term problem in protection treatments, culture media that promote the precipitation of calcite (i.e., M-3P) should be chosen to protect porous limestone.

The treated stone showed in all cases a weight increase with respect to the weight of the original stone (Fig. 3a), demonstrating that a newly precipitated calcium carbonate overgrowth formed on the slabs during the treatment. Such weight increases ranged from 0.27 to 0.33 g in M-3 runs and from 0.14 to 0.23 g in M-3P runs, and reached a maximum of 0.1 g in CC runs. The higher weight increases occurred in non-sterile stone immersed in *M. xanthus*-inoculated culture media, then in non-sterile stone immersed in sterile culture media, then in sterile stone immersed in *M. xanthus*-inoculated culture media, and, finally, in sterile stone immersed in sterile culture media. These data are in accordance with those of the evolution of $Ca_{T(aq)}$. The overgrowth of the treated stone represented a 3.0–3.2% increase over the original weight of the stone in M-3 runs, a 1.7–2.3% increase in M-3P runs, and an 0.7% increase in CC runs.

Back-scattered electron imaging (BSE) of cross-sections of the treated slabs shows calcium carbonate overgrowths with an average thickness of about 50 μm in M-3 runs, of about 30 μm in M-3P runs, and of about 10 μm in CC runs. A representative BSE image showing the original calcarenite (substrate) and the overgrowth formed by a layer of newly precipitated vaterite in CC culture medium with a thickness ranging from 10 to 30 μm is shown in Fig. 3b. Such overgrowth is considerably greater than those obtained by Le Métayer-Levrel et al. (1999), which are only a few micrometers thick. Moreover, in our experiments, the new cement was rooted in the original stone down to a depth of up to 3 mm, as can be seen in detail in Fig. 3c, where newly precipitated vaterite is found in a pore of the substrate at a depth of 3 mm. Fig. 3c also shows details of the

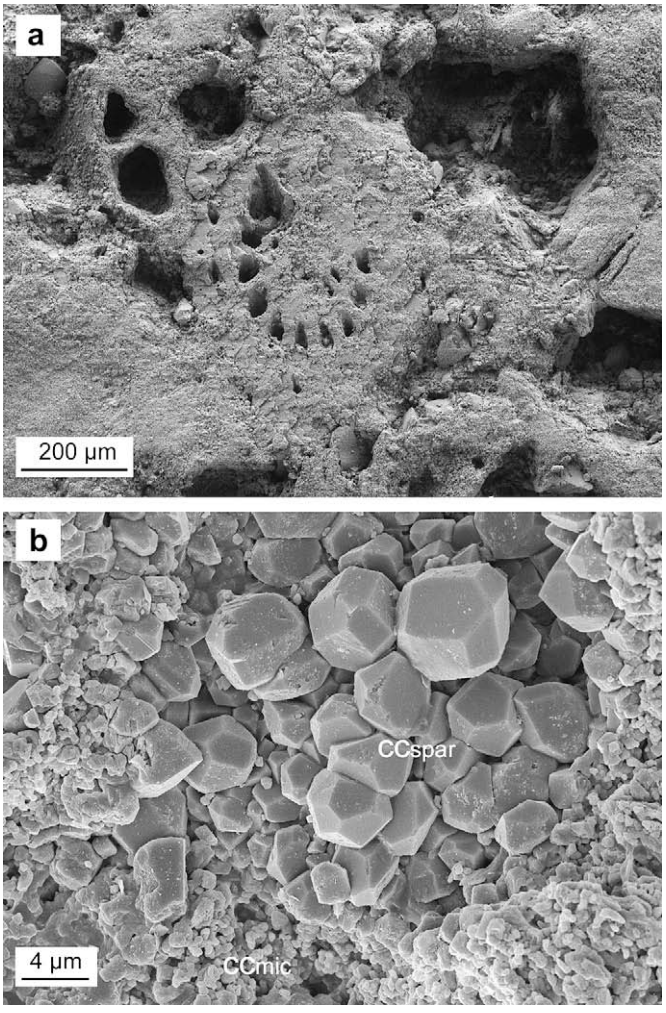

Fig. 4. - Representative SEM photomicrographs of quarry porous limestone before bacterial treatment: (a) low magnification image displaying intra-particle pores within microfossils, as well as large inter-particle pores; and (b) detail of micrite (cc_{mic}) and sparite (cc_{spar}) calcite crystals in the calcarenite.

bacterial vaterite structures, which are similar to those reported by Rodriguez-Navarro et al. (2007): i.e., they are spherulitic, hollow-core shells, the hollow representing the mold of the original bacterial cell located at the core.

Fig. 4 shows representative SEM photomicrographs of the surface of untreated stone slabs. The calcarenite is made up of calcite microfossils, and it displays large intra- and inter-particle pores (Fig. 4a). Two types of calcite crystals were observed (Fig. 4b): (a) micrite, characterized by subangular grains with size $\leq$2 μm; and (b) sparite crystals with size $\geq$4 μm. Note that most calcite crystals do not have the typical rhombohedral habit but, rather, a combination of different growth forms (Fig. 4b), a morphological feature that will help differentiate between the original calcite in the stone and the bacterial carbonates, as will be shown below. Similar morphological features were observed upon immersion of the sterile calcarenite slabs in sterile culture media (Fig. 5a–c). In the presence of *M. xanthus* both sterile and non-sterile slabs, as well as in the case of non-sterile stones immersed in sterile media,

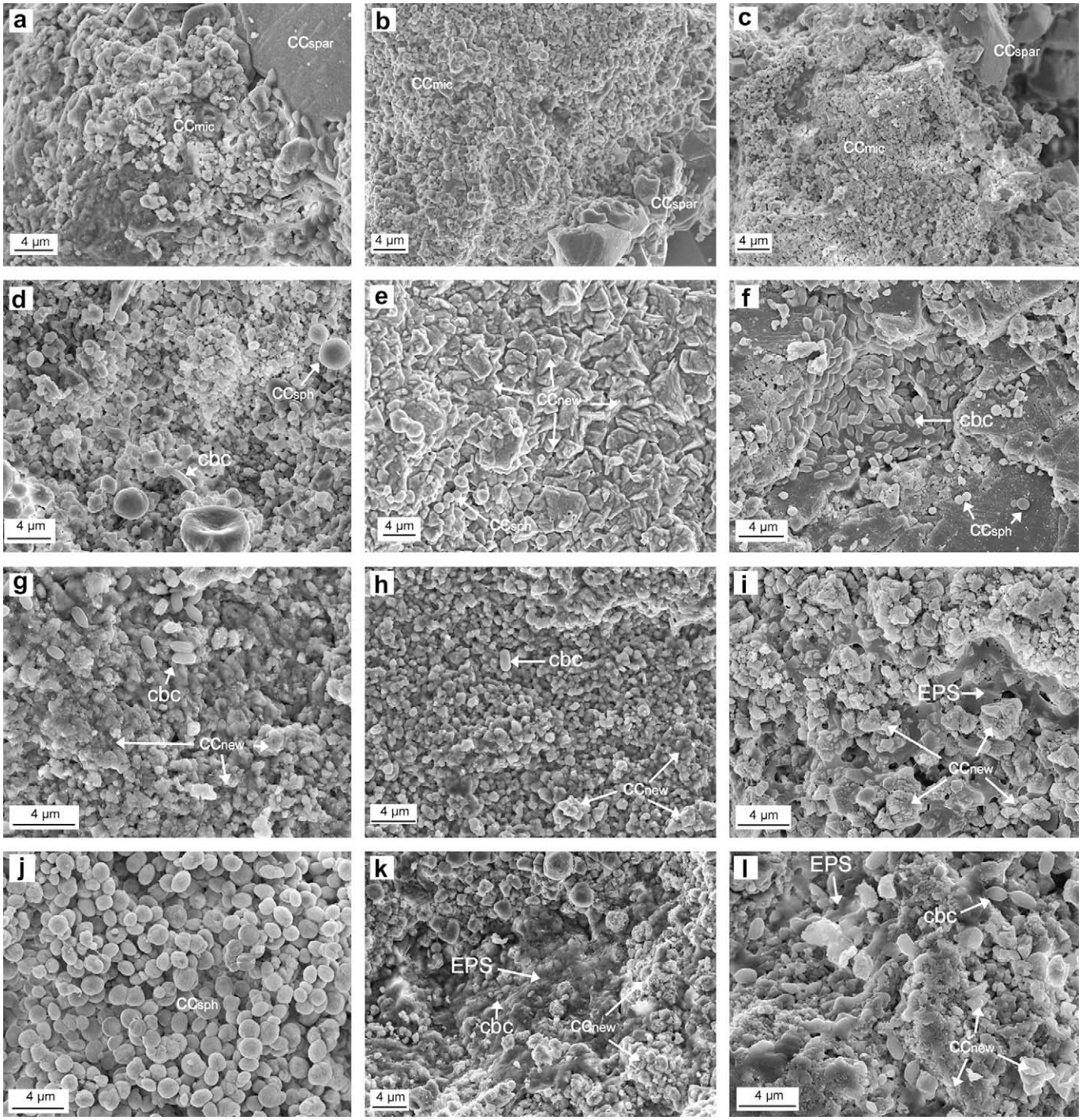

Fig. 5. SEM photomicrographs of treated sterile quarry calcarenite immersed in sterile culture media: (a) M-3, (b) M-3P (c) CC; sterile quarry calcarenite immersed in *M. xanthus*-inoculated culture media: (d) M-3, (e) M-3P, (f) CC; non-sterile quarry calcarenite immersed in sterile culture media: (g) M-3, (h) M-3P, (i) CC; and non-sterile quarry calcarenite immersed in *M. xanthus*-inoculated culture media: (j) M-3, (k) M-3P, (l) CC. Legend: cc_{spar}: sparite; cc_{mic}: micrite; cbc: calcified bacterial cells; cc_{new}: newly formed bacterial calcite rhombohedra; cc_{sph}: bacteria carbonate (vaterite) spherulites; EPS: exopolymeric substances.

carbonate overgrowths developed. Such overgrowths were made up of micron-sized aggregates of either spherulitic or rod-shaped carbonate crystals, or micron-sized carbonate rhombohedra (Fig. 5d–l). These distinctive morphological features are typically observed following bacterially induced carbonate precipitation (Rodriguez-Navarro et al., 2003, 2007; Ben Chekroun et al., 2004), and have been used to identify bacterial carbonates (Ben Chekroun et al., 2004). The carbonate overgrowth was homogenously distributed on the surface of the slabs; limited exopolysacharide (EPS) production was observed (e.g., Fig. 5i, k). Abundant spherulitic and rod-shaped crystals and scarce rhombohedra were observed in samples cultured in M-3 medium (Fig. 5d, g, and j) and CC medium (Fig. 5f, i, and l), while rhombohedral-shaped crystals were more abundant in samples cultured in M-3P medium (Fig. 5e, h, and k). That holds true irrespective of the presence of *M. xanthus* and of the pre-treatment of the stone (sterile/non-sterile). Combining these observations with XRD results and with the usual crystal habits for calcite and vaterite described in the literature (i.e., Lippmann, 1973), it may be inferred that rhombohedral crystals are probably calcite while spherules correspond to vaterite. While some bacterial carbonates are easily identified by their distinctive morphology (spherulitic or rod-shaped), identification of bacterial calcite rhombohedra formed on the calcarenite is not trivial. However, it has been observed that bacterial calcite typically appears as compact aggregates of rhombohedra with sizes ranging from 2 to 4 μm (Rodriguez-Navarro et al., 2003), similar to those depicted in Fig. 5e. Such features, plus the limited presence of biofilms covering the newly formed rhombohedra (see Fig. 5i and k), help identify bacterial carbonate cements.

Treated stone slabs were in all cases more resistant to mechanical stress (i.e., more consolidated) than non-treated stone slabs. After sonication, the weight loss of treated stones was less than 0.30%, while untreated stones lost up to 0.85% of the initial weight (here referred to as the weight at the beginning of the sonication treatment). These results show that the sole action of *M. xanthus*, the microbial community, and even the culture media alone was able to increase the mechanical resistance of the stone. Of the treated stone slabs, those sterilized prior to the precipitation experiments showed less resistance to sonication (weight losses from 0.21% to 0.30% of the initial weight, depending on the culture media) than those not previously sterilized (weight losses from 0.14 to 0.20% of the initial weight, depending on the culture media; see Fig. 6a–c). The presence of *M. xanthus* seems to be important for the consolidation of the stone in M3-P culture medium, since minimum weight losses were observed in *M. xanthus*-inoculated runs (Fig. 6b). This observation is in accordance with that of Rodriguez-Navarro et al.'s (2003) study of sterile quarry porous limestone. Our results show that weight increases of the stone were always higher than weight losses, even at the end of the sonication treatment of five cycles of 5 min of sonication per cycle. Overall, sonication test results demonstrate that the new cement created by the microbial community and/or by the combined action of the microbial

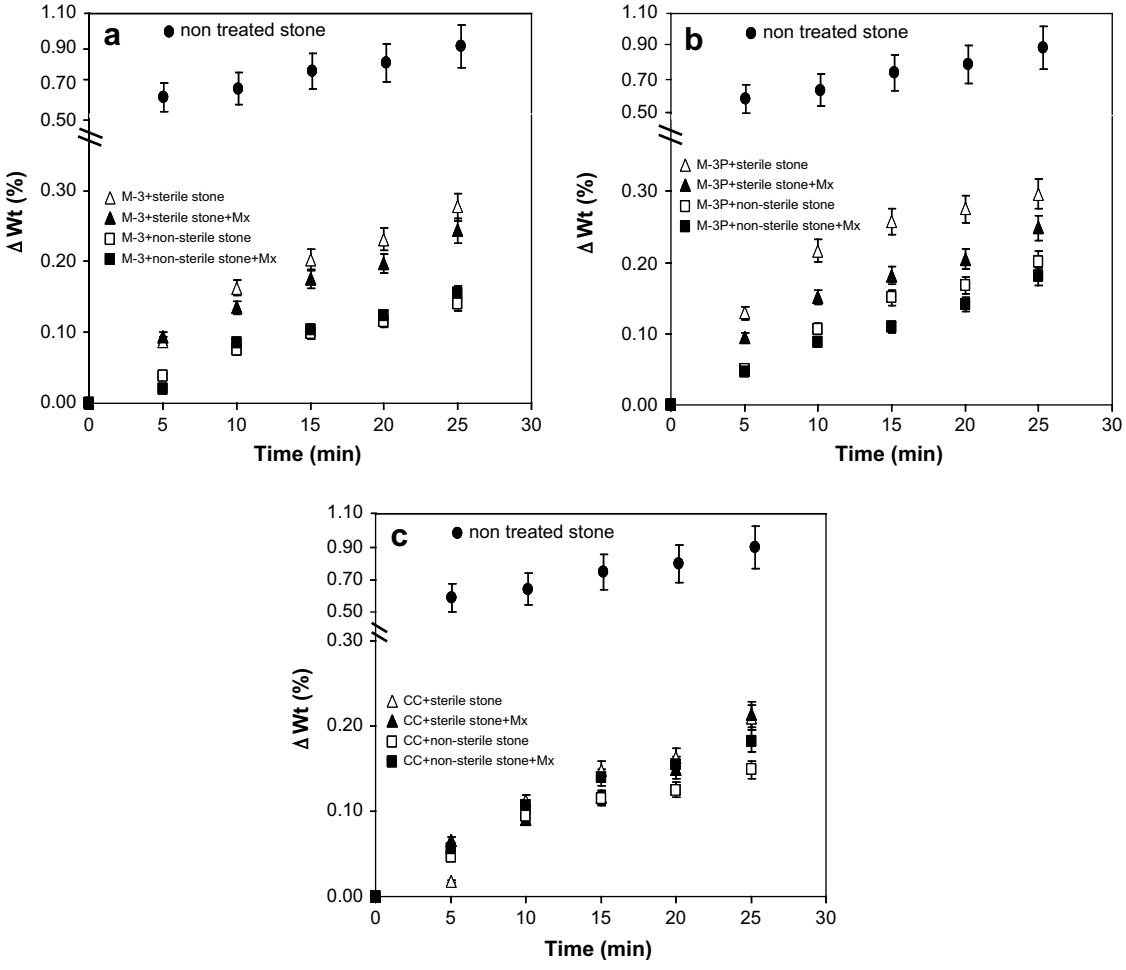

Fig. 6. - Weight loss of sterile and non-sterile porous limestone stones immersed in sterile and inoculated with *M. xanthus*: (a) M-3, (b) M-3P and (c) CC culture media. The lower the weight loss, the greater the consolidation. Also shows the weight loss of non-treated calcarenite (raw calcarenite non-treated).

community and *M. xanthus* was more resistant than that created by the sole action of either *M. xanthus* or the culture media. Moreover, the new cement was not only deposited on top of the surface, but it was rooted both in the surface and deeply into the porous system of the stone. In summary, the proposed treatment effectively consolidates the original stone, increasing its resistance to mechanical stress and to erosion, making the treated stone more durable.

One important aspect in the protection of ornamental stone is that the new cement has to maintain the porosity of the original stone (Clifton, 1980). In this regard and according to our measurements of slab porosity, treated non-sterilized slabs maintained the porosity and pore size distribution of the original stone (pore size distribution: ~0.1 μm, ~3 μm, and ~21 μm), although the size of the bigger pores was slightly reduced in those stones immersed in CC culture medium (pore size distribution: ~0.2 μm, ~3 μm, and ~17 μm). Therefore, the proper choice of culture medium is also important to ensure that the porosity of the treated stone is not altered. The fact that the non-sterilized treated stones preserve the original stone porosity is critical to being able to use the tested treatment to protect ornamental stone in situ. On the other hand, treated sterile stone showed a decrease in the size of the bigger pores when compared with that of the original stone (~0.2 μm, ~3 μm, and ~11–16 μm). Therefore, our results show that the stone sterilization process (steaming with flowing vapor) alters the stone porosity. Thus, special care must be taken in protection and/or restoration processes that include a pre-treatment of the stone to be consolidated (e.g., using standard vapor steam cleaning), to avoid excessive alteration of such stone. It is worth noting that the porosity of the stone is maintained regardless of the presence or absence of *M. xanthus*.

4. Conclusions

In summary, the results of this study show that the culture media tested were able to activate only calcium carbonate producing-bacteria within the microbial community of the porous limestone (calcarenite) tested. This activation was possible even though the culture media did not contain carbohydrates as a source of carbon. This is important, since such an exclusion of carbohydrates from the composition of the culture medium may avoid problems related to acid production. Culture media enhanced the growth of chemo-organotrophic bacteria that are able to use amino acids as a source of carbon, nitrogen, and energy and thus, induce the alkalinization of the culture media by oxidative deamination of such amino acids. The bacteria activated from the natural microbial community of the stone were able to induce the precipitation of a $CaCO_3$ cement that was compatible with the substrate. This new cement consolidated the porous limestone without pore plugging.

Although the success of this method was only demonstrated on calcarenite from the Escúzar quarry, in southern Spain, the method may also be successfully employed in stone from a wider range of locations because (1) carbonate producing-bacteria are commonly found and (2) calcarenite from different quarries have similar characteristics. This method may be relevant to the Mediterranean Basin, where calcarenite is one of the most common and widely used ornamental stones, and where physico-chemical factors are so similar from one location to another that activation and growth of similar microorganisms are enabled. The proposed method could also be applicable to carbonate stone used in modern buildings.

One of the advantages of this method is that it does not introduce any new microorganism to stone that is been treated. This may be less risky than the method used at present to consolidate ornamental stone through the application of a bacteria-inoculated culture medium (Castanier et al., 2000).

Acknowledgments

This work was financed by grants MAT 2005-03994 and MAT2006-05411 from the Spanish government (DGI). The study was supported by Research Groups BIO 103, NMR 179, and FQM 195 (Junta de Andalucía). CJL and GP wish to thank projects CGL2004-03910 and CGL2007-63859 and "Hertha-Firnberg-Nachwuchsstelle (T137)" from FWF (Austrian Science Fund), respectively. We thank the personnel of the CIC (University of Granada) for their assistance in AAS and SEM analyses. Thanks goes to Dr. Koestler for his editing of the manuscript and for his commentaries that have greatly improved the paper.

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