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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 medium, a more user-friendly method is the *in situ* application of a sterile culture medium 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 medium with the characteristics specified in the present study.
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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 (Tiano et al., 1999). 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 *Myxococcus 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 bacterium 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* decays without forming a resistant stage when nutrient feeding is discontinued. This considerably reduces the probability of undesirable uncontrolled growth when

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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 where microbial community is not eliminated.

Decayed stones in sculptural and architectural heritage are already colonized by microbial communities whose members have a potential for mineral precipitation (e.g. Urzi et al., 1999). Microorganisms can induce extracellular precipitation of calcium carbonate through autotrophic and heterotrophic pathways (see, e.g. Castanier et al., 2000). In fact, it has been observed that the number of bacteria taxa capable of producing calcium carbonate is considerably high, among others: sulphate-reducing bacteria and cyanobacteria (e.g. Warthmann et al., 2000), *zBacillus* (e.g. Castanier et al., 2000; Baskar et al., 2006), myxobacteria (e.g. Rodriguez-Navarro et al., 2003) and *Pseudomonas* (e.g. 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 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. 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 *Myxococcus xanthus* (strain 422, Spanish Type Culture Collection, Burjasot, Valencia, Spain). Liquid culture media used for inoculum (CT) and biomineralization experiments (M-3 and M-3P) are described in Rodriguez-Navarro et al. (2003). Inoculum 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}. Liquid M-3 and M-3P culture media were filtered through a Millipore membrane (0.4 μm) before sterilization. Solid M-3 and M-3P media were prepared by adding 2% purified agar (Difco) to filtered M-3 and M-3P.

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 are often removed, thus exposing the sub-surficial layer. The stone used in this study corresponds to the sub-surficial layer of the pinnacle. X-ray diffraction (XRD) analyses show that the stone was >95% calcite with <5% quartz and gypsum. Stone slabs with $2 \times 5 \times 0.5$ 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 (Sykes, 1969; flowing steam at 100 °C for 1 h, four d in a row; between the steaming steps the Petri dishes were kept at room temperature).

2.2. Methods

A volume of 100 ml of filtered M-3 liquid culture medium were placed in 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 six Erlenmeyer flasks following the same distribution as the non-sterile ones. Therefore, there were three replica of each experiment. The same procedure holds true for M3-P culture medium. Erlenmeyer flasks were incubated at 28 °C for 30 d, at 5.97 rad s^{-1} (Certomat R). Evaporation rate was measured by weighting the Erlenmeyer flask every 24 h interval. At predetermined time intervals (0, 1, 3, 5, 7, 10, 15 and 30 d) an aliquot of 7 ml of culture media was withdrawn from the Erlenmeyer flasks under aseptic conditions, filtered through a Millipore membrane (0.2 μm) and kept at −4 °C in sealed vials for chemical analysis. At the end of the experiment (30 d), a volume of 2 ml of the culture media was withdrawn from the Erlenmeyer flasks under aseptic conditions, centrifuged at 1570 rad s^{-1} for 5 min. After withdrawing the supernatant, the resulting pellet was stored at −80 °C 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.

2.3. Analyses

Culture media 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 (Perkin–Elmer 1100B), after acidifying the samples with 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, following the instructions of the manufacturer. Based on repeated

measurements, experimental errors were: ±0.05 for pH, ±0.05 mM for $Ca_{T(aq)}$ and $NH_{3(aq)}$ and ±0.08 mM for phosphate.

Treated stone slabs were analyzed by XRD (Philips PW1547 difractometer). Small fractions of the stone slabs were then separated and gold-coated prior to observation by scanning electron microscopy (SEM) (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 stones when they were sonicated in deionized water for 5 min intervals, five times in succession (50 kHz ultrasonic bath, Ultrasons, 200 W; J.P. Selecta), following the methodology described in Rodriguez-Navarro et al. (2003). This treatment represents wind erosion and vibrations and measures consolidation by means of the loss of small and movable grains. Based on repeated measurements, analytical error was ±10%.

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

2.4. Phylogenetic identification of the microbiota grown in the culture media

The autochthonous bacteria associated with altered ornamental stone were studied using a culture-independent approach. DNA extraction protocol was described by Schabereiter-Gurtner et al. (2001). Polymerase chain reaction (PCR) was carried out in 25 μl volume containing 12.5 pmol of each primer, a final 200 μM concentration 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 (Roche Diagnostics, Mannheim, Germany), 5% dymethylsulphoxide and 0.5 U 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).

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 Denaturing Gradient Gel Electrophoresis (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, 1990) for the European Molecular Biology Laboratory (EMBL) database to search for close evolutionary relatives.

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 those 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 ($a^{2+}_{Ca}a^{2-}_{CO3}$). Saturation state (Ω) with respect to the particular mineral phase (vaterite or calcite) is defined as: $\Omega = \mathrm{IAP}\ K^{-1}_{sp}$, 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. 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 d of each experiment on Petri dishes containing solid sterile M-3 and M3-P. Bacterial population was 10^8–10^9 colony-forming units per ml, that holding true for each bacterium growing in the solid culture media.

Bacterial 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 (i.e. equine fecal contam-

ination, contaminated groundwater). 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 (at pH 8.0) (Fig. 1a

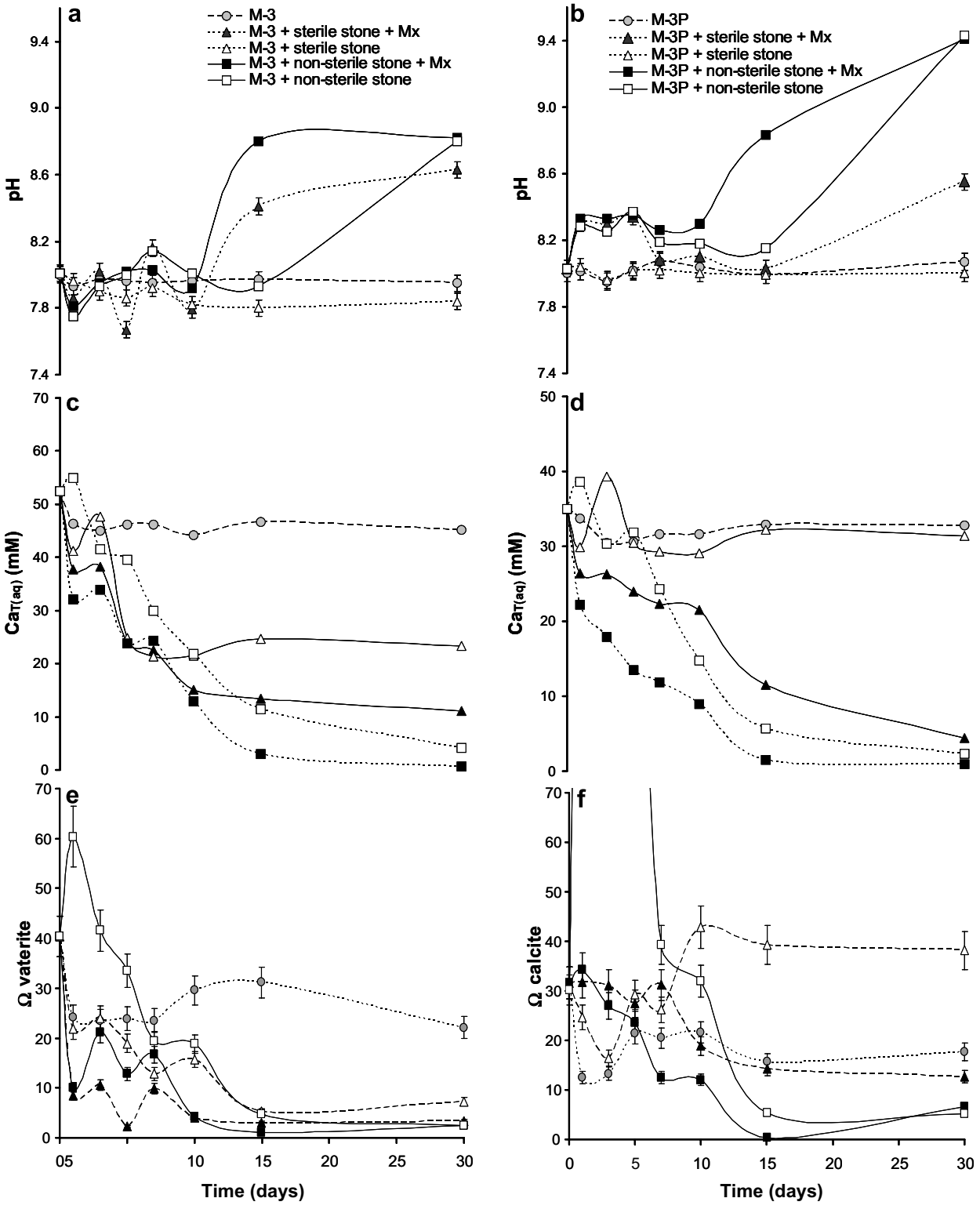

Fig. 1. Evolution over time of the pH, $Ca_{T(aq)}$ and supersaturation for the media M-3 (a, c, e) and M-3P (b, d, f), respectively. Error bars for (c) and (d) are smaller than the symbols.

and b). 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 day of the experiments and rose after 15 d 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 (Fig. 1a and b). The final pH values for the experiments containing non-sterile stones immersed in both sterile and *M. xanthus*-inoculated culture media were 8.8 for M-3 and 9.4 for M-3P. In these runs, the pH increase occurred mainly after the 10–15 d of the experiments (Fig. 1a and b).

$Ca_{T(aq)}$ decreased throughout the experiment in all runs. 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

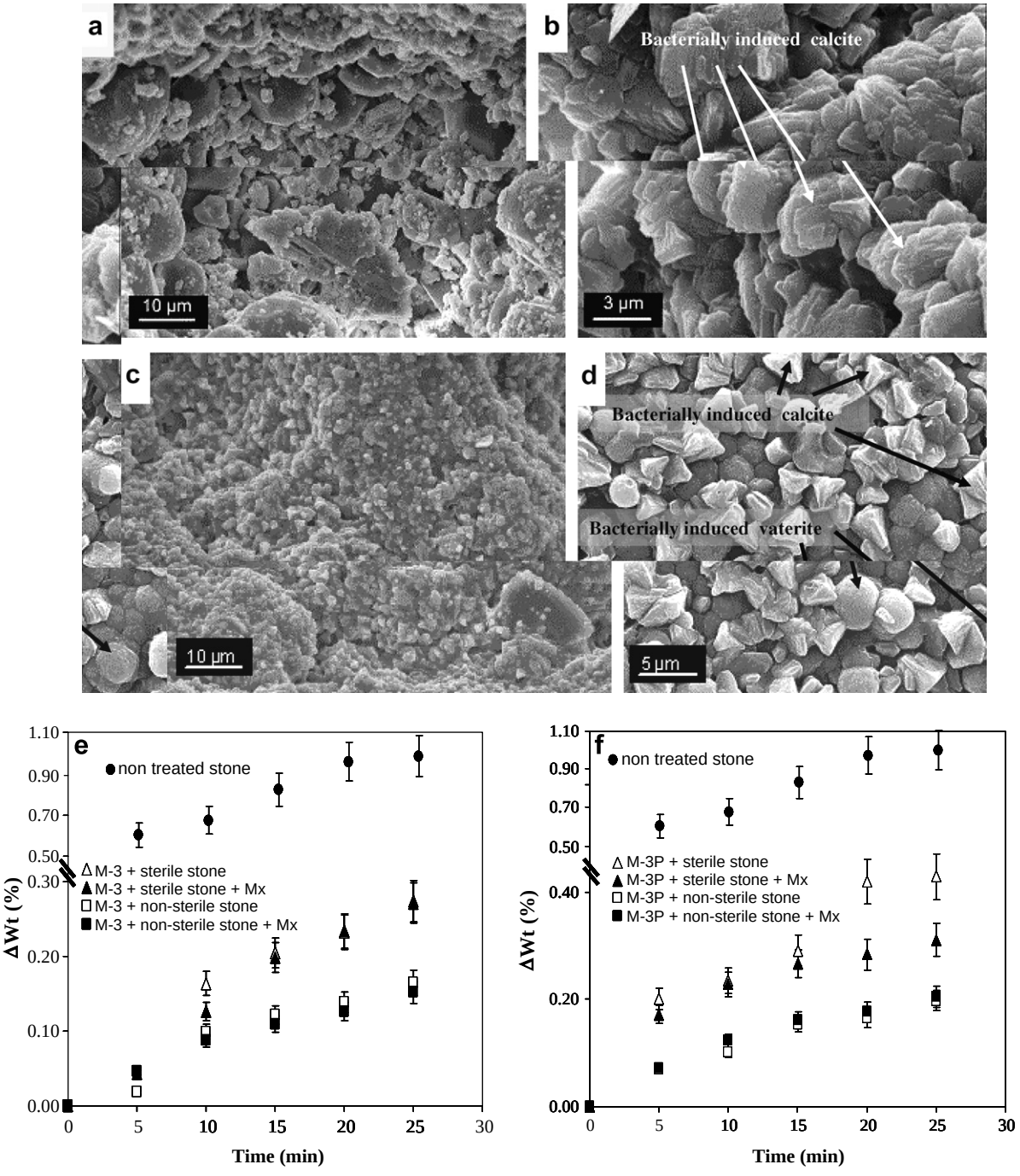

Fig. 2. SEM photomicrographs of: (a) non-treated porous decayed limestone; (b) detail of *M. xanthus*-induced calcite precipitated on sterile decayed porous limestone immersed in sterile M-3P; (c) calcium carbonate precipitated on non-sterile porous limestone immersed in sterile M-3; and (d) calcium carbonate precipitated on non-sterile porous limestone immersed in *M. xanthus*-inoculated M-3. 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.