Consolidation of degraded ornamental porous limestone stone by calcium carbonate precipitation induced by the microbiota inhabiting the stone

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Abstract

Although it has already been shown that calcareous stone can be consolidated by using a bacterially-inoculated culture media, a more user-friendly method is the *in situ* application of a sterile culture media that is able to activate, among the microbial community of the stone, those bacteria with a potential for calcium carbonate precipitation. In order to test this new method for stone consolidation, non-sterilized decayed porous limestone was immersed in sterile nutritional media. Results were compared to those of the runs in which stone sterilized prior to the treatment was used. The effects of the microbial community on stone consolidation were determined by recording the evolution of the culture media chemistry. The treated stone was tested for mechanical resistance and porosity. Results demonstrate that the tested media were able to activate bacteria from the microbial community of the stone. As a consequence of the growth of these bacteria, an alkalinization occurred that resulted in calcium carbonate precipitation. The new precipitate was compatible with the substrate and consolidated the stone without pore plugging. Therefore, a good candidate to *in situ* consolidate decayed porous limestone is the application of a sterile culture media with the characteristics specified in the present study.

Key words : Bacterial biomineralization, stone conservation, *Myxococcus xanthus*, calcium carbonate.

1.- INTRODUCTION

Bacterially induced mineralization has recently emerged as a method for protecting and consolidating decayed ornamental stone, which offers noticeable advantages compared to traditional restoration procedures. Castanier et al. (2000) found that *Bacillus cereus* was able to induce extracellular precipitation of calcium carbonate on decayed limestones. Rodriguez-Navarro et al. (2003) tested the ability of *M. xanthus* to induce calcium carbonate precipitation on sterilized porous limestone, finding that: i) a coherent carbonate cement of 10-50 μm coated the treated stones; ii) the new cement was compatible with the substrate; and iii) this cement was rooted down to a depth of ~1 mm while at the same time stone porosity remained completely unaltered. The higher depth reached compared to Castanier´s method is probably linked to the gliding motility that displays *M. xanthus*, which allows the bacteria to move deeply into stone pores, thus promoting both surface and in-depth consolidation. Another advantage is that, in the experimental conditions tested, *M. xanthus* dies without forming a resistant stage when nutrient feeding is discontinued. This considerably reduces the probability of undesirable uncontrolled growth when the nutrient supply is accidentally restored. The newly formed bacterial cement was more resistant to mechanical stress, i.e. more consolidated, than the original carbonate. All of these advantages over traditional organic and inorganic protection and consolidation treatments have opened an array of practical options for the conservation of ornamental stone. However, no studies have yet focused on the consequences for stone consolidation of the application of an *M. xanthus*-inoculated culture medium to a degraded stone whose microbial community is not eliminated.

Moreover, decayed stones in sculptural and architectural heritage are colonized by microbial communities whose members have a potential for mineral precipitation

(Urzi et al., 1999). Microorganisms can induce extracellular precipitation of calcium carbonate through autoprophic and heterotrophic pathways (Castanier et al., 2000). In fact, it has been observed that the number of bacteria capable of producing calcium carbonate is considerably high, among others: sulphate-reducing bacteria and cianobacteria (Wright, 1999), *Bacillus* (Castanier et al., 2000; Baskar et al., 2006), Myxobacteria (Rodríguez Navarro et al., 2003) and *Pseudomonas* (Baskar et al. 2006). Therefore, a more user-friendly method to *in situ* consolidate degraded ornamental stone could be the application of a culture medium that activates, from within the microbial community, those bacteria that are able to induce the precipitation of calcium carbonate. This procedure is easier than the use of a bacterially-inoculated medium since difficulties linked to the need of a specialized person/equipment to work with microorganisms and/or or technical requirements to ensure optimal growth conditions would be avoided.

The aim of this study is to determine the effects of the application of a culture medium on the consolidation of decayed limestone whose natural microbial community has not been eliminated. To this end, sterilized and non-sterilized stone slabs were immersed in sterile culture media and in culture media inoculated with *M. xanthus*. Detailed analyses of the microbial community were not performed, since the behavior and/or the potential for carbonate precipitation of isolated individuals is not the focus of the present study and can be considered in future experiments. Our results would make it possible to design a new and more easily implemented procedure for *in situ* consolidation of decayed ornamental stone.

2.- MATERIAL AND METHODS

2.1.- Materials

The microorganism used was *M. xanthus* (strain number 422 provided by the Spanish Type Culture Collection, Burjasot, Valencia, Spain). For inoculum preparation, *M. xanthus* was cultured in liquid medium CT (Rodríguez- Navarro et al., 2003). The culture was incubated on a shaker (18.85 rad·s^{-1}) for 48 h at 28 °C to reach a cell density of ~3×10^{8} cells·mL^{-1}.

Biomineralization tests were conducted in two liquid media described in (Rodríguez- Navarro et al., 2003). Bacto Casitone (Difco) was the carbon and nitrogen source in all media.

The stone used was porous limestone collected from a large, thoroughly decayed pinnacle, once part of the Granada Cathedral complex and recently substituted during a conservation intervention. In restoration treatments, the surficial layer and black crust is often removed, thus exposing the sub-surficial layer. The stone used in this study corresponds to the sub-surficial layer of the pinnacle. XRD analyses show that the stone was >95 % calcite with < 5% quartz and gypsum. Stone slabs with 2 x 5 x 0.5 cm in size were cut out of the pinnacle using a diamond saw. Twelve stone slabs were sterilized and other twelve were not sterilized. To avoid an excessive alteration of the original stone, stone slabs were sterilized by Tyndallization. These slabs were steamed in flowing steam at 100°C for 1 hour, in Petri dishes covered with lids, four days in a row. Between the steaming steps the Petri dishes were kept at room temperature to allow the remaining endospores to germinate.

2.2.- Methods

2.2.1.- Experimental set-up and methods

A volume of 100 ml of filtered M-3 liquid culture medium were placed on 12 Erlenmeyer flasks. Culture medium was sterilized by autoclaving for 20 min at 120 °C. Once sterilized, six of the Erlenmeyer flasks were inoculated with 2 ml of *M. xanthus* culture. Three non-sterile stone slabs were immersed (one per flask) in three Erlenmeyer flasks containing *M. xanthus*-inoculated medium and other three non-sterile slabs were immersed in three Erlenmeyer flasks containing sterile M-3. Six sterile stone slabs were immersed in the remaining 6 Erlenmeyer flasks following the same distribution as the non-sterile ones. Therefore, there were three replica of each experiment. The same procedure hold true for M3-P culture medium**.** Erlenmeyer flasks were incubated at 28°C for 30 days, at 5.97 $rad \cdot s^{-1}$ (Certomat R). Evaporation rate was measured by weighting the Erlenmeyer flask every 24h interval. 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, filtered through a 0.2 μm pore size Millipore membrane and kept under refrigeration in sealed vials for chemical analysis. At the end of the experiment (30 days), a volume of two milliliters of the culture media was withdrawn from the Erlenmeyer flasks under aseptic conditions, centrifuged at 15,000 rpm for 5 minutes. After withdrawing the supernatant, the resulting pellet was stored at –80ºC for further molecular analysis 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.

2.2.2.- Chemical analyses of the culture media

Solution pH was measured with a combination pH electrode (Crison micropHmeter 2001). Total calcium concentration in solution, $Ca_{T(aq)}$, was determined by Atomic Absorption Spectrophotometry (AAS, Perkin - Elmer 1100B). In order to prevent further precipitation of solid carbonate, samples were acidified using HCl. $NH_{3(aq)}$ and phosphate concentration in the culture media was measured using the HACH DR 850 colorimeter and the Salicylate and Amino Acids methods, respectively. Based on repeated measurements, experimental error for pH was ± 0.05, for $Ca_{T(aq)}$ and $NH_{3(aq)}$ ± 0.05 mM and for phosphate ± 0.08 mM.

2.2.3.- Analyses of stone slabs

Treated stone slabs were analyzed by X-ray diffraction (Philips PW1547 difractometer). Small fractions of the stone slabs were then separated and gold-coated prior to observation by Scanning Electron Microscopy (Leo Gemini LV 1530). Consolidation tests were carried out on the remaining fractions of the treated stone slabs by means of measuring the weight loss of the stone when it was sonicated for different periods. This treatment represents wind erosion and vibrations and measures consolidation by means of the loss of small and movable grains (Rodríguez-Navarro et al., 2003). The stones were sonicated in deionized water for 5 min intervals, five times in succession (50-kHz ultrasonic bath, Ultrasons model, 200 W; J. P. Selecta). Samples were collected, dried for 24h in an 80°C oven, and weighed after each 5-min sonication cycle. Based on repeated measurements, analytical error was ± 10 %.

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

2.2.4.- Phylogenetic identification of the microbiota grown in the culture media

The autochthonous bacteria associated with altered ornamental stone was studied using a culture-independent approach. DNA extraction protocol was described by

Schabereiter-Gurtner et al. (2001). DNA was further purified using the QIAamp Viral RNA Mini Kit (Qiagen). PCR reactions were carried out in 25 µl volume containing 12.5 pmol of each primer, 200 µM of each deoxyribonucleoside triphosphate (MBI Fermentas), 2.5 µl of 10x 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% dymethylsulphoxide (DMSO) and 0.5U of Taq DNA polymerase (Roche Diagnostics, Mannheim, Germany). A volume of 1,5-2 µl of the DNA extraction was used as template DNA. PCR was performed in a Robocycler (Stratagene, La Jolla, CA).

16S rDNA of eubacteria was amplified using the primers pair 341f (Muyzer et al., 1993) and 907r (Muyzer et al., 1995). The thermocycling program was: 5 min denaturation at 95°C, 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.

Clone libraries were constructed by cloning 4 µl of the purified PCR product amplified with primers 341f and 907r. PCR products were purified by using the QIAquick PCR purification kit (QIAGEN). Cloning was performed with the pTZ57R/T Vector (InsT/Aclone™ PCR product cloning kit, MBI Fermentas). The ligation product was transformed into *E. coli* XLI-Blue and plated on LB medium containing ampicilline, tetracycline, X-Gal and IPTG (Sambrook et al., 1989).

The clone libraries were screened by PCR using the standard M13 primers. The thermocycling program was the same as aforementioned with the following variations: 35 cycles at the denaturation stage and 54°C of temperature of annealing. 8 µl of the PCR products were analysed by electrophoresis in 2% (wt·vol^{-1}) agarose gels.

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. The rDNA inserts were purified and sequenced as previously described by the afore-mentioned authors. The sequences were compared with known sequences using the FASTA search option (Pearson, 1994) for the EMBL database to search for close evolutionary relatives.

2.2.5.- Calculations

Activities and activity coefficients for all aqueous species were calculated using the EQ3/6 program (Wolery, 1992) from measured values of $Ca_{T(aq)}$, pH, phosphate and $NH_{3(aq)}$ and calculated values of alkalinity, acetate and $K^+_{(aq)}$ (24.2 mM). Carbonate alkalinity could not be measured using acid-titration methods, since the acetate present in the culture media acts as a buffer. Carbonate alkalinity and acetate concentration at each time interval were adjusted by means of charge balance. The former were also adjusted in order to comply with the condition that the pH values calculated by the program had to be identical to the pH values measured during the experiments. The amount of acetate was assumed to vary within a maximum of 5 % of the initial amount, since it has not been described that *M. xanthus* uses or produces any extracellular acetate. For runs containing sterile culture media, the concentration of acetate was considered constant over time (120.6 mM). Ion activity products (IAP) at each time interval were calculated as the product of the activity of calcium and carbonate in solution ($a_{Ca2+} \times a_{CO32-}$). Saturation state (Ω) with respect to the particular mineral phase (vaterite or calcite) is defined as: $\Omega = IAP \cdot K_{sp}^{-1}$, where K_{sp} is the solubility product ($\log K_{ps,\ vaterite} = -7.913$ and $\log K_{ps,\ calcite} = -8.48$; (Plummer and Busenberg, 1982). Error in saturation values were calculated from experimental errors in pH, $Ca_{T(aq)}$, $NH_{3(aq)}$ and phosphate and were estimated to be ± 10 %.

3.- RESULTS

Microbial growth was detected in the experiments inoculated with *M. xanthus* and in the experiments non-inoculated with *M. xanthus* containing non-sterile stone. That holds true for both culture media. The culture media in runs containing non-sterile stone became highly dense as a consequence of intense microbial growth. Measurements of optical density were not performed due to the considerable amount of crystals suspended in the culture media, which would have made it impossible to determinate the number of cells·mL^{-1}. Instead, measurements of the size of the microbial community activated by the culture media were performed by inoculating samples of the culture media taken at 30 days of each experiment on Petri dishes containing solid sterile M-3 and M3-P. Bacterial population, both in the absence and presence of *M. xanthus,* was ~ 10^8 - 10^9 UFC·mL^{-1}, that holding true for each bacterium growing in the solid culture media.

Sequence results obtained from inserted clones showed percent of similarities in between 97.7% and 99.6 % with sequences from the EMBL. Sequences obtained from non-treated stone were phylogenetically affiliated with *Sphingomonadaceae* (*Sphingomonas and Novosphingobium)*, *Imtechium assamiensis*, *Comamonadaceae* (*Acidovorax* and *Diaphorobacter*), Actinobacteria, *Corynebacterineae,* with uncultured bacterium clones inhabiting a Dolomite aquifer and with different uncultured bacterium clones related with degradation process (equine fecal contamination, contaminated groundwater and activating sludge). Sequences obtained from experiments containing non-sterilized stone were phylogenetically affiliated with cultivated members of the gamma-proteobacteria, *Moraxellaceae* (*Psychrobacter sp., Acinetobacter*) and *Pseudomonadaceae* (*Pseudomonas sp)* and with members of the Clostridiales, *Clostridiaceae* (*Alkaliphilus crotonoxidans*) and of the Bacillales, *Paenibacillaceae* (*Brevibacillus sp.*).

The pH values of the sterile culture media containing no stone slabs remained almost constant (Figs. 1a and 1b). The same holds true when the culture media contained sterile stone slabs. However, this was not the case when sterile stone slabs were immersed in *M. xanthus*-inoculated culture media. In this case, pH values oscillated within the first days of the experiments and drastically rose after 15 days to final values of 8.63 and 8.55 for M-3 and M-3P, respectively. For non-sterile stone runs, final pH values were higher compared to those corresponding to runs containing sterile stone (Figures 1a and 1b). The final pH values for the experiments containing non-sterile stones immersed in both sterile and *M. xanthus*-inoculated culture media were of ~ 8.8 for M-3 and ~ 9.4 for M-3P. In these runs, the pH increase occurred mainly after the 10-15 day of the experiments (Figs. 1a and 1b).

$Ca_{T(aq)}$ decreased throughout the experiment in all the runs for both M-3 and M-3P. The higher decreases occurred in the following sequence (higher to lower): first, on non-sterile stones immersed in *M. xanthus* inoculated culture media; second, on non-sterile stones immersed in sterile media; third, on sterile stones immersed in *M. xanthus* inoculated media and finally, on sterile stone immersed in sterile media (Figs. 1c and 1d). The amount of new calcium carbonate, measured as the difference between the initial and final values of $Ca_{T(aq)}$, was higher in M-3 culture medium compared to that in M-3P. The IAP values were about one order of magnitude higher in M-3 runs ($\sim 10^{-7}$) compared to those in M-3P runs ($\sim 10^{-8}$). Supersaturation values became lower in most experimental runs throughout the experiment. However, it is noticeable that such decreases show fluctuations, mostly within the first ten days of the experiments, slightly varying thereafter (Figs. 1e and 1f). At the end of the experiment, the lowest supersaturation values correspond to experiments containing non-sterile stone slabs, irrespectively of the culture media and/or the presence of *M. xanthus*.

Vaterite precipitated on stone slabs immersed in M-3, while calcite precipitated on stone slabs immersed in M-3P, regardeless the presence of *M. xanthus.* Little new precipitation was detected by SEM on sterile stone slabs immersed in sterile media. However, SEM observation of the sterile samples immersed in *M. xanthus*- inoculated M-3 or M-3P evidence the formation of a newly formed cement (Fig. 2b). Spherulitic or needles shaped crystals were observed in samples cultured in M-3. Rombohedra was more abundant in samples cultured in M-3P (Fig. 2b). This holds true for non-sterile stones immersed in M-3 and M-3P, both sterile and inoculated with *M. xanthus* (Figs. 2c and 2d). In this latter case, a massive precipitation of calcium carbonate was noticeable. Limited biofilm formation was detected by SEM in all experiments. Thin sections and SEM analyses of the treated slabs show overgrowths ranging within the interval 30 - 400 μm. Moreover, new cement was rooted in the original stone down to a depth of about 1-2 mm.

Treated slabs were in all cases more resistant than non-treated ones, since the former showed a maximum weight loss after sonication of 0.27 % for M-3 runs and 0.43 % for M-3P runs, while non-treated stone slabs showed weight losses within the range of 0.63 % to 0.93 % (Figs. 2e and 2f). Within treated stone slabs, those sterilized showed less resistance to sonication than those non-sterilized. The weight loss in all cases at the end of ultrasound treatment was under 40 mg, which is one order of magnitude lower than the mass of overgrowth precipitated (ranging from 200 mg to ~1 g). In M-3 runs, sterilized slabs lost 0.27 % of the initial weight while non-sterile ones lost ~0.16%, regardless the presence of *M. xanthus*. In M-3P runs, sterile stone slabs immersed in sterile culture medium lost 0.43 % of the initial weight, while those immersed in culture medium inoculated with *M. xanthus* lost 0.31 %. For non-sterile stone, the weight loss was 0.21 % regardless of the presence of *M. xanthus.*

The pore sizes and distribution of the treated stone were highly similar to that of the non-treated stone (0.1, 5 and 26 μm), regardless of the treatment procedure (culture medium, sterilization of the slab, inoculation with *M. xanthus*). A slight shift to lower pore sizes was detected in treated sterile slabs (0.2, 5, ~15 and ~20 μm) and was more noticeable in the biggest pores.

4.- DISCUSSION

Culture media were designed to potentiate bacterial growth that induces the precipitation of calcium carbonate, while at the same time avoiding, due to the nature of their metabolic activity, the production of acids that can actually dissolve the stone. With this idea, on the first place, activated microorganisms must alkalinize the culture media to create favorable conditions for calcium carbonate precipitation. With this end, bacto-casitone was introduced as a source of carbon and nitrogen, thus favoring alkalinization due to the oxidative deamination of amino acids that results in a release of CO_2 and ammonia. The release of ammonia increases the pH of the culture media creating an alkaline environment and thus favoring calcium carbonate precipitation, according to the following reactions:

$$NH_{3(g)} + H_2O \Leftrightarrow NH_4^+{}_{(aq)} + OH^-{}_{(aq)} \quad (1)$$

$$CO_{2(g)} + H_2O_{(l)} \Leftrightarrow H_2CO_{3(aq)} \Leftrightarrow HCO_3^-{}_{(aq)} + H^+{}_{(aq)} \Leftrightarrow CO_3^{2-}{}_{(aq)} + 2H^+{}_{(aq)} \quad (2)$$

$$Ca^{2+}{}_{(aq)} + CO_3^{2-}{}_{(aq)} \Leftrightarrow CaCO_{3(s)} \quad (3)$$

The probability of acid production was drastically reduced by avoiding carbohydrates as a carbon supply. The acids which the use of carbohydrates could have produced were therefore completely excluded, while the growth of the bacteria that are able to make use of amino-acids as sources of carbon and nitrogen was enhanced.

Moreover, calcium source was introduced as calcium acetate, to allow the pair acetic/acetate to form and to act as a buffer against pH decreases.

The identified stone microbial community is chemoorganotrophic and can grow in culture media containing aminoacids: nutrient agar (*Brevibacillus brevis*; Shida et al. (1997) and *Bacillus*; Sneath (1986)), TSA (*Psychrobacter*; Bozal et al. (2003) and *Pseudomonas*), SM (*Alkaliphilus*; Takai et al. (2001)) and BHI (*Acinetobacter*; Dominguez et al. (2000)). Such bacteria can also grow within the pH ranges and temperature of our experiments. Therefore, the culture media and the physical-chemical conditions of our experiments are thus compatible, not only with the growth of the afore-mentioned bacteria, but they also provided adequate conditions for the culture media alkalinization that resulted in calcium carbonate precipitation, as it was observed in our results. Some of these bacteria have been previously reported to produce calcium carbonate in other media and in nature: *Pseudomonas* and *Bacillus* (Castanier et al., 2000; Baskar et al., 2006).

Regarding the microbial community bacteria that have been identified in the non-treated stone, the habitats/characteristics of these bacteria (deep sediments, dolomite formations, degradation processes: i.e. equine fecal contaminated groundwater, aromatic hydrocarbons (*Sphingomonas*, Takeuchi et al., 1999) and polyhydroxyalkanoates (Comamonadaceae, Hiraishi and Khan, 2003) are consistent, firstly, with the geological/hydrological setting of the quarry from which our porous limestone was extracted and also, with the exposure of the ornamental stone to urban contaminants. The quarry from which the stone was extracted is infiltrated by waters previously percolated through saline strata. These waters also crosscut a nearby dolomitic formation in lateral contact and overlapping the porous limestone formation (Trias Alpujarride Dolostones). It is therefore reasonable to find microorganisms isolated from dolomite formations. Finally, the old quarry was used during an interim period as a corral yard for horses and mules, therefore, the growth of bacteria associated with fecal contaminated groundwater is plausible. The development of bacteria associated to the degradation of aromatic hydrocarbons and polyhydroxyalkanoates is also consistent due to the stone exposure to urban contaminants.

Going deeper in the alkalinization of the culture media, the effect of the activated bacteria, as well as that of *M. xanthus* can be followed by the evolution of the solution pH. Solution pH may increase as a result of: (i) bacterial metabolism, (ii) $CO_{2(g)}$ degassing and (iii) dissolution of solid carbonate, while pH values decrease as a consequence of calcium carbonate precipitation. *M. xanthus* metabolic activity produces CO_2 and NH_3. Extracellular ammonia release increases pH and, therefore, $CO_{3(aq)}^{2-}$ concentration. When a sufficient supersaturation with respect to a particular calcium carbonate phase is reached, the precipitation of such a phase is induced (Rodríguez-Navarro et al., 2003). The microbial community metabolism reinforces this effect, being that the bacteria activated in the microbial community can use aminoacids and thus induce alkalinization as a result of the NH_3 release. Finally, it is worth mentioned the strong alkalinization induced by the sole action of *M. xanthus*, which, in M-3, is comparable to that created by the added efforts of the microorganisms from the microbial community. Furthermore, other factors like the degassing of $CO_{2(g)}$ from the culture media and dissolution of the slab also may account for pH increases. Based on the measured evaporation rate (0.5 mL·day^{-1}), $CO_{2(g)}$ degassing occurred during the entire experiment while dissolution of the slab only occurred in experiments containing sterile stone immersed in sterile culture media and only within the first stages of the experiment (according to $Ca_{T(aq)}$ data). Therefore, changes in pH over time are mainly related to bacterial metabolism and calcium carbonate precipitation. The higher pH values of the M-3P experiments are related to an enhanced bacterial metabolism or cell density in this culture medium. This is in agreement with the higher ammonia concentration detected over time in M-3P (~55 mM versus ~50 mM in M-3). Moreover, the higher calcium carbonate precipitation that occurred in M-3 is also probably responsible for the lower pH values observed in this medium.

Heterogeneous precipitation of solid carbonate occurred on all slabs during the time course experiments, as indicated by the gradual decrease in $Ca_{T(aq)}$ values. The most intense precipitation of calcium carbonate that occurred in experiments containing bacterial cells can be explained by the added effects of the metabolic activity of the cells as well as by the role of bacteria as nuclei for crystallization (Rodríguez-Navarro et al., 2003). The higher the number of cells, the higher the number of crystallization nuclei and the faster the precipitation of calcium carbonate occurs. It has been shown that bacteria act as a highly reactive geochemical interface (Rodríguez-Navarro et al., 2003). Bacteria probably linked to the stone surface by their extracellular polymeric substances, which attached Ca^{2+}. Since Ca^{2+} is not likely to be used in large quantities by intracellular microbial metabolic processes (Rosen et al. 1987), it accumulates outside the cell and bonds to carbonate ions, resulting in $CaCO_3$ precipitation, both as calcite or vaterite.

Considering that vaterite has a higher solubility than the more stable calcite (Ogino et al., 1987), it should be expected that the durability of the newly formed carbonate cement and the degree of consolidation (by means of a lower loss of small and loose grains) achieved upon vaterite precipitation would be lower than that of bacterial calcite. However, our results show similar degrees of consolidation in both cases (Figs. 2e and 2f). Several parameters like stability of the new precipitate, epitaxial growth, crystal size and biofilm formation accounts for the stone consolidation.

Regarding the first parameter, vaterite is stabilized by the incorporation of organics within the new precipitate (Rodríguez-Navarro et al., unpublished results). In contrast, structural matching between bacterial calcite and the limestone substrate enables epitaxial growth while this is not the case when the new cement is vaterite. However, ripening of the new precipitate occurred and results in the formation of bigger crystal, and it is particularly important in M-3. The higher IAP values detected in M-3 culture medium compared to those of M-3P, on top of being consistent with the precipitation of vaterite in the most saturated solution (Plummer and Busenberg, 1982), can also trigger a more intense nucleation of small crystals, more unstable, that dissolve with time giving rise to larger crystals, following an Ostwald Ripening process (Ogino et al., 1987). Such effect, obviously, enhances stone consolidation by means of creating less small loose grains. The lower IAP observed in M-3P is due to the presence of phosphate cations, which compete with carbonate ions to bind with Ca^{2+}.

The Ripening process is in accordance with fluctuation in the supersaturation values in both M-3 and M-3P culture media (Figs. 1e and 1f). Supersaturation values rise as a consequence of increases in pH and alkalinity induced by bacterial metabolism and/or dissolution of previously formed calcium carbonate, while such values become lower when calcium, carbonate and/or pH decrease due to the precipitation of calcium carbonate. While the individual effects of bacterial metabolism and calcium carbonate dissolution cannot be disentangled in bacterially-bearing runs, the dissolution of a previously formed calcium carbonate and re-precipitation can be observed in the fluctuations of the saturation values in those runs containing sterile stone immersed in sterile culture media (Figs. 1e and 1f). The Ostwald Ripening process may therefore also account for the higher degree of consolidation of sterile stone immersed in sterile culture media compared to non-treated stone.

Even though the microbial community induced the most intense calcium carbonate precipitation, it is noticeable that the sole effect of *M. xanthus* accounts for the 80-85 % of the total precipitation. The inoculum size of *M. xanthus* at the beginning of the experiment (~10^6 cells·mL^{-1}) enables the precipitation of calcium carbonate since the very first stages of the experiment while bacteria from the microbial community requires a period of time to become activated by the application of the sterile culture media and to produce calcium carbonate. Therefore, the presence of *M. xanthus* could be an advantage in those restoration interventions in which time is an issue and fast formation of calcium carbonate is required. However, once bacteria from the microbial community are activated, their effects become more noticeable than that of *M. xanthus*. Being their generation time much faster than that of *M. xanthus*, at a given time, activated bacteria reach a higher cell number than that reached by *M. xanthus*, thus enhancing metabolic activity and the number of nuclei for heterogenous nucleation.

These results show that, the culture media used in this study are able to activate bacteria from the microbial community that induced the precipitation of calcium carbonate on porous limestone. Such precipitate was compatible with the limestone substrate and consolidated the stone without pore plugging. Calcium carbonate precipitation was slightly enhanced when the culture media was inoculated with *M. xanthus*. According to our experiments, the application of a culture medium with the characteristics specified in this study is a more user-friendly to the *in situ* consolidation of decayed ornamental stone than methods used so far based on the application of a bacterially-inoculated culture media.

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Figure captions

Figure 1.- Evolution over time of the pH, total aqueous calcium concentration ($Ca_{T(aq)}$) and supersaturation for a, c, e) M-3 and b, d, f) M-3P , respectively. Error bars for Figs. 1c and 1d are smaller than the symbols.

Figure 2.- SEM photomicrographs of: a) non-treated porous decayed limestone; b) detail of *M. xanthus*-induced calcite precipitated on sterile decayed porous limestone immersed in sterile M-3P; c) calcium carbonate precipitated on non-sterile porous limestone immersed in sterile M-3, and d) calcium carbonate precipitated on non-sterile porous limestone immersed in *M. xanthus*-inoculated M-3P. Weight loss after sonication of non-treated porous limestone and that treated with e) M-3 and f) M-3P both sterile and inoculated with *M. xanthus*. Wt = weight loss.

Figure 1

Figure 1

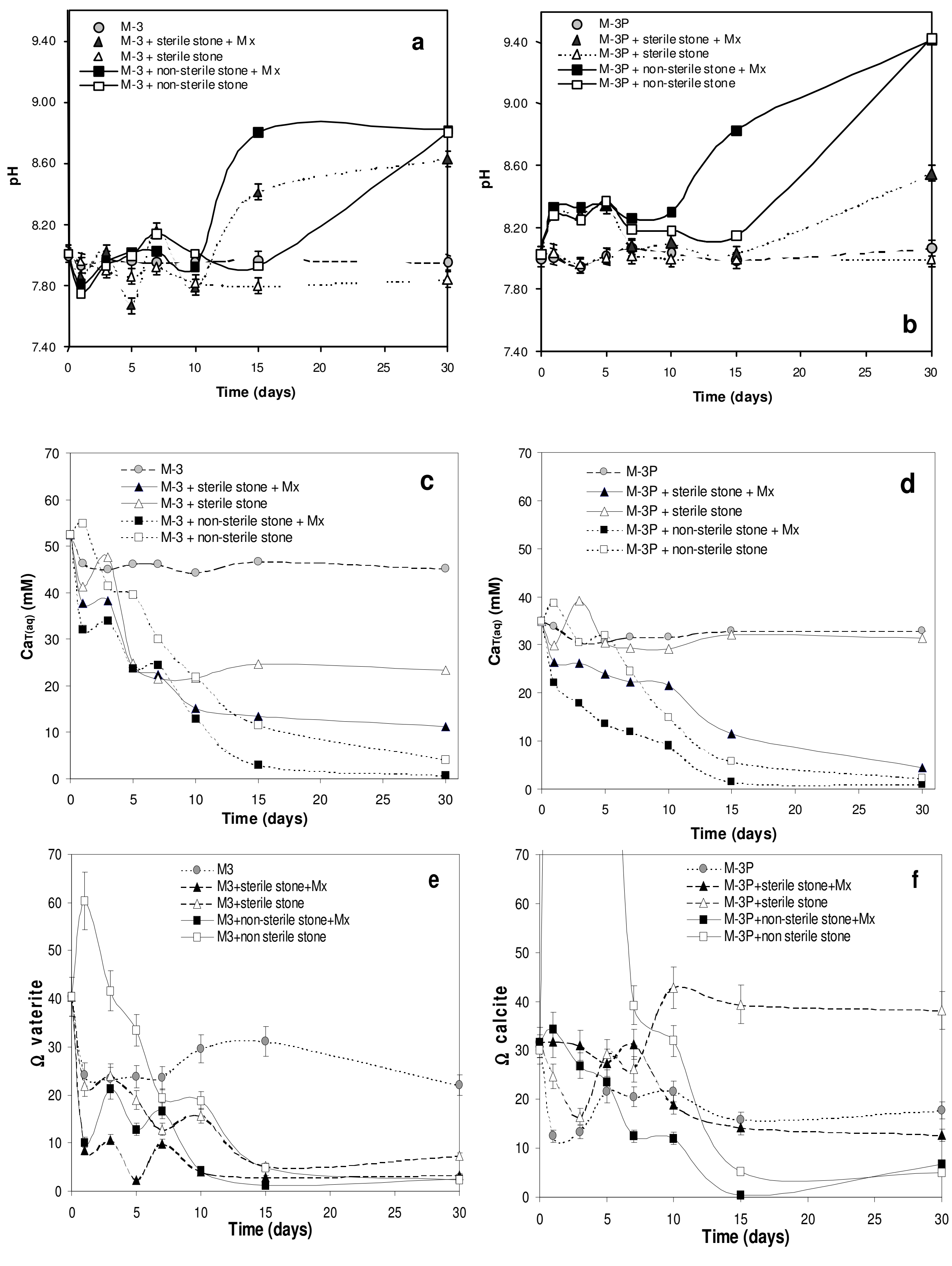

Figure 2

Figure 2