# REGENERATIVE HYDROGEL-BASED MICROBIAL MORTARS: INVESTIGATION OF VIABILITY AND STRENGTH IN SUCCESSIVE MATERIAL GENERATIONS

S. L. Williams[1], J. Artier[2,3], J. Qiu[4], C. Heveran[4], A. Nagarajan[2,3], M. Hubler[4], S. M. Cook[4], J. C. Cameron[2,3,5], W. V. Srubar III[1,4*]

[1] Materials Science and Engineering Program, University of Colorado Boulder, 027 UCB, Boulder, CO 80309, USA

[2] Renewable and Sustainable Energy Institute, University of Colorado Boulder, 027 UCB SEEC N321, Boulder, CO 80303, USA

[3] Department of Biochemistry, University of Colorado Boulder, 596 UCB, Boulder, CO 80303, USA

[4] Department of Civil, Environmental and Architectural Engineering, University of Colorado Boulder, UCB 428 ECOT 441, Boulder, CO 80309, USA

[5] National Renewable Energy Laboratory 15013 Denver West Parkway, Golden, CO 80401, USA

*Corresponding author; e-mail: wsrubar@colorado.edu

**Abstract**

Over the past 20 years, considerable research efforts have been aimed at improving properties of cement-based materials using microbial-induced calcium carbonate precipitation (MICCP). Generally, it has been shown that MICCP can reduce permeability and increase compressive strength in these systems by filling pores and sealing microcracks. Previous studies have also interrogated the potential for embedded microorganisms to survive long-term and promote crack-sealing *in situ* during the material's service life with some success; however, it is known that the long-term viability of microorganisms in cement-based systems is threatened by harsh environmental conditions that are intrinsic to ordinary portland cement (OPC) concrete, including high pH, elevated temperature during hydration, and nutrient depletion. In this study, *Synechococcus sp.* PCC 7002, a cyanobacterium capable of MICCP, was used to create a cement-free, hydrogel-based "living" mortar. These mortars were examined for their microstructural properties, cell viability, and propensity to regenerate when subjected to temperature gradients. Scanning electron microscopy revealed that the bio-based hydrogel acted both as the primary binding agent and as a scaffold for microbial activity. Results suggested that these hydrogel-based microbial mortars generally exhibited greater property tunability and more favorable environmental conditions for beneficial microbial activity and viability than has been observed in cement-based mortars.

**Keywords:**

Microbial induced calcium carbonate precipitation (MICCP), Hydrogel, Mortar, Regeneration, *Synechococcus sp.* PCC 7002

## 1 INTRODUCTION

Microbial induced calcium carbonate precipitation (MICCP) has received interest in recent years as a possible method to improve the properties of building materials [Bundur 2015]. Specifically, MICCP has been shown to reduce permeability [Achal 2011a] and remediate cracks [Joshi 2017; Wang 2014] in concrete as well as improve the engineering properties of soil [DeJong 2006]. Although numerous diverse microorganisms are capable of MICCP, most studies to date have focused on ureolytic bacteria (e.g., *Sporosarcina pasteurii*). Ureolytic bacteria possess the urease enzyme, which catalyzes hydrolysis of urea to yield ammonia and carbon dioxide. These products can subsequently participate in acid-base reactions which serve to elevate the pH such that $CaCO_3$ precipitation is favored [Stocks-Fischer 1999].

However, more recently, researchers have examined the potential of other natural microbial processes to trigger MICCP. One such process is the carbon-concentrating mechanism (CCM) found in cyanobacteria, a unique phylum of photosynthetic bacteria that are prolific biocatalysts for carbonate mineralization. Interestingly, cyanobacteria possess two different modes for $CO_2$ utilization – $CO_2$ fixation via photosynthesis and carbonate mineralization enabled by the CCM [Kamennaya 2012]. The CCM comprises at least four $CO_2$ uptake mechanisms which can increase the intracellular $CO_2$ concentration by up to 1,000x as compared to the surrounding environment [Badger 2003]. This concentration gradient results in a localized rise in pH and supersaturation of precursor minerals at the cell exterior, both of which are favorable for MICCP.

In this study, *Synechococcus sp*. PCC 7002, a common cyanobacterium, was employed to promote MICCP in hydrogel-based mortars. Cell viability and microstructure were assessed for these mortars along with their ability to regenerate when subjected to specific temperature triggers designed to direct metabolic activity. This was accomplished by propagating *Synechococcus sp*. cells from the initial batch of mortar (along with adding abiotic ingredients) to yield three successive material generations. These regenerated mortars were also assessed for viability and microstructure, and these data were used to compare properties across the four generations of mortar.

## 2 MATERIALS

### 2.1 Microorganism, Media, and Hydrogel

Cyanobacterium *Synechococcus sp.* PCC 7002 was selected for this study, and *Tab. 1* lists the compositions of A+ and ALS-gel media. A+ media was used to grow the required density of cells prior to mortar mixing. ALS-gel medium was developed by modifying A+ medium such that it contained sufficient nutrients to maintain cell viability but also provided the necessary precursor minerals for MICCP and acted as a structural scaffold. This was accomplished by adding 0.1M $CaCl_2$•$H_2O$ and 0.1M $NaHCO_3$ to the medium as precursors for $CaCO_3$ precipitation and by adding gelatin, a bio-based hydrogel with properties that can be tuned by adjusting temperature and moisture state.

*Tab. 1: Compositions of A+ and ALS-gel media.*

| | **A+ (g/L)** | **ALS-gel (g/L)** |
|---|---|---|
| NaCl | 18.00 | - |
| $MgSO_4$•7H2O | 5.00 | 5.00 |
| $Na_2EDTA$•$2H_2O$ | 0.03 | 0.03 |
| KCl | 0.60 | 0.60 |
| $NaNO_3$ | 1.00 | 1.00 |
| $KH_2PO_4$ | 0.05 | 0.05 |
| Tris HCl (pH 8.2) | 1.00 | 1.00 |
| **$CaCl_2$•$2H_2O$** | **0.37** | **14.70** |
| **$NaHCO_3$** | **-** | **8.40** |
| **Gelatin** | **-** | **100.00** |
| *Trace components* | | |
| $H_3BO_3$ | 0.11 | 0.11 |
| $ZnCl_2$ | 0.06 | 0.06 |
| $MoO_3$ | 0.69 | 0.69 |
| $C_6H_8FeNO_7$ | 0.09 | 0.09 |
| $MnCl_2$ | 5.72 | 5.72 |
| $CuSO_4$ | 13.30 | 13.30 |
| $CoCl_2$ | 2.60 | 2.60 |

### 2.2 Sand

A local river sand (Boulder, CO, USA) was used as the fine aggregate for the gelatin mortars. Prior to mixing, the sand was washed once with diluted hydrochloric acid and then several more times with DDI water until the pH stabilized. The washed sand was dried in an oven at 50°C for at least 24 hours, after which it was stored at ambient conditions.

## 3 METHODS

### 3.1 Sample Preparation and Conditioning

*Preculture*

A preculture protocol was established to ensure consistent cell viability and $CaCO_3$ precipitation in the mortars. *Synechococcus sp.* was grown at 37°C, 200-rpm, and 180-µE in 50-mL of A+ media in 125-mL flasks until the late exponential growth phase was reached (*Fig. 1A*). At this time, the cells were harvested by centrifugation (*Fig. 1B*) at 4300 x g for 12 minutes and resuspended in 50-mL batches of ALS-gel media in 125mL flasks at a concentration of $OD_{750}$ = 0.3. These flasks were then incubated at 37°C, 200-rpm, and 180-µE for an additional 10 hours (*Fig. 1C*) to allow sufficient time for MICCP to occur prior to mixing.

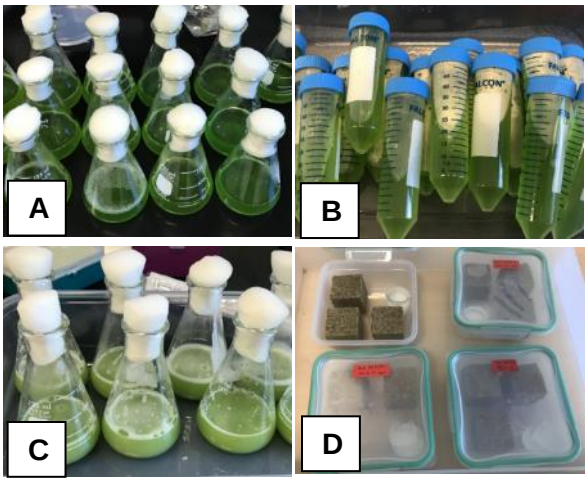


*Fig. 1: Photographs from 0th generation mortar casting. Preculture:* Synechococcus sp. *(A) in A+ medium in the late exponential growth phase, (B) immediately prior to centrifugation, and (C) immediately after incubation in ALS-gel medium. (D) 0th generation mortar cubes stored at either 50% or 100% relative humidity.*

*Gelatin Mortars: 0th generation*

Two types of gelatin mortars were examined:

1. Cyanobacterial mortar: ALS-gel medium containing *Synechococcus sp.* cells mixed with sand;
2. Control mortar: ALS-gel media without cells mixed with sand.

At a binder-to-sand ratio of 0.3 (v/w), ingredients were mixed semi-continuously by hand for one hour before casting in 2x2in cube molds at 4°C. Mortar cubes were demolded after 8 hours and stored at 4°C in either 50% or 100% relative humidity (RH) chambers (*Fig. 1D*).

*Gelatin Mortars: Regeneration*

To interrogate whether 7-day old cyanobacterial mortar cubes could exhibit regenerative behavior when subjected to temperature gradients, three subsequent "generations" of specimens were fabricated according to the following protocol:

1. High-Temp Trigger: Mortar cubes designated for regeneration were placed individually into 500-mL beakers and incubated at 37°C and 200-rpm for one hour. During this time, the contents of the beakers were melted until they

resembled a medium viscosity liquid-sand suspension

2. Liquid Abiotic Addition: 54-mL of ALS-gel medium were added to each beaker.
3. Biotic Propagation: The beakers were incubated at 37°C, 200-rpm, and 180-µE for 6 hours. The doubling time of *Synechococcus sp.* PCC 7002 has been reported in the range of 3-4 hours depending upon the growth conditions [Ludwig 2011]. Thus, 6 hours was considered sufficient for propagation of the biotic component.
4. Solid Abiotic Addition: 180g of sand were added to each beaker.
5. Low-Temp Trigger: Regenerated mortars were cast in 2x2in cube molds at 4°C. Cubes were demolded after 8 hours and stored at 4°C at either 50% or 100% RH.

### 3.2 Most Probable Number (MPN) Viability

The MPN method was utilized to quantify *Synechococcus sp.* PCC 7002 viability in cyanobacterial mortar cubes. For the 0$^{th}$ generation, viability was measured at 0, 7, 14, and 30 days. For the 1$^{st}$, 2$^{nd}$, and 3$^{rd}$ generations, viability was measured at 0 and 7 days. To obtain suitable aliquots for the MPN assay, whole mortar cubes were individually placed in 500-mL beakers and incubated at 37°C and 200-rpm for one hour until they resembled a liquid-sand suspension. Test tubes with A+ media were inoculated from the beakers and serially diluted from $10^{-1}$ to $10^{-4}$. Replicates (n=5) were prepared at each dilution rate. The test tubes were incubated at 37°C, 200 rpm, and 180 µE for seven days, at which time positive (i.e., green) tubes were counted. These data were then used to obtain MPN values and 95% confidence intervals from standard tables [Blodgett 2018]. The MPN assay was also performed on 0$^{th}$ generation control mortar cubes at 30 days to eliminate the possibility of "false positive" tubes that might occur due to contamination.

### 3.3 Scanning Electron Microscopy (SEM)

Microstructure of the gelatin mortars was evaluated using a JEOL JSM-6480LV SEM. Mortar cubes were fractured with a pestle; the fracture surfaces were mounted on carbon tape and sputter-coated with a 10-nm coating of platinum. The SEM was operated at an accelerating voltage of 5kV, working distance of 6mm, and a spot size of 30. Fractured mortar surfaces were visualized at 100x and 3,000x for each sample.

## 4 RESULTS AND DISCUSSION

### 4.1 MPN Viability

To accomplish the "biotic propagation" step in the regeneration protocol, it was imperative to show that *Synechococcus sp.* cells could remain viable inside the material matrix for an extended period of time. From *Fig. 2*, approximately 69% of the initial inoculum survived up to 14 days in cyanobacterial mortar kept at either 50% or 100% RH. Moreover, approximately 9% of the initial inoculum survived up to 30 days in cyanobacterial mortar kept at 50% RH, and 14% of the initial inoculum survived up to 30 days in cyanobacterial mortar kept at 100% RH.

It was also necessary to assess cell viability retention in the subsequent generations of material to determine whether the "biotic propagation" step could be repeated. *Fig. 3A* and *Fig. 3B* show that a considerable percentage of the initial inoculum of *Synechococcus sp.* remained viable in 0$^{th}$, 1$^{st}$, 2$^{nd}$, and 3$^{rd}$ generation mortar cubes for up to 7 days at 50% and 100% RH, respectively.

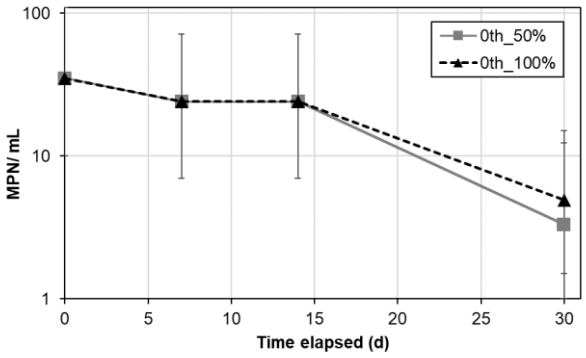


*Fig. 2: Most probable number viability of* Synechococcus sp. PCC 7002 *in 0$^{th}$ generation gelatin mortar cubes at 0, 7, 14, and 30 days. Specimens were stored at 4°C and either 50% or 100% RH.*

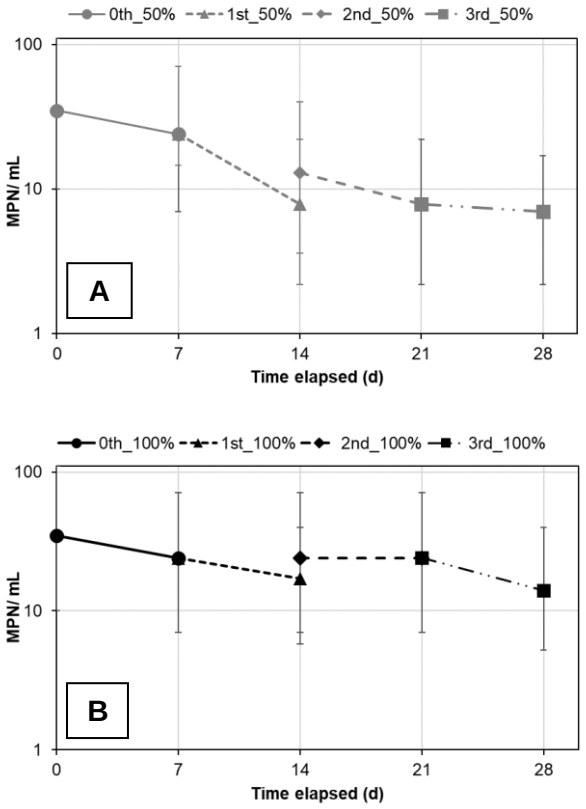


*Fig. 3: Most probable number viability of* Synechococcus sp. PCC 7002 *in 0$^{th}$, 1$^{st}$, 2$^{nd}$, and 3$^{rd}$ generation gelatin mortar cubes at 0 and 7 days. Specimens were stored at 4°C and either (A) 50% or (B) 100% relative humidity.*

It should be noted that the viability retention reported herein of *Synechococcus sp*. in cement-free, cyanobacterial mortars (*Figs. 2 and 3*) is considerably greater than similar cell viability retention data that has been reported in the literature for cement-based materials. This could be attributed to harsh conditions inside cement paste that are intrinsic to the material, including high pH, elevated temperature during hydration, and nutrient depletion. Achal [2011b] reported that just 0.1% of vegetative (i.e., metabolically active) *Bacillus megaterium* cells remained viable for an

in aged bacterial mortar; similarly, Bundur [2017] reported that 0.4% of vegetative *Sporosarcina pasteurii* cells remained viable in bacterial cement paste at 28 days. Jonkers [2006, 2011] examined the viability of endospores (i.e., metabolically inactive) in bacterial mortar and reported that just 2% of the initial inoculum were detected in mortar mixed with either *Bacillus cohnii* or *Bacillus halodurans* at 10 days, while 7% of the initial inoculum were detected in mortar mixed with *Bacillus pseudofirmus* at 10 days.

### 4.2 SEM

SEM was utilized to compare microstructural characteristics between control and cyanobacterial mortars in the 0$^{th}$ generation as well as to compare the 3$^{rd}$ generation cyanobacterial mortars to the 0$^{th}$ generation cyanobacterial mortars.

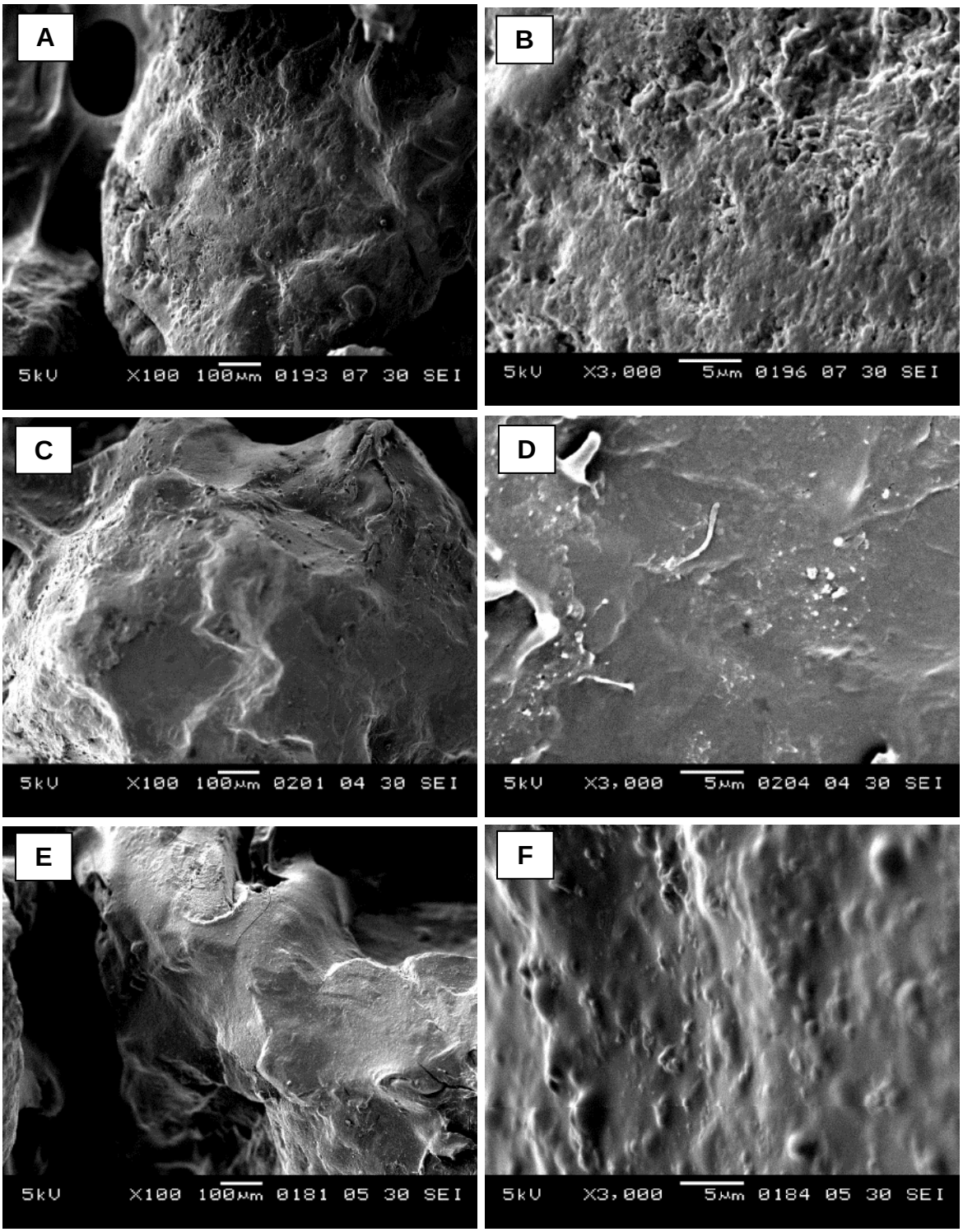


Fig. 4: SEM images of 0$^{th}$ generation control mortar at (A) low magnification and (B) high magnification; 0$^{th}$ generation cyanobacterial mortar at (C) low magnification and (D) high magnification; 3$^{rd}$ generation cyanobacterial mortar at (E) low and (F) high magnification. Specimens were stored at 4°C and 50% RH.

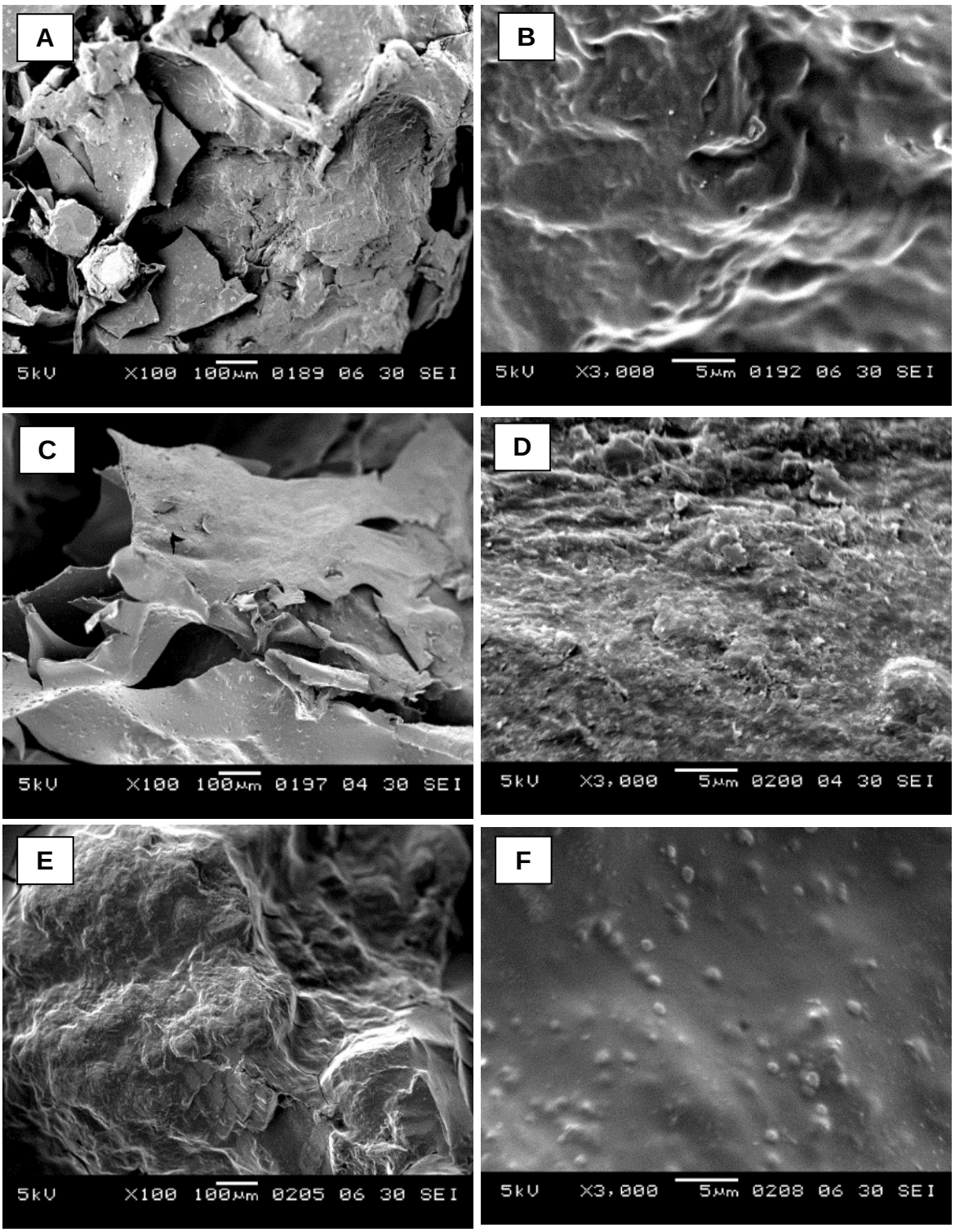


Fig. 5: SEM images of 0th generation control mortar at (A) low magnification and (B) high magnification; 0th generation cyanobacterial mortar at (C) low magnification and (D) high magnification; 3rd generation cyanobacterial mortar at (E) low magnification and (F) high magnification. Specimens were stored at 4°C and 100% relative humidity.

*Fig. 4* shows representative images of 0th generation control mortar, 0th generation cyanobacterial mortar, and 3rd generation cyanobacterial mortar stored at 50% RH. *Fig. 5* shows representative images of 0th generation control mortar, 0th generation cyanobacterial mortar, and 3rd generation cyanobacterial mortar stored at 100% RH.

In general, fracture surfaces of the control mortars appeared smooth and glassy while the fracture surfaces of the cyanobacterial mortars exhibited a rougher texture (compare *Figs. 5B* and *5F*). This could be attributed to reinforcement of the gelatin matrix by inorganic crystalline $CaCO_3$ produced via MICCP by *Synechococcus sp* cells.

## 5 CONCLUSIONS

*Synechococcus sp.* PCC 7002 was used to create a gelatin-based "living" mortar from which the biotic component can be propagated to inoculate new generations of material. This was accomplished by subjecting the mortars to specific temperature triggers that were used to direct metabolic behavior. The low-temp trigger (4°C) induced metabolic dormancy in *Synechococcus sp.* cells that proved necessary for long-term viability retention. Conversely, the high-temp trigger (37°C) was efficient at promoting metabolic activity in cells that were previously rendered dormant by the low-temp trigger. Approximately 69% of the initial inoculum were found to be viable at 14 days in 0th generation cyanobacterial mortar kept at either 50% or 100% RH. Moreover, at 30 days, approximately 9% of the initial inoculum survived in 0th generation cyanobacterial mortar kept at 50% RH and 14% of the initial inoculum survived in 0th generation cyanobacterial mortar kept at 100% RH. Viability was also assessed for 1st, 2nd, and 3rd generation cyanobacterial cubes, and it was determined that a considerable percentage of cells remained viable at 7 days in each generation. Finally, SEM was utilized to compare microstructural characteristics between fracture surfaces of control mortars and cyanobacterial mortars. Cyanobacterial mortar fracture surfaces exhibited a rougher texture than the control mortars, which was attributed to MICCP by *Synechococcus sp*. PCC 7002.

## 6 ACKNOWLEDGMENTS

The work depicted is sponsored by the Defense Advanced Research Projects Agency (Agreement HR0011-17-2-0039). The content does not necessarily reflect the position or the policy of the Government, and no official endorsement should be inferred.

## 7 REFERENCES

[Achal 2011a] Achal, V.; Mukherjee, A.; Reddy, M; Effect of calcifying bacteria on permeation properties of concrete structures. J Ind Microbiol Biotechnol, 2011, 38, 9, 1229-1234.

[Achal 2011b] Achal, V.; Pana, X.; Özyurt, N.; Improved strength and durability of fly ash-amended concrete by microbial calcite precipitation. Ecol Eng, 2011, 37, 4, 554-559.

[Badger 2003] Badger, M.; Price, G.; $CO_2$ concentrating mechanisms in cyanobacteria: molecular components, their diversity and evolution. J Exp Bot, 2003, 54, 383, 609-622.

[Blodgett 2018] Blodgett, R.; Appendix 2: Most Probable Number Determination from Serial Dilutions. In United States Food & Drug Administration, Bacteriological Analytical Manual (BAM), 2018.

[Bundur 2015] Bundur, Z.; Kirisits, M.; Ferron, R.; Biomineralized cement-based materials: Impact of inoculating vegetative bacterial cells on hydration and strength. Cement and Concrete Research, 2015, 67, 237-245.

[Bundur 2017] Bundur, Z.; Bae, S.; Kirisits, M.; Ferron, R; Biomineralization in Self-Healing Cement-Based Materials: Investigating the Temporal Evolution of Microbial Metabolic State and Material Porosity. J Mat Civil Eng, 2017, 29, 8.

[DeJong 2006] DeJong, J.; Fritzges, M.; Nüsslein, K.; Microbially Induced Cementation to Control Sand Response to Undrained Shear. J Geotech Geoenviron Eng, 2006, 132, 11.

[Jonkers 2006] Jonkers, H.; Schlangen, E.; Self-healing of cracked concrete: A bacterial approach. In Proceedings of the 6th International Association of Fracture Mechanics for Concrete and Concrete Structures Conference, Catania, 2006.

[Jonkers 2010] Jonkers, H.; Thijssena, D.; Muyzer, B;, Copuroglu, O.; Schlangen, E.; Application of bacteria as self-healing agent for the development of sustainable concrete. Ecol Eng, 2010, 36, 2, 230-235.

[Joshi 2017] Joshi, S.; Goyal, S.; Mukherjee, A.; Reddy, M; Microbial healing of cracks in concrete: a review. J Ind Microbiol Biotechnol, 2017, 44, 11, 1511-1525.

[Kamennaya 2012] Kamennaya, N.; Ajo-Franklin, C.; Northen, T.; Jansson, C.; Cyanobacteria as Biocatalysts for Carbonate Mineralization. Minerals, 2012, 2, 4, 338-364.

[Ludwig 2011] Ludwig, M.; Bryant, D.; Transcription Profiling of the Model Cyanobacterium *Synechococcus sp.* Strain PCC 7002 by Next-Gen (SOLiD™) Sequencing of cDNA. Front Microbiol, 2011, 2, 41.

[Stocks-Fischer 1999] Stocks-Fischer, S.; Galinat, J.; Bang, S.; Microbiological precipitation of CaCO3. Soil Biol Biochem, 1999, 31, 11, 1563-1571.

[Wang 2014] Wang, J.; Soens, H.; Verstraete, W.; De Belie, N.; Self-healing concrete by use of microencapsulated bacterial spores. Cem Concrete Res, 2014, 56, 139-152.