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2 Microbiologically Induced Calcium Carbonate
3 Precipitates using TRLFS

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7 **ABSTRACT**

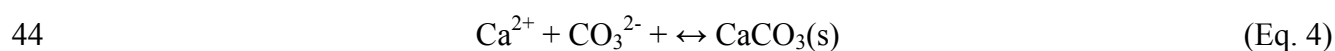
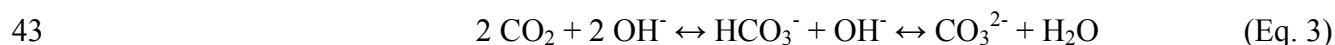
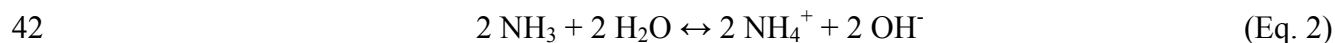
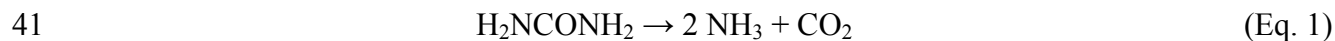
8 The microbial induced biomineralization of calcium carbonate using the ureolytic bacterium
9 *Sporosarcina pasteurii* in the presence of trivalent europium, a substitute for trivalent actinides,
10 was investigated by time resolved laser-induced fluorescence spectroscopy (TRLFS) and a
11 variety of physicochemical techniques. Results showed that the bacterial-driven hydrolysis of
12 urea provides favorable conditions for CaCO_3 precipitation and Eu^{3+} uptake due to subsequent
13 increases in NH_4^+ and pH in the local environment. Precipitate morphologies were characteristic
14 of biogenically formed CaCO_3 and consistent with the respective mineral phase compositions.
15 The formation of vaterite with some calcite was observed after one day, calcite with some
16 vaterite after one week, and pure calcite after two weeks. The presence of organic material
17 associated with the mineral was also identified and quantified. TRLFS was used to track the

18 interaction and speciation of Eu^{3+} as a molecular probe with the mineral as a function of time.
19 Initially, Eu^{3+} is incorporated into the vaterite phase, while during CaCO_3 phase transformation
20 Eu^{3+} speciation changes resulting in several species incorporated in the calcite phase either
21 substituting at the Ca^{2+} site or in a previously unidentified, low-symmetry site. Comparison of
22 the biogenic precipitates to an abiotic sample shows mineral origin can affect Eu^{3+} speciation
23 within the mineral.

24 INTRODUCTION

25 Biomineralization is the process by which living organisms produce minerals. This phenomenon
26 occurs universally throughout the biological kingdom, yielding a variety of minerals with
27 different compositions, morphologies, and properties. There are three categories of
28 biomineralization: biologically-controlled, biologically-influenced, and biologically induced
29 mineralization.¹⁻³ For the latter, an array of microorganisms has been reported to induce the
30 precipitation of calcium carbonate minerals, i.e., carbonatogenesis, also referred to as microbial
31 induced calcium carbonate precipitation (MICP).⁴ Over 40 different microorganisms have been
32 reported to induce CaCO_3 precipitation.⁵ Organisms associated with the nitrogen cycle that are
33 able to induce biomineralization, e.g., ammonification of amino acids, nitrate reduction, or urea
34 hydrolysis, have been the most studied.⁶⁻⁸ Hydrolyzing urea using ureolytic bacteria has proven
35 to be a simple and efficient method for precipitating CaCO_3 . Mechanistically, when urea
36 (H_2NCONH_2) is metabolized it is converted to NH_3 and dissolved inorganic carbon (Eq. 1),
37 which equilibrate in water increasing the alkalinity (Eq. 2 and 3). The increased pH of the
38 solution, is buffered by the formation of NH_4^+ ($\text{pK}_a = 9.24$), but ultimately leads to precipitation

39 of CaCO_3 in the presence of Ca^{2+} (Eq. 4).^{6,9-12} Previous studies have shown that varying
40 polymorphs of CaCO_3 , typically vaterite and calcite, can be produced using this technique.¹³



45 MICP has gained much interest as a geobiological tool for addressing a variety of environmental,
46 agricultural, and architectural problems.^{4,14-16} For example, these include soil and water
47 remediation, formation of underground barriers, lake and reservoir sealing, concrete and
48 limestone restoration, and CO_2 sequestration.^{4,15} From an environmental remediation focus, the
49 use of MICP has been suggested for the sequestration, retardation, or even complete removal of
50 toxic and harmful contaminants. These contaminants include toxic metals^{17,18} and radioactive
51 elements, e.g., U and ^{90}Sr .¹³ Particularly concerning these radioelements, most studies have been
52 aimed at the cleanup and removal of ^{90}Sr from contaminated nuclear legacy sites, which include
53 the proposed use of bioengineering techniques such as pulse-flow injection.¹⁹⁻²¹ Results have
54 shown effective Sr^{2+} uptake and immobilization into biogenic CaCO_3 . Using XAS Fujita et al.
55 observed the formation of a solid solution with Sr^{2+} substitution at the Ca^{2+} site within calcite
56 lattice.²⁰

57 The long-lived, highly radiotoxic transuranic elements, i.e., neptunium, plutonium, americium,
58 and curium, as well as some lanthanide fission products, e.g. ^{155}Eu ,²² are a major environmental
59 concern. They are man-made elements or nuclides, resulting from nuclear processes that have

60 been or will be introduced into the biosphere during geologic disposal, nuclear accidents, and in
61 nuclear weapons fallout.^{23,24,25} The progressively widespread use of lanthanides in technological
62 devices and as medical imaging agents also increases possibilities of contamination of the
63 environment.²⁶ Additionally, some trivalent lanthanides/actinides exhibit similar size and
64 coordination geometry to Ca^{2+} , which allows these elements to follow Ca^{2+} in biological systems,
65 e.g. by incorporation into bones [Holliday et al.].²⁷ When considering their radio- and
66 chemotoxicities, this behavior can be particularly harmful for biota, thus schemes for their reuse
67 and/or disposal should be developed.

68 Previous studies have determined that trivalent americium²⁸ and curium²⁹ as well as their
69 homologous lanthanides³⁰⁻³² are strongly partitioned into phases of CaCO_3 . The interactions of
70 these elements with CaCO_3 are often dominated by surface sorption or incorporation, where
71 incorporation can occur either in the bulk structure or at the Ca^{2+} position in the crystal lattice via
72 substitution.³²⁻³⁴ Although an appreciable amount of natural calcite is of biogenic origin and can
73 host a substantial amount of Eu^{3+} ,³⁵ never have the interactions of trivalent rare earth or actinide
74 elements with biogenic CaCO_3 been systematically studied. Comparatively, the bio(apatite)
75 system has been investigated, and it was revealed that these elements interact with biogenic
76 apatite considerably different than with the inorganically derived mineral. Results showed that
77 Eu^{3+} was found incorporated in the grain boundaries of the lattice and not at either of the Ca^{2+}
78 sites within the mineral as was previously reported. This difference in structural localization can
79 have significant implications when considering actinide sequestration in human bone.^{36,37} With
80 CaCO_3 ubiquitously occurring throughout the bio- and geosphere, found in shells and skeletal
81 components and in the mineralogy of many nuclear repositories, this raises the question if similar

82 effects may be found for biogenic CaCO_3 , i.e., if there are differences in how trivalent actinides
83 and lanthanides interact with abiotic and biotic CaCO_3 .

84 Time resolved laser-induced fluorescence (TRLFS) is an important and versatile tool for
85 studying mineral interactions with luminescent trivalent actinides and lanthanides, i.e., Eu, Am,
86 and Cm.^{23,28,29,38,39} This characterization tool allows the site-selective excitation of a fluorescent
87 probe in low concentrations ($\sim 10^{-7}$ M Eu^{3+}) to determine discrete species present with a mineral.
88 From this, detailed information of probe symmetry from characteristic emission band splittings
89 and extent of hydration from measured fluorescent decay lifetimes can be determined for the
90 differentiation of species that are either surface sorbed or incorporated in the mineral.⁴⁰ In turn,
91 this may help to evaluate the stability of the formed solid solution more accurately as well as the
92 geochemical properties of trivalent actinides and lanthanides associated with CaCO_3 .

93 The aim of this study was to gain a better understanding of the process of biomineralization of
94 CaCO_3 and how trivalent actinide or lanthanide elements could potentially interact with it.
95 Calcium carbonate was precipitated via ureolysis using *Sporosarcina pasteurii*, an archetypal
96 ureolytic bacterium, in the presence of Eu^{3+} and studied using a variety of analytical techniques.
97 TRLFS was used to investigate the behavior of Eu^{3+} with the resulting mineral. The effect of
98 biogenic components incorporated into the mineral on Eu^{3+} speciation was compared to an
99 abiotic sample synthesized under similar conditions.

100 **EXPERIMENTAL**

101 **Culturing and Inoculation.** The strain *Sporosarcina pasteurii* (DSM33, ATC11859) was
102 cultured in CASO medium (Merck 105458) (15 g/L peptone from casein, 5 g/L peptone from
103 soymeal, 5 g/L NaCl, pH = 7.3) amended with 20 g/L urea at 30 °C on a rotary shaker (80 rpm)

104 overnight. For the inoculation, cultures were transferred into fresh media and grown at 30 °C for
105 approximately 15 hours. The bacteria were harvested from a 50 mL aliquot using a rotary
106 centrifuge (8,820 rcf) for 15 minutes. The supernatant was removed and the resulting cell pellet
107 was resuspended and washed two times with 50 mL of medium containing 20 g/L urea, 10 g/L
108 NH₄Cl, and 2.12 g/L Na₂CO₃ in MilliQ filtered water and pH adjusted to 5.6. The cell pellet was
109 then diluted in the same medium until an optical density reading at 600 nm (OD₆₀₀) using a
110 spectrophotometer (Cary 50 Bio) with a 1 cm path length of 0.40 ± 0.02.

111 **Mineralizing Medium.** The CaCO₃ mineralizing medium used was similar to that reported by
112 Stocks-Fischer (1999).⁶ This was prepared by mixing of two solutions: a first solution of 3.0 g
113 bacterial nutrient broth in 800 mL of MilliQ filtered water at pH adjusted to 5.6 with the
114 dropwise addition of concentrated HCl(aq), which was then autoclaved. A second solution was
115 prepared by dissolving 20 g urea, 10 g NH₄Cl, and 2.12 g Na₂CO₃ in 180 mL of MilliQ filtered
116 water and pH adjusted to 5.6. To this solution 3.7 g CaCl₂·2H₂O and a 100 µL aliquot of a
117 1.0x10⁻³M solution of EuCl₃·6H₂O were added, the pH was readjusted again to 5.6, and the final
118 volume adjusted to 200 mL. The second solution was filter sterilized through a 0.2 µm filter and
119 combined with the first for a final stock solution containing 3.g/L nutrient broth, 20 g/L urea, 10
120 g/L NH₄Cl, and 2.12 g/L Na₂CO₃, 3.7 g/L CaCl₂·2H₂O, and 1.0x10⁻⁷ M EuCl₃·6H₂O.

121 **CaCO₃ Precipitation Experiments with Europium.** Experiments were performed using sterile
122 handling techniques under aerobic conditions. The mineralizing medium stock (150 mL) was
123 transferred into two different 250 mL Erlenmeyer flasks each, and 1 mL of the previously
124 described inoculum containing *S. pasteurii* was added to one of the flasks for a calculated
125 starting OD₆₀₀ = 0.003 and a 1 mL aliquot of medium containing no bacteria added to the
126 control. The bacterial and abiotic control flasks were kept at 30 °C on a rotary shaker (80 rpm).

127 Samples (1 mL) were collected from both flasks at varying times throughout the duration of the
128 experiment into 1.5 mL centrifuge tubes, centrifuged at 16,100 rcf at 25 °C for 5 minutes, and the
129 supernatants were removed. The pH was determined immediately, while samples for inductively
130 coupled plasma mass spectrometry (ICP-MS) measurements and determination of $[\text{NH}_4^+]$ were
131 stored at 4 °C until measured. In addition, mineral formed in the flasks containing bacteria was
132 sampled with a pipette during the duration of the experiment at different times and monitored
133 with light microscopy. Experiments were run for durations of either one day, one week, or two
134 weeks. All experiments were performed in triplicate.

135 At the end of each experiment, the supernatant containing the biomass was removed from the
136 precipitate by decantation. Subsequently, the reaction flask containing was scraped with a
137 sterilized glass rod to remove any adhered precipitate and the precipitate was transferred into a
138 sterile centrifuge tube. The precipitate was washed twice with MilliQ water and twice with
139 acetone, lightly capped, and air-dried. Precipitate from the triplicate samples for one day, one
140 week, and two weeks were then characterized using a variety of physicochemical instrumental
141 methods unless stated otherwise.

142 Because it was of interest to distinguish between the impact of biogenic components and that of
143 the growth medium on the interaction of Eu^{3+} with the mineral, an abiotic precipitate was
144 synthesized for comparison with the biogenic CaCO_3 . In the absence of bacteria, mineralization
145 was induced by dropwise titration of the Eu^{3+} -containing mineralizing medium (150 mL) with an
146 NaOH solution until pH \sim 9.2. The resulting sample was incubated for 10 days at 30 °C on a
147 rotary shaker (80 rpm) and the precipitate was harvested using the same techniques as above.
148 The resulting sample, which will be referred to as the abiotic precipitate, was analyzed by
149 TRLFS

150 **Characterization Methods.** *Time Resolved Laser-Induced Fluorescence (TRLFS).* TRLFS can
151 be used for determining the local coordination environment of the Eu^{3+} ion. The non-
152 degenerative ($J = 0$) excitation of the ground state ${}^7\text{F}_0$ to the excited state ${}^5\text{D}_0$ yields a single peak
153 for each respective species. Excitation spectra can be produced by probing the sample with
154 varying excitation wavelengths and measuring the integrated fluorescence yield. For the Eu^{3+}
155 ${}^7\text{F}_0 \rightarrow {}^5\text{D}_0$ transition an appropriate excitation wavelength window ranges from 577 nm to
156 582 nm. Direct excitation of each species yields a characteristic emission spectrum and
157 fluorescence lifetime. Splitting patterns of the (${}^5\text{D}_0 \rightarrow {}^7\text{F}_1$) and (${}^5\text{D}_0 \rightarrow {}^7\text{F}_2$) bands can be used to
158 determine the local site symmetry of the Eu^{3+} ion which can be compared to the crystallography
159 of the host mineral.⁴⁰ Higher splitting patterns of these transitions are indicative of lower site
160 symmetry with a maximum splitting equal to $(2J + 1)$.⁴⁰

161 Fluorescence lifetimes can be empirically correlated to the degree of hydration where shorter
162 lifetimes, i.e., $< 1700 \mu\text{sec}$, are a result of non-radiative energy transfer to OH-oscillator
163 vibrations, and longer lifetimes, i.e., $\geq 1700 \mu\text{sec}$, indicate no bound water associated with the
164 Eu^{3+} ion. Employing Horrocks and Sudnick's equation (Eq. 5) the decay constant k ($1/\tau$)
165 correlates to the amount of quenching water molecules where τ is in milliseconds.⁴¹

$$166 \quad n(\text{H}_2\text{O}) = 1.07k - 0.62 \quad (\text{Eq. 5})$$

167 The measured lifetime and degree of hydration provide insight into the speciation of Eu^{3+} either
168 in solution or in the presence of a solid interface. For example, the Eu^{3+} aquo-ion yields a low
169 fluorescence lifetime, i.e., $\tau = 110 \mu\text{sec}$, that equates to 9 H_2O in its inner coordination sphere,
170 whereas when it is fully incorporated into the crystal lattice of a mineral, such as calcite, longer
171 lifetimes are obtained, i.e., $\tau > 3600 \mu\text{sec}$, equivalent to 0 H_2O present.³²

172 TRLFS was performed using a pulsed (10 Hz) Nd:YAG (Spectra Physics, 532 nm) pumped dye
173 laser (Radiant Dyes). The laser dye Pyrrhomethene 580 was used for direct excitation of Eu^{3+} in
174 the range from 575–582 nm. The laser wavelength and power were monitored using a wavemeter
175 (High Finesse WS-5) ($>10^{-5}$ nm accuracy) and power meter (Newport 1918-R), respectively.
176 Fluorescence measurements were detected by an optical multichannel analyzer that consists of a
177 monochromator with 300/600/1200 lines/mm gratings (Acton SpectraPro 300i) and an
178 intensified CCD (Princeton Instruments). Maximum resolution at 300 and 1200 lines/mm was
179 measured to be 0.9 and 0.2 nm, respectively. The detection system was calibrated with a neon
180 lamp (Pen Ray 6032). The samples were cooled to ~ 10 K by a helium refrigerated cryostat
181 (CTI-cryogenics) to improve resolution. To avoid camera exposure to the direct laser beam, the
182 minimum gate delay between laser pulse and camera gating was set to 1.0 μs . The gate width of
183 the camera was fixed at 10 ms to ensure the collection of the entire fluorescence signal.
184 Fluorescence lifetime measurements were made with a delay time step between 50 and 150 μs
185 and a total of 100 steps were taken for each lifetime measurement.

186 *Microscopy and Mineral Composition Analysis.* Precipitates for light microscopy imaging were
187 directly sampled from solution, separated from the supernatant, and washed with sterile MilliQ
188 water. The samples were resuspended in water (~ 4 μL) and pipetted onto glass slide, covered
189 with a glass coverslip, and imaged using an Olympus BX61 light microscope. Samples for
190 scanning electron microscopy (SEM)/energy dispersive X-ray spectroscopy (EDX) were
191 prepared by transferring the precipitate in MilliQ water onto a silicon wafer and dried in a
192 desiccator at ambient temperature. The samples were imaged with a Zeiss EVO 50 SEM
193 equipped with a Bruker AXS QUANTAX 200 EDX. Mineral phase composition was determined
194 with a Bruker D8 powder X-ray diffractometer or a RIGAKU MiniFlex 600. Precipitates were

195 pulverized in an agate mortar and the resulting powders were evenly dispersed on a low-
196 background silicon wafer and measured from 18° to $50^\circ 2\theta$ with a step size of $0.02^\circ 2\theta$. Thermal
197 gravimetric analyses (TGA) were conducted on one representative of the triplicate samples from
198 the one day, one week, and two week experiments. Samples were measured in a Mettler Toledo
199 TGA with a TG 50 furnace unit. A sample of 20 to 40 mg were heated in a ceramic crucible
200 under a nitrogen atmosphere (100 ml/h) over the range of 35 to 850 °C at a rate of 10 °C/sec.
201 Multi-point N_2 -BET surface area of the precipitate after two weeks (Brunauer-Emmett-Teller
202 theory) was determined using a surface area and pore size analyzer (mod. Coulter SA 3100,
203 Beckman Coulter, Fullerton, USA). Weighed quantities of the resulting precipitates were
204 dissolved in 30% HNO_3 , and the Eu^{3+} concentration for each sample was determined using ICP-
205 MS (NexION 350 X, PerkinElmer).

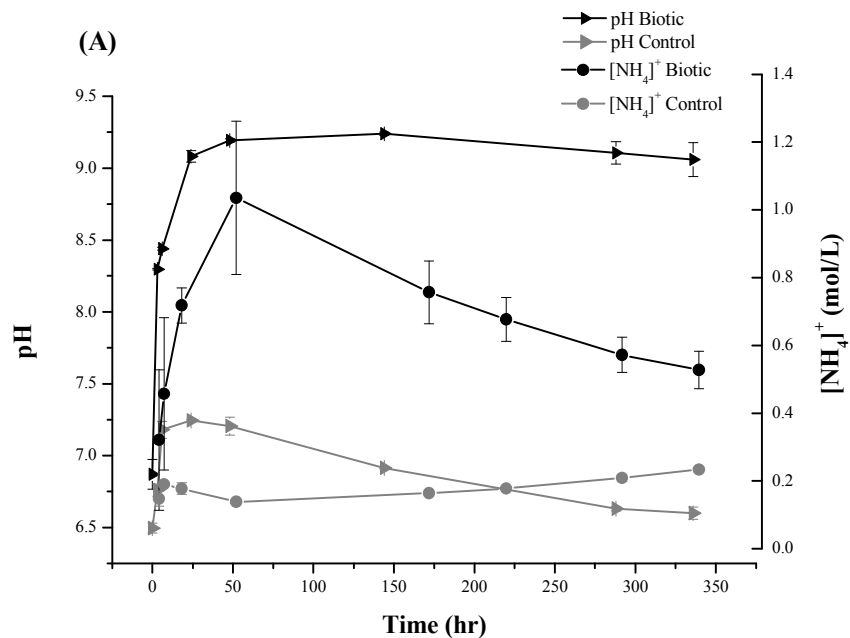
206 *Solution Chemistry Analysis.* The pH was measured using a Mettler Toledo Inlab® electrode and
207 calibrated with pH = 4, 6, and 9 standard solutions (WTW, Weilheim, Germany). Ammonium-
208 ammonia concentration was determined using an ammonium ion selective electrode with
209 Nernstian response and measured against a calibration curve (WTW, Weilheim, Germany).
210 Supernatants collected were acidified with HNO_3 (1 %), and Eu^{3+} and Ca^{2+} concentrations were
211 determined using an ICP-MS. All measurements were made in triplicates.

212 RESULTS

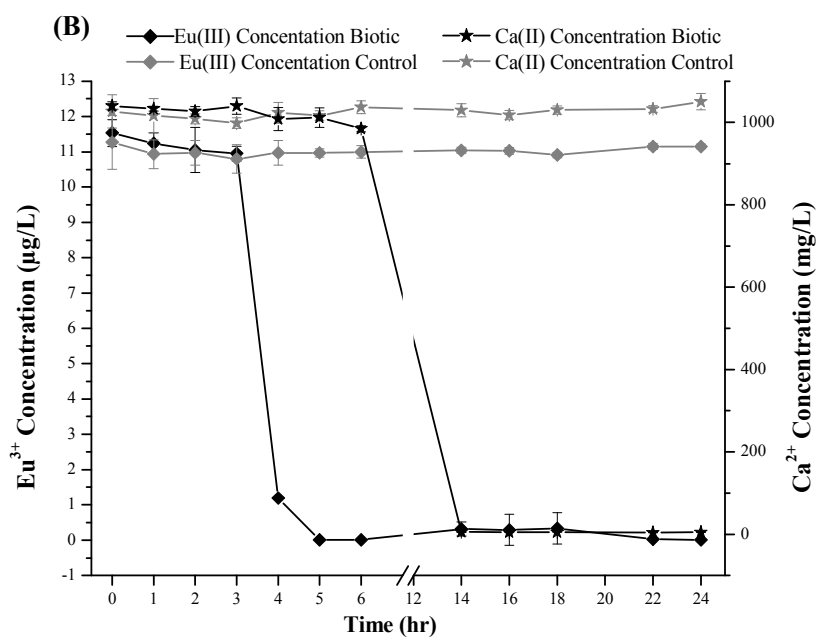
213 **Biom mineralization Precipitation Experiments.** In mineralizing medium containing Eu^{3+} and
214 urea, *S. pasteurii* demonstrated ureolytic behavior consistent with the literature.^{6,13,20} As shown in
215 Figure 1A, pH and NH_4^+ concentrations increased rapidly within the first 6 hours and reached
216 maximum values after 48 hours of 9.2 and 1.0 mol/L, respectively. The decrease of NH_4^+

217 concentrations over time was caused by the release of $\text{NH}_3(\text{g})$ in equilibrium with NH_4^+ at this
218 pH. An initial increase in pH of the abiotic control was observed reaching $\text{pH} = 7.2$ after 24
219 hours, although no significant amount of paralleled NH_4^+ production was detected. Notably,
220 induced mineral precipitation was consistently observed in the bacterial samples under these
221 conditions after 3 to 4 hours of reaction time between the pH range of 7.8 and 8.0. For the abiotic
222 control samples, no mineral precipitation was observed throughout the duration of the
223 experiment. This trend was also evident when tracking soluble Ca^{2+} and Eu^{3+} concentrations.

224



225



226

227 **Figure 1.** (A) Measured pH and total NH_4^+ in solution in biotic (black triangles and black circles,

228 respectively) and control (grey triangles and grey circles, respectively) cultures as a function of

229 time. (B) Eu^{3+} and Ca^{2+} concentrations measured in the solution in biotic (black diamonds and

230 black stars, respectively) and control (grey diamonds and grey stars, respectively) cultures as a

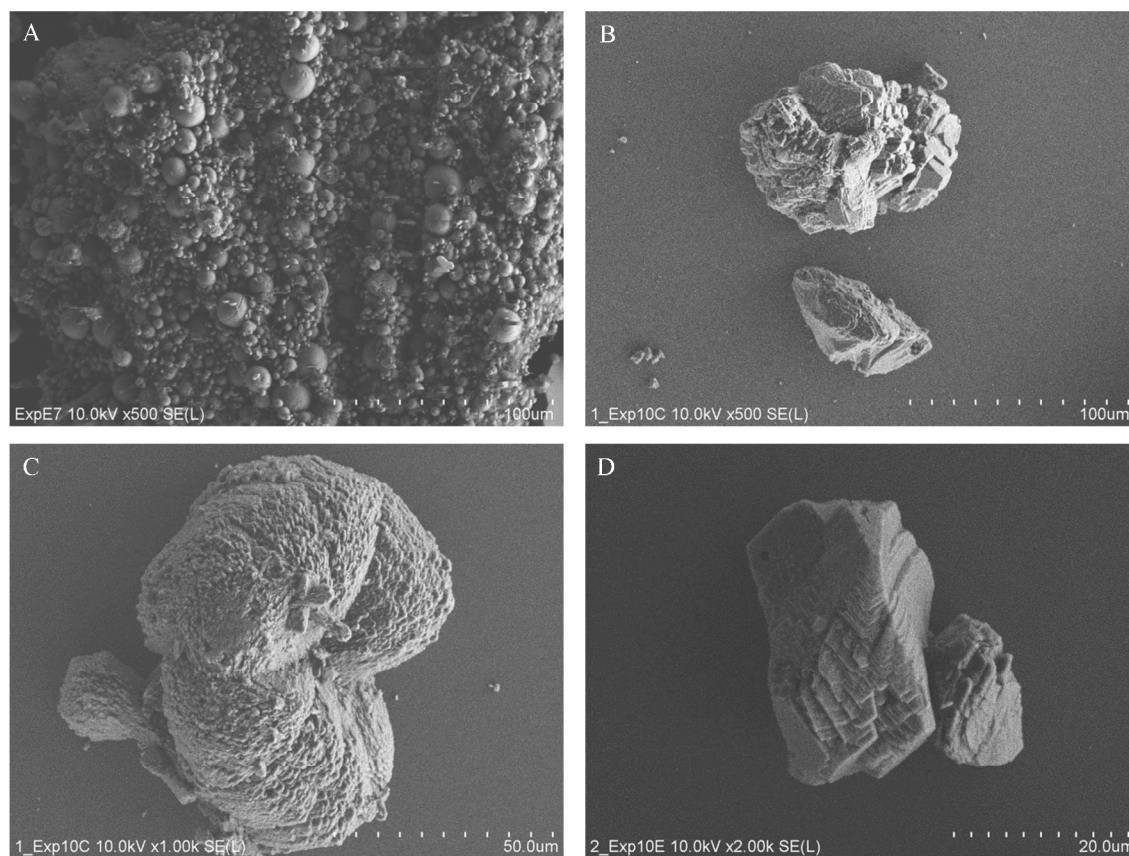
231 function of time. Points represent the average of triplicate measurements and error bars represent
232 the standard deviation.

233 The change in soluble Eu^{3+} and Ca^{2+} concentrations was tracked and measured by ICP-MS as
234 shown in Figure 1B. In the cultures containing *S. pasteurii*, no drastic consumption of either Ca^{2+}
235 or Eu^{3+} was observed in the first 3 hours of the experiment. After this “induction phase,”
236 europium concentration in solution decreased rapidly. To note, the earlier onset of the decrease
237 in Eu^{3+} does indicate precipitation of a Eu^{3+} solid phase. A significant decrease in Ca^{2+}
238 concentration did not occur until later between the 6th and 14th hours of the experiment.
239 Concentrations of Eu^{3+} and Ca^{2+} reached 0.01 $\mu\text{g/L}$ and 4.35 mg/L and were stabilized after 5
240 and 14 hours, respectively, and no leaching of Eu^{3+} was observed following precipitation.
241 Conversely, in the abiotic control the concentrations of both elements remained constant and the
242 formation of any precipitates was not observed.

243 **Mineral Characterization. Particle Formation and Morphology.** Light microscopy was used to
244 monitor the precipitation of biogenic CaCO_3 examining morphology, size, and distribution of the
245 mineral. Figure S1 (Supplementary information) displays images of the formation and
246 crystallization of biomineralized CaCO_3 at various times over a two-week period. Initial samples
247 collected after three hours show small, undefined precipitates surrounding and closely associated
248 with the bacteria. After six hours, precipitates were observed with discrete shapes ranging in size
249 from 10 to 40 μm . After one day, precipitates ranging up to 50 μm were present exhibiting
250 framboidal morphology. After one and two weeks, precipitates were found $\leq 50 \mu\text{m}$ in diameter
251 with defined faces and edges. The average surface area per mass of the bulk precipitate collected
252 after two weeks was measured by BET to be $0.42 \pm 0.02 \text{ m}^2/\text{g}$.

253 SEM was employed to better understand the formation and development of the precipitate
254 morphology from particle to aggregate level. In Figure 2A, the image of precipitate harvested
255 after one day shows small particles with distinct spherical shapes consistent with the vaterite
256 morphology. Other morphologies, e.g., anhedral irregular, hemi-spherulite, and discoid, that have
257 been reported⁴³ for biogenic carbonates were also found here. On the surfaces of some of the
258 precipitates, pitting resembling bacteria morphology was observed. After one and two weeks
259 (Figure 2B-D), precipitate morphologies were more euhedral/subhedral and resembled those
260 reported for calcite with characteristic rhombohedral faces. In addition, particle size distribution
261 of these precipitates ranged from approximately 2 to 150 μm . EDX analyses of the precipitates
262 (Figure S2) yielded C, O, and Ca as expected for CaCO_3 . Comparatively small amounts of P and
263 Mg were also identified that most likely originate from the cellular composition of the bacteria.
264 The sensitivity of the EDX analyses was not high enough to detect Eu^{3+} at the concentration
265 used. A weighed quantity of each of the precipitates was dissolved and the Eu^{3+} concentration
266 was measured by ICP-MS. The concentrations after one day, one week, and two weeks were
267 $470 \pm 47 \mu\text{g g}^{-1}$, $546 \pm 55 \mu\text{g g}^{-1}$, and $531 \pm 53 \mu\text{g g}^{-1} \text{Eu}^{3+}$, respectively.

268

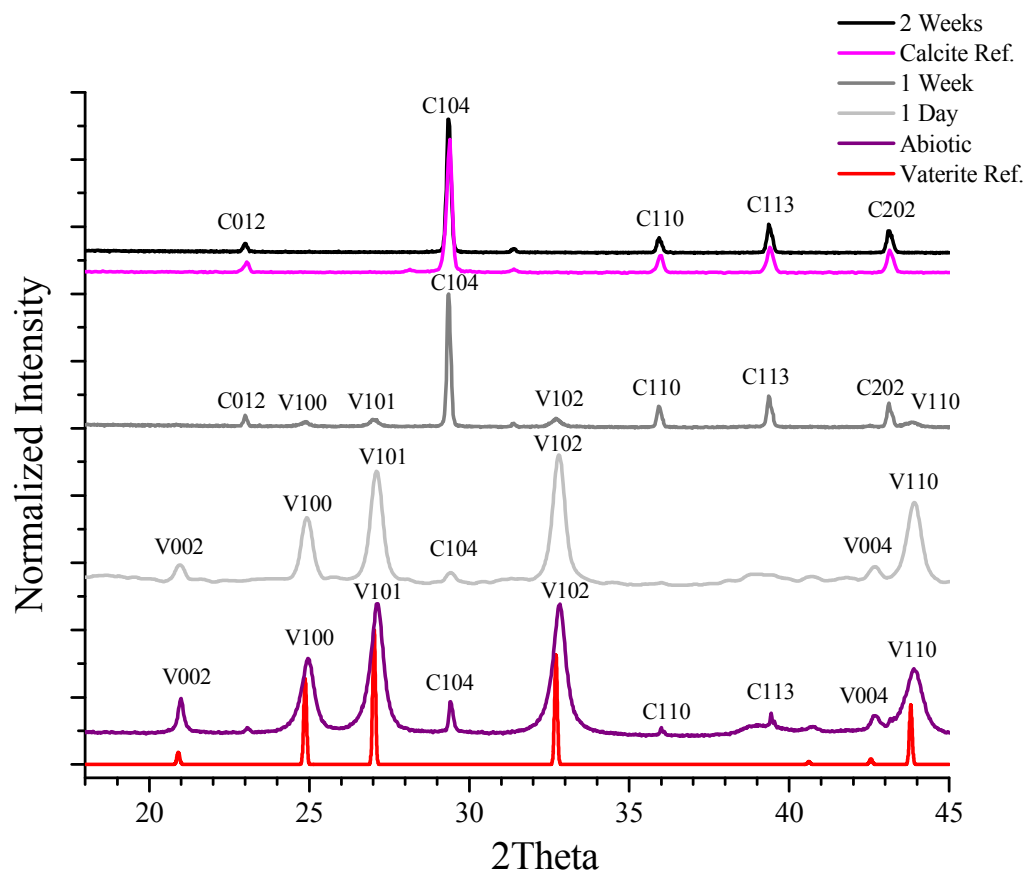


269

270 **Figure 2.** SEM images of biogenic CaCO₃ at various times: A) 24 hours, B and C) 1 week, and
271 D) 2 weeks. Scale bars provided at the lower right hand of each image.

272 *Particle Composition Analysis.* The mineralogical compositions as a function of time were
273 characterized by powder XRD. As shown in Figure 3, the relative abundance of the identified
274 phases, i.e., vaterite and calcite, of the biogenic CaCO₃ changed over time. Vaterite was the
275 predominant phase found after 24 hours, and although the (104) peak of calcite was also
276 observed, it was very weak indicating calcite was a minor phase at this time. After one week the
277 sample also exhibited a mixture of vaterite and calcite, but with calcite as the major constituent.
278 Using the XRD method reported by Dickinson and McGrath⁴⁴ for estimating relative phase
279 compositions, the samples were found with 9 ± 5 wt% calcite and 91 ± 5 wt % vaterite after one
280 day and 85 ± 5 wt% calcite and 15 ± 5 wt% vaterite after one week. The complete transformation

281 to calcite was observed after 2 weeks. The phases present at the respective times were consistent
282 with morphologies observed by imaging techniques (Figure 2). For the abiotic precipitate after
283 10 days, a relative phase composition of 14 ± 5 wt% calcite and 86 ± 5 wt% vaterite was
284 determined.

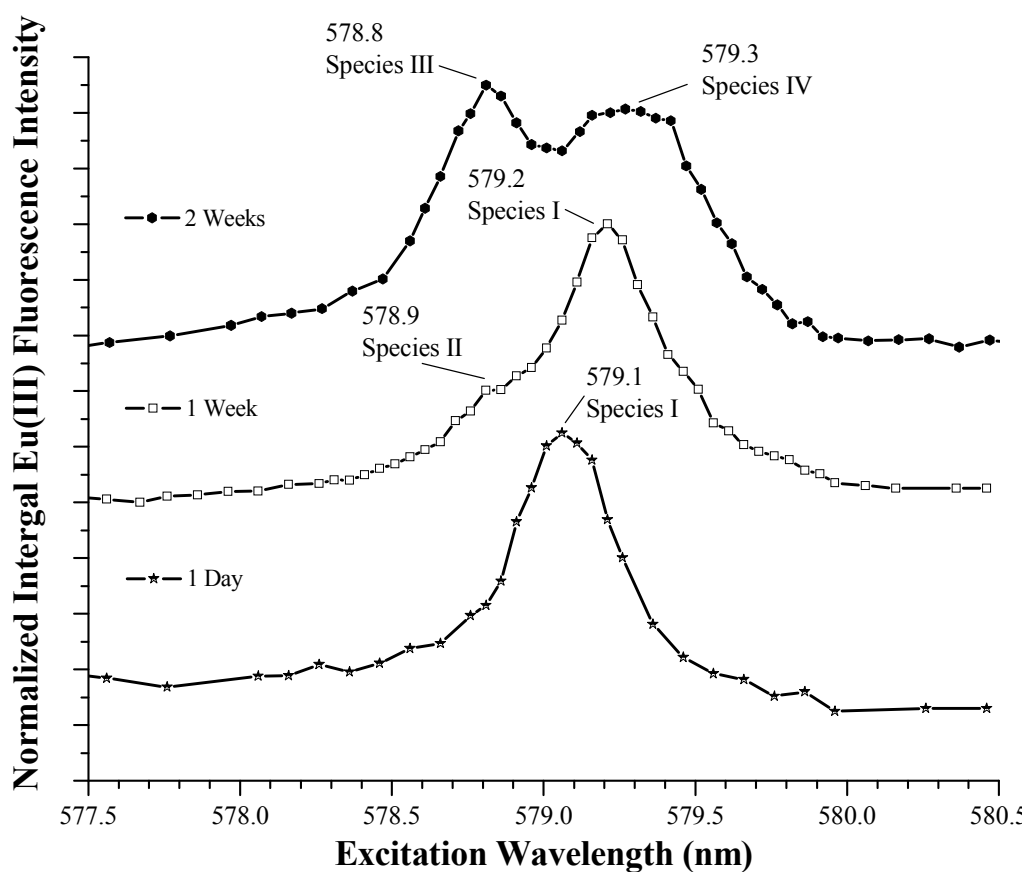


285
286 **Figure 3.** A comparison of XRD powder patterns of biom mineralized CaCO_3 from *S. pasteurii*
287 after one day, one week, and two weeks, and abiotic CaCO_3 after 10 days. Peaks are labeled with
288 the calcite (C) or vaterite (V) and the respective Miller indices [HKL].

289 Thermal gravimetric analysis was used to determine the amount of remaining organic material in
290 the precipitate after recovery and washing. Figure S4 displays the thermal behavior of samples
291 precipitated at different times versus a calcite standard. In comparison with an abiotic calcite

292 standard, which showed no significant loss of mass before ~ 750 °C, a two-step change in mass
293 in this range was observed for the biogenic CaCO_3 samples. This can be attributed to the loss of
294 water associated with the mineral up to 200 °C, and the loss of organic material from 200 to
295 650 °C.⁴⁵ From this, it was determined that the sample after one day contained about 6 wt%
296 organic matter, whereas the one and two week samples contained about 2 wt%. Final
297 decomposition temperatures of CaCO_3 to CaO and CO_2 were approximately 795, 818, and
298 837 °C for the CaCO_3 after one day, two weeks, and one week, respectively. These
299 decomposition temperatures were notably lower than the calcite standard measured at 842 °C.
300 This has been attributed to the presence of crystal defects due to crystal grain growth speed and
301 the inclusion of trace elements, e.g., Mg^{2+} , into the lattice during crystallization, which can
302 distort and lower the stability of the crystal lattice.^{46,47}

303 **Europium TRLFS: Analysis and Speciation.** *Precipitate After One Day.* The excitation
304 spectrum of the Eu^{3+} doped biogenic CaCO_3 after one day is shown in Figure 4. In the spectrum,
305 only a single absorption maximum was observed at 579.1 nm corresponding to one species,
306 which will be designated as Species I. The luminescence lifetime of Species I (Table 1, Figure
307 S4) was measured after selective excitation at 579.1 nm and characterized by monoexponential
308 behavior with a value of $\tau = 4550 \pm 580$ μsec . This lifetime value is consistent with 0
309 coordinating H_2O molecules and incorporation of Eu^{3+} in the mineral phase. For the emission
310 spectrum (Figure 5), a two-fold splitting of the $^5\text{D}_0 \rightarrow ^7\text{F}_1$ and a three-fold splitting of the
311 $^5\text{D}_0 \rightarrow ^7\text{F}_2$ transitions were observed. Comparatively, the emission spectrum and lifetime are
312 consistent with the Eu^{3+} incorporated vaterite species substituted at the Ca^{2+} site.³²



313
 314 **Figure 4.** The ${}^7F_0 \rightarrow {}^5D_0$ excitation spectra of the Eu^{3+} containing biogenic CaCO_3 produced by
 315 *S. pasteurii*.

316 *Precipitate After One Week.* After one week, two different excitation species were observed
 317 (Figure 4). The speciation here is still dominated by Species I, which exhibited an identical
 318 emission spectrum and lifetime compared to the same species after one day. Its excitation
 319 maximum had shifted, however, to 579.2 nm. This is explained by spectral overlap with a second
 320 species as a much less intense shoulder at 578.9 nm.

321 The shoulder located at 578.9 nm is unique and must be indicative of the presence of a second
 322 species, which will be referred to as Species II. Excitation at 578.9 nm yielded a characteristic

323 biexponential fluorescence decay behavior, a property when multiple species are simultaneously
324 excited. A biexponential fit was used and a short and a long component could be derived as
325 $\tau_1 = 1100 \pm 140 \mu\text{sec}$ and $\tau_2 = 4070 \pm 530 \mu\text{sec}$, respectively. The long component is identical
326 within the errors of measurement for the lifetime of Species I, hence the short lifetime must be
327 characteristic for Species II. Using Horrock's equation, the value of the short lifetime is
328 equivalent to $\sim 0.5 \text{ H}_2\text{O}$ molecules coordinated to the Eu^{3+} . This slight hydration suggests that
329 Species II occurs within the solution-solid interface, although primarily still bound within the
330 mineral. Species II can be compared with the transition species identified by Schmidt et. al
331 shown in Table 1.³² The emission spectrum (Figure 5) of Species II is characterized by both a ${}^7\text{F}_1$
332 and a ${}^7\text{F}_2$ band with a four-fold splitting. In particular, the magnitude of splitting of the ${}^7\text{F}_1$ band
333 exceeds the maximum number of possible $(2J + 1)$ sublevels, which again indicates that multiple
334 species have been simultaneously excited at this wavelength. These results are also consistent
335 with the multiexponential lifetime decay pattern.

336

337 **Table 1.** Fluorescence emission lifetimes of Eu^{3+} species found in biogenic and abiotic
 338 precipitates synthesized in mineralization medium (*italics*, this study) vs. abiotic vaterite³² and
 339 calcite⁴⁹.

Species	$\lambda_{\text{excit.}}$ (nm)	Lifetime (μsec)	$n(\text{H}_2\text{O})$
<i>Species I, One day</i>	579.1	4550 \pm 580	0
<i>Species I, One week</i>	579.2	4330 \pm 560	0
<i>Species II</i>	578.9	1100 \pm 140	0.5
<i>Species III</i>	578.8	2780 \pm 360	0
<i>Species IV</i>	579.3	3380 \pm 300	0
<i>Abiotic Precipitate</i>	579.5	2040 \pm 260	0
*Vaterite Incorporated	579.3	4069 \pm 244	0 [32]
*Transition Species	579.1	1106 \pm 133	0.5 [32]
**Calcite Sorption A	578.1	412 \pm 49	1-2 [49]
**Calcite Incorporated B	578.4	4368 \pm 524	0 [49]
**Calcite Incorporated C	579.6	4352 \pm 261	0 [49]

340 * Vaterite samples were precipitated by bubbling $\text{CO}_2(\text{g})$ through a CaCl_2 (1 M) and NH_4OH (2
 341 M) solution containing 20 ppm Eu^{3+} and suspended in a CaCO_3 saturated solution for vaterite to
 342 calcite transformation.

343 ** Calcite samples were produced in a mixed-flow reactor with a calcite seed crystal and input
 344 solutions of $\text{Ca}(\text{ClO}_4)_2$ (2 mM), NaHCO_3 (1.8 mM)/ Na_2CO_3 (0.15 mM), and Eu^{3+} ($\sim 10^{-7}$ M).
 345

346 *Precipitate After Two Weeks.* Two defined excitation peaks were identified in the sample after
 347 two weeks shown in Figure 4: a peak at 578.8 nm and a broad peak centered at 579.3 nm. The
 348 species at 578.8 nm exhibited a long lifetime of 2780 \pm 360 μs with a monoexponential decay
 349 profile. This value corresponds to a complete loss of hydration. The correlating emission
 350 spectrum shown in Figure 5 is characterized by a six-fold split ${}^7\text{F}_1$ band and a ${}^7\text{F}_2$ band with a
 351 minimum of five-fold splitting. Again, this magnitude of splitting is consistent with the
 352 excitation of multiple species. Notably, this species at 578.8 nm is unique from the other species
 353 identified here and those in prior TRLFS studies of the Eu^{3+} - CaCO_3 system, hence it will be

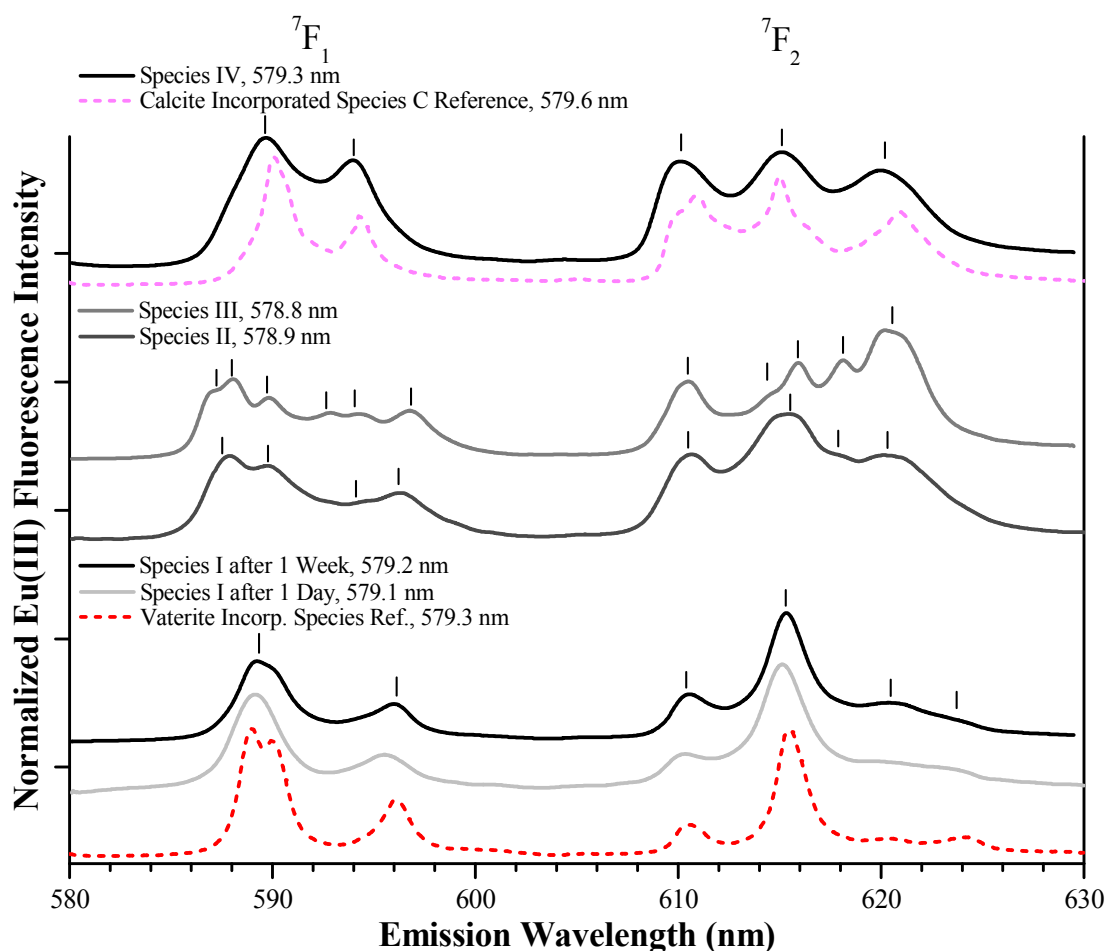
354 referred to as Species III. It is speculated that Species III represents Eu^{3+} incorporated within a
355 low symmetry site in the calcite, although its precise local environment cannot be specified.

356 The lifetime of the species at 579.3 nm was measured with a value of 3380 ± 300 μsec , which is
357 consistent with a complete loss of hydration and Eu^{3+} incorporation. A two-fold splitting and
358 three-fold splitting of the ${}^7\text{F}_1$ and ${}^7\text{F}_2$ band, respectively, are observed in the emission spectrum
359 (Figure 5). This splitting pattern is characteristic of Eu^{3+} incorporated into the trigonal C_{3i} Ca^{2+}
360 site within the calcite lattice.^{32-34,49} The lifetime value and emission spectrum of this species is
361 significantly different to the other species in this study, and therefore will be referred to as
362 Species IV. However, it is noted that Species IV is the same species as Species C identified in
363 previous TRLFS investigations of Eu^{3+} with calcite (Table 1 and Figure 5), which has been
364 described as Eu^{3+} substituting Ca^{2+} on a nearly undistorted lattice site.^{32-34,49} Because of the
365 broadness of the peak, excitations at 579.2 nm and 579.5 nm were also performed to ensure only
366 one species was present. The results from excitation at these wavelengths were identical with
367 those at 579.3 nm.

368 It is noteworthy that no calcite surface sorption species were observed in the precipitates after
369 one and two weeks. The second calcite incorporation species, Species B, which has been
370 reported in the literature, was also not identified (Table 1).^{32,33,49}

371 *Abiotic Precipitate After 10 Days.* As recent investigations have shown, the growth medium may
372 have a significant impact on the calcite surface,⁴⁸ and the uptake of trivalent cations by the
373 mineral.³⁹ To confirm the findings made in this study were due to microbial activity and not the
374 growth medium, mineral was precipitated from the growth medium in the absence *S. pasteurii*
375 and analyzed by TRLFS. The excitation spectrum (Figure S5) of the abiotic precipitate after 10

376 days was measured and a single, broad peak centered at approximately 579.5 nm was identified.
377 Excitation at 579.5 nm yielded a luminescent lifetime of 2040 ± 260 μsec (Table 1), which
378 equates to 0 H_2O molecules in the coordinating sphere the Eu^{3+} species and incorporation into
379 the mineral. The emission spectrum shown in Figure S6 is characterized by a three-fold split ${}^7\text{F}_1$
380 band and a ${}^7\text{F}_2$ band with a minimum of four-fold splitting. From the lifetime and emission data,
381 this Eu^{3+} species can be interpreted as being incorporated into the bulk of the mineral, although
382 in a low-symmetry site. Notably, this species is unique in comparison to Species III and the other
383 species identified in this study as well as those identified in previous TRIFS studies. Therefore,
384 it is difficult to speculate if Eu^{3+} incorporation occurs in the vaterite or calcite phase, however,
385 these results demonstrate the bacteria can affect Eu^{3+} speciation differently than just the medium.



386

387 **Figure 5.** The $^5D_0 \rightarrow ^7F_{1,2}$ emission spectra collected from the site-specific excitation of Eu^{3+} in
388 biomineralized CaCO_3 . Black dash marks indicate splitting peaks.

389

390 **DISCUSSION**

391 In the presence of Eu^{3+} , *S. pasteurii* demonstrated capable ureolytic activity under the
392 experimental conditions consistent with the literature.^{6,13,20} This yielded a significant increase in
393 alkalinity of the growth medium, which could be measured by both pH and $[\text{NH}_4^+]$. The NH_4^+
394 resulting from ureolysis buffered the medium at pH = 9.2, a pH substantially more alkaline than
395 necessary for CaCO_3 precipitation, i.e., pH ~ 8.3.⁶ The uptake of free Ca^{2+} and Eu^{3+} ions
396 coincided with CaCO_3 precipitation and both were essentially depleted from solution after 14
397 hours. TRLFS results showed no surface sorbed Eu^{3+} species present with either the vaterite or
398 calcite after one day. Surface sorbed species have been reported in batch and mixed-flow studies
399 with inorganic CaCO_3 where surface interactions of Eu^{3+} with the mineral occur before
400 incorporation.^{32,34,49} This suggests that the Eu^{3+} was either co-precipitated with the CaCO_3 or
401 completely incorporated into the vaterite after one day. After the onset of precipitation, the
402 bacterium and small aggregates of the mineral were in close association, providing evidence for
403 bacterial involvement during the mineralization process.⁶ The resulting precipitates were
404 characteristic of biogenic vaterite and calcite and contained between 2 to 6 weight percent of
405 organic material as well as trace element inclusions such as Mg and P.

406 At least up to one week, Eu^{3+} was found preferentially incorporated in the vaterite phase (i.e.,
407 Species I), or to a lesser degree as a transition species (i.e., Species II), at the mineral-solution
408 interface. Notably, no Eu^{3+} was found associated with the calcite phase, even with it being the

409 predominate phase present in the mineral composition after one week. These trends suggest that
410 the incorporation of Eu^{3+} in biogenic vaterite undergoes slower phase transformation to calcite in
411 comparison to vaterite without Eu^{3+} . The presence of a hydrated transition species confirms that
412 the phase transformation from vaterite to calcite is a solution-mediated process and is consistent
413 with the literature.³²

414 In this study, no sorption species were identified within the timeframe of the experiment. The
415 formation of surface sorbed species with calcite has been identified in mix-flowed reactor and
416 batch studies resulting from mineral dissolution-reprecipitation under steady-state and mineral
417 growth conditions.^{32,34,49} This is likely attributed to the lower mineral solubility,⁸ which would
418 also prevent significant leaching of either Ca^{2+} or Eu^{3+} after mineral precipitation, observed
419 under these conditions. Previous studies have shown that MICP minerals can behave notably
420 different than their abiotic counterparts due to organic and trace element inclusions and high
421 variability in mineral morphologies and surface topologies originating from their biogenic
422 formation.^{50,51}

423 A new Eu^{3+} species, Species III, was identified in the calcite phase incorporated in a low-
424 symmetry environment. This species was not identified in the abiotic sample, nor has it been
425 reported in previous Eu^{3+} TRLFS studies with calcite. The literature has shown that the origin of
426 the mineral can significantly impact the possible interactions of doping elements within the
427 mineral, such as for the bioapatite system.^{36,37} Considering the mineral composition, it is
428 speculated that Species III could reside in the organic matrix, organic-mineral interface, and/or
429 another low-symmetry coordination environment in the calcite. However, the presence of
430 Species IV, which is incorporated at the Ca^{2+} site in the lattice, also demonstrates that the
431 substitution of Eu^{3+} for Ca^{2+} is still thermodynamically favored under these conditions.^{32-34,49}

432 Ultimately, both Species III and Species IV are incorporated and essentially sequestered within
433 the calcite precipitate. When considering contaminate retardation or removal in natural or
434 engineered environmental systems, this geochemical behavior is quite favorable.

435 In the abiotic precipitate, a mixture of vaterite and calcite was obtained, and a single low-
436 symmetry, incorporated species was identified by TRLFS. However, because this species has not
437 been identified in previous studies or in the biogenic samples produced here, it cannot be
438 definitively stated in which phase this species is incorporated. It is well-known that growth
439 medium and synthetic conditions can have a significant impact on mineral phase, morphology,
440 stability, etc.⁵² during CaCO₃ precipitation, whereas the effect they might have on speciation of
441 co-contaminants present during mineral formation has been considerably less studied. Although,
442 recent investigations have shown that nitrate present even in trace amounts ($\sim 10^{-7}$ M) in the
443 medium can effectively alter Eu³⁺ interactions and speciation with CaCO₃.³⁹ Future research
444 should be aimed at also better understanding these phenomena.

445 The results presented here demonstrate that biomineralized CaCO₃ arising from MICP is capable
446 of sequestering Eu³⁺, and hence this system should also behave similarly for other trivalent
447 lanthanides and trivalent actinides. These findings reiterate that the interactions of these elements
448 with minerals can be affected by the provenance and the conditions under which the mineral is
449 formed. Thus, the unique interaction of biomineralized CaCO₃ with Eu³⁺ solidifies the need to
450 further explore the role of biologically formed minerals in the near and far field of nuclear
451 repository sites and how they interact with (radio)contaminants. It also demonstrates the
452 importance of molecular level studies of diverse systems to be able to reliably describe (and
453 predict) the behavior of biogeochemical systems.

454 ASSOCIATED CONTENT

455 **Supporting Information.** Light microscope images of precipitates and graphs of TGA, EDX,
456 TRLFS lifetime measurements of biogenic CaCO₃ with Eu³⁺ samples, and TRLFS excitation
457 spectrum of calcite produced without bacteria are provided. This material is available free of
458 charge via the Internet at <http://pubs.acs.org>.

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462 **Notes**

463 The authors declare no competing financial interest.

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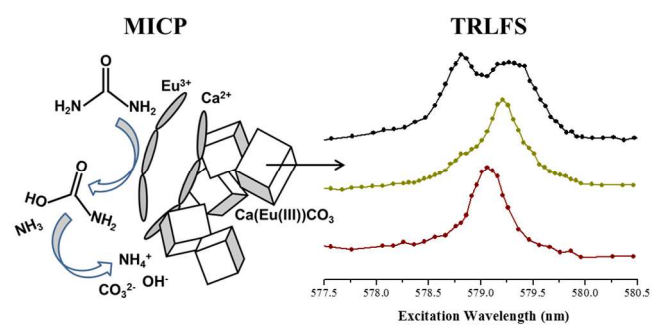
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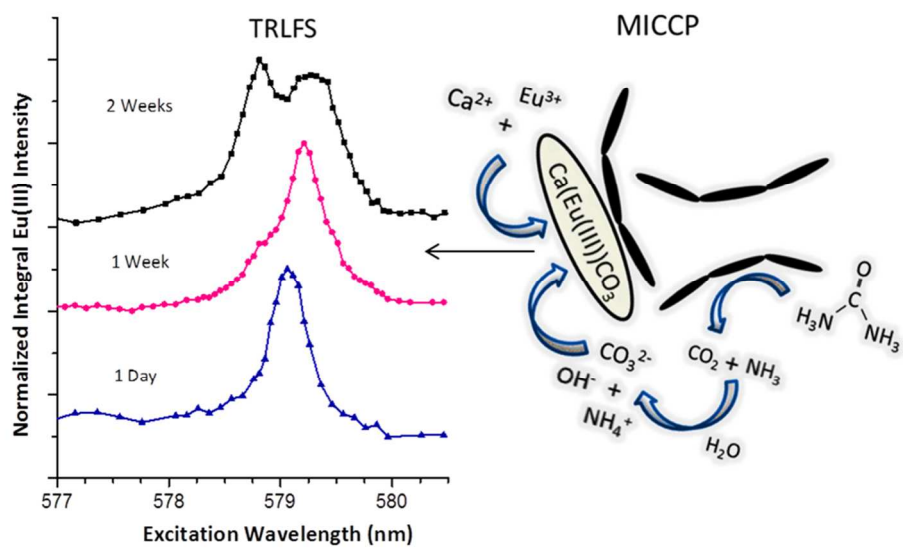
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606 TOC



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TOC graphic

227x131mm (150 x 150 DPI)