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HAL Authorization

1 Cyanobacterial formation of intracellular Ca-carbonates 2 in undersaturated solutions

3 Running title: Cyanobacterial carbonatogenesis in undersaturated solutions

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25 **Abstract**

26 Cyanobacteria have long been thought to induce the formation of Ca-carbonates as secondary
27 byproducts of their metabolic activity, by shifting the chemical composition of their
28 extracellular environment to conditions favoring mineral precipitation. Some cyanobacterial
29 species forming Ca-carbonates intracellularly were recently discovered. However, the
30 environmental conditions under which this intracellular biomineralization process can occur
31 and the impact of cyanobacterial species forming Ca-carbonates intracellularly on extracellular
32 carbonatogenesis are not known. Here, we show that these cyanobacteria can form Ca-
33 carbonates intracellularly while growing in extracellular solutions undersaturated with respect
34 to all Ca-carbonate phases, i.e., conditions thermodynamically unfavorable to mineral
35 precipitation. This shows that intracellular Ca-carbonate biomineralization is an active process,
36 i.e., it costs energy provided by the cells. The cost of energy may be due to the active
37 accumulation of Ca intracellularly. Moreover, unlike cyanobacterial strains that have been
38 usually considered before by studies on Ca-carbonate biomineralization, cyanobacteria forming
39 intracellular carbonates may slow down or hamper extracellular carbonatogenesis, by
40 decreasing the saturation index of their extracellular solution following the buffering of the
41 concentration of extracellular calcium to low levels.

42

43

44 **Introduction**

45 Biomineralization of CaCO₃ by cyanobacteria has been thoroughly studied because of its
46 consequences for the formation of ancient biogenic carbonate deposits and its impact on the
47 global geochemical cycles of carbon and calcium (Riding, 2000; Jansson & Northen, 2010;
48 Gérard *et al.*, 2013; Bundeleva *et al.*, 2014). CaCO₃ biomineralization by cyanobacteria is
49 usually believed to result from their photosynthetic activity (Merz, 1992). More precisely,

50 cyanobacteria actively import inorganic carbon, mostly as HCO_3^- (Miller & Colman, 1980).
51 Intracellular conversion of HCO_3^- to CO_2 by carbonic anhydrases followed by CO_2 fixation by
52 Ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) and the associated import of H^+
53 to regulate intracellular pH result in the increase of extracellular pH, which favors extracellular
54 CaCO_3 precipitation (Verrecchia *et al.*, 1995; Badger & Price, 2003; Riding, 2006). The crucial
55 role of carbonic anhydrases in carbonate biomineralization has been repeatedly noticed, not
56 only for cyanobacteria but also for other calcifying organisms (e.g. in sponges, Jackson *et al.*,
57 2007). At least in some cyanobacteria, $\text{Ca}^{2+}/\text{H}^+$ transmembrane exchangers regulate the
58 intracellular concentration of Ca^{2+} at low levels by exporting Ca extracellularly, favoring
59 further CaCO_3 biomineralization (Jiang *et al.*, 2013). Therefore, for many years, CaCO_3
60 biomineralization by cyanobacteria has been considered as exclusively extracellular and
61 dependent on the chemical conditions prevailing in the extracellular environments of
62 cyanobacterial cells. However, several species of cyanobacteria forming intracellular CaCO_3
63 granules were recently discovered (Couradeau *et al.*, 2012; Benzerara *et al.*, 2014; Moreira *et*
64 *al.*, 2017). Couradeau *et al.* (2012) tentatively suggested that these CaCO_3 granules may serve
65 as ballasts increasing cell density and favoring a benthic mode of life. Alternatively, CaCO_3
66 granules may buffer intracellular pH. Finally, the formation of intracellular carbonates may
67 have no biological function and may just be a byproduct of photosynthesis in cyanobacteria that
68 do not regulate well intracellular pH and/or Ca^{2+} concentrations. Cyanobacteria forming
69 intracellular CaCO_3 were found in various environments, including lakes, soils, karstic ponds
70 and hydrothermal settings across the world (Benzerara *et al.*, 2014; Ragon *et al.*, 2014; Saghäi
71 *et al.*, 2015). This suggests that diverse environmental conditions may allow intracellular
72 calcification but these have yet to be explored further. Moreover, it raises the question of
73 whether ancient cyanobacteria used to induce calcium carbonate precipitation intracellularly or
74 extracellularly. This is important in order to figure out what fossil traces of these

75 microorganisms may be expected in the geological record (Riding, 2012). Indeed,
76 cyanobacteria favoring extracellular carbonatogenesis can get encrusted by the resulting
77 minerals under some conditions, forming calcimicrobes (e.g., Arp et al., 2001; Couradeau *et*
78 *al.*, 2013). In contrast, this may not be the case for cyanobacteria forming carbonates
79 intracellularly (Riding, 2012).

80 At least two different mechanisms of intracellular biomineralization may exist based on the
81 observation of two different distribution patterns of the intracellular CaCO₃ granules in cells
82 (Li *et al.*, 2016): 1) in one clade of cyanobacteria, CaCO₃ granules are mostly located at the
83 poles of the cells and nucleate at the division septum where cells divide. In this case, the
84 involvement of division proteins in the nucleation of ACC has been speculated. 2) In other
85 clades, CaCO₃ granules do not show this polar distribution and are scattered or form chains
86 within the cytoplasm (Li *et al.*, 2016). In all clades, granules are composed of amorphous
87 calcium carbonates (ACC) as determined by transmission electron microscopy selected area
88 electron diffraction (Benzerara et al., 2014). How these mineral phases with a relatively high
89 solubility form in the cytoplasm, supposedly undersaturated with ACC, remains enigmatic
90 (Cam *et al.*, 2015). It has been speculated that unlike cyanobacteria forming extracellular
91 carbonates, intracellularly calcifying cyanobacteria may decrease the saturation of the
92 extracellular solution with respect to CaCO₃ phases or at least not affect it at all (Couradeau *et*
93 *al.*, 2012). However, this assumption lacks experimental evidence. Moreover, it is not clear how
94 the different species of intracellularly calcifying cyanobacteria, especially those showing
95 different biomineralization patterns, may affect the supersaturation of their environment.

96 Here, we followed the changes with time of the chemical composition of the culture media of
97 three strains of cyanobacteria forming intracellular carbonates: *Gloeomargarita lithophora*
98 strain C7, *Thermosynechococcus elongatus* strain BP-1 and *Cyanothece* sp. strain PCC 7425
99 and one strain not forming intracellular carbonates: *Gloeocapsa* sp. strain PCC 73106. CaCO₃

100 granules are scattered within the cytoplasm in *G. lithophora* and *Cyanothece sp.* while they are
101 located at cell poles in *T. elongatus*. We measured cell growth and chemical parameters such
102 as concentrations of dissolved Ca^{2+} and HCO_3^- and pH to assess the saturation of the
103 extracellular solution with various CaCO_3 phases. Moreover, we analyzed the intracellular
104 distribution of Ca by electron microscopy analyses. This allows defining the environmental
105 conditions necessary for intracellular calcification and how intracellularly calcifying
106 cyanobacteria may affect their local environments.

107

108 **MATERIALS AND METHODS**

109 **Cyanobacterial strains and culture conditions**

110 Four cyanobacterial strains were cultured. Three strains encompassing most of the phylogenetic
111 diversity of cyanobacteria that have been shown to form intracellular Ca-carbonates (Benzerara
112 *et al.*, 2014) were studied: *Gloeomargarita lithophora* strain C7 enriched from an alkaline crater
113 lake in Mexico as described by Moreira *et al.* (2017) and showing CaCO_3 granules scattered in
114 the cytoplasm; the axenic strains *Cyanothece sp.* strain PCC 7425 isolated from a rice field in
115 Senegal, described by (Rippka *et al.*, 1979) and (Porta *et al.*, 1999) and showing CaCO_3
116 granules scattered in the cytoplasm; the axenic strain *Thermosynechococcus elongatus* strain
117 BP-1 isolated from a hot spring in Japan, described by Yamaoka *et al.* (1978) and Nakamura *et*
118 *al.* (2002) and showing CaCO_3 granules at the poles. One strain not forming intracellular
119 carbonates (Benzerara *et al.*, 2014) was used as a comparison for Ca uptake: the axenic strain
120 *Gloeocapsa sp.* strain PCC 73106 isolated from a *Sphagnum* bog in Switzerland and described
121 by (Rippka *et al.*, 1979). The choice of this strain as a control was motivated by the fact that
122 *Gloeocapsa* was previously studied for its capabilities to induce extracellular carbonatogenesis
123 (Bundeleva *et al.*, 2014) and *Gloeocapsa sp.* strain PCC 73106 can be cultured in the same
124 medium as the three strains forming intracellular carbonates. It should be noted that

125 extracellular CaCO₃ precipitation by *Gloeocapsa sp.* was observed in solutions different from
126 the BG11 medium used in the present study, in particular with much higher initial
127 concentrations of dissolved Ca: 5-10 mM in Bundeleva et al. (2014) vs. 250 μM, here.
128 Strains were cultured in triplicates in medium BG-11 (Rippka *et al.*, 1979), under continuous
129 light (5-10 μmol.m².s⁻¹) at 45 °C for *T. elongatus* and 30 °C for *G. lithophora*, *Cyanothece sp.*
130 and *Gloeocapsa sp.* BG-11 is classically used to culture freshwater cyanobacterial strains. Its
131 composition is available on <http://cyanobacteria.web.pasteur.fr/>. It mostly contains Na⁺ and
132 NO₃⁻ with ~180 μM of orthophosphates and ~250 μM of calcium. Evaporation was
133 compensated by daily addition of sterile milli-Q water. The optical density (OD) of the cultures
134 was measured at 730 nm to assess cell growth. The combined measurement of OD and cell
135 counting on one sample allowed to derive the relationships between OD and cell density as
136 9×10^7 , 3×10^7 , 2.5×10^7 and 6.4×10^7 cells.mL⁻¹.OD unit⁻¹ for *G. lithophora*, *Cyanothece sp.*,
137 *T. elongatus* and *Gloeocapsa sp.*, respectively. Similarly, the relationships between OD and cell
138 dry mass were 3.6×10^{-4} , 2.4×10^{-4} and 3.2×10^{-4} g.mL⁻¹.OD unit⁻¹ for *G. lithophora*,
139 *Cyanothece sp.* and *T. elongatus*, respectively.

140

141 **Bulk chemical analyses of solutions**

142 For chemical analyses, culture samples were centrifuged at 5,000 g for 10 min. The pH was
143 measured in the supernatants. Variations of pH in non-inoculated sterile controls were less than
144 0.1. Supernatants were systematically filtered at 0.22 μm.

145 The concentration of dissolved calcium in filtered supernatants was measured by colorimetry
146 based on the method described by (Moorehead & Biggs, 1974). Twenty-five microliters of
147 filtered sample were added to 1 mL of a mix solution containing o-cresolphthalein complexone,
148 hydrochloric acid, 8-hydroxyquinoline and 2-amino-2-methyl-1-propanol. The OD of the
149 resulting solution was measured at 570 nm. Standard solutions of calcium at 1, 0.5, 0.25, 0.125,

150 0.1 and 0.05 mM were used for calibration. The detection limit for colorimetry measurements
151 was 0.02 mM. Uptake rates normalized by the number of cells were determined for each time
152 step as followed:

$$153 \frac{[Ca^{2+}]_{t-1} - [Ca^{2+}]_t}{\text{average cell density between } t_{-1} \text{ and } t} / (t - t_{-1})$$

154 Where t and t₋₁ were consecutive sampling times, and the average density between t₋₁ and t was
155 obtained as the arithmetic average of cell density derived from OD measurements. An
156 alternative approach to measuring removal of dissolved Ca from the solution, would have been
157 to measure Ca in the cells. This would involve an additional step of chemical extraction of Ca
158 from the cells. We tested that these two approaches provide similar result (data not shown).

159 The total alkalinity is a form of mass-conservation relationship for hydrogen ion and is defined
160 as: “the number of moles of hydrogen ion equivalent to the excess of proton acceptors (bases
161 formed from weak acids with a dissociation constant $K \leq 10^{-4.5}$, at 25°C and zero ionic strength)
162 over proton donors (acids with $K > 10^{-4.5}$) in one kilogram of sample” (Sarazin *et al.*, 1999).

163 Here, alkalinity (Alk) was defined as:

$$164 \text{Alk} = [\text{HCO}_3^-] + 2[\text{CO}_3^{2-}] + [\text{HPO}_4^{2-}] + 2[\text{PO}_4^{3-}] + [\text{NH}_3] + [\text{OH}^-]$$

165 Alkalinity was measured by colorimetry. Five hundred microliters of filtered sample were
166 added to 500 μL of a solution composed of formic acid at 3.5 mM and bromothymol blue at
167 30 $\text{mg}\cdot\text{L}^{-1}$. The OD of the solution was measured at 590 nm and was related to the alkalinity by
168 a second order relation (Sarazin *et al.*, 1999). The calibration was performed using standard
169 NaHCO_3 solutions with concentrations between 0.5 and 3.5 mM with steps of 0.25.

170 Dissolved inorganic phosphorus (DIP) and ammonium (ΣNH_3) concentrations were measured
171 by continuous flow colorimetry on a QuAAtro Axflow (Seal Analytical). Concentrations of
172 dissolved chloride, sulfate, nitrite and nitrate were measured by ion-exchange chromatography
173 using an ICS1100 Thermofisher on a Ionpac thermo AS14 column with an eluant composed of
174 3.5 mM of Na_2CO_3 and 1 mM of NaHCO_3 with a flow rate of 1.2 $\text{mL}\cdot\text{min}^{-1}$. Nitrites and nitrates

175 were measured using a UVD340U detector. Concentrations of HCO_3^- were deduced from pH,
176 alkalinity, dissolved phosphorus and ammonium measurements.

177 Concentrations of major dissolved cations, including Ca were measured using a Thermo
178 Scientific™ iCAP™ 6200 inductively coupled plasma atomic emission spectrometer (ICP-
179 AES) equipped with a Cetac ASX-520 autosampler. For ICP-AES analyzes, 300 μL of filtered
180 supernatants were acidified with 10 mL of 2 % HNO_3 . Measurements of Ca concentrations by
181 ICP-AES and colorimetry were correlated along a 1:1 line with consistency better than 85 %
182 (r^2 for the regression) above 50 μM .

183 **Speciation and saturation indices calculations**

184 The Visual MINTEQ (3.0) software (Gustafsson, 2013) with the Davies method (Davies *et al.*,
185 1962) was used to calculate the concentrations of species in the culture medium based on bulk
186 chemical analyses. The cultures were supposed to be in free exchange with the atmosphere with
187 a partial CO_2 pressure of 380 ppm following the procedure by (Siong and Asaeda, 2009). The
188 saturation index was defined as:

$$189 \quad \text{SI} = -\log [(\text{CO}_3^{2-})(\text{Ca}^{2+})/\text{Ks}]$$

190 Where () denotes the activity and Ks the solubility of a given phase. Saturation indices were
191 calculated for all Ca-carbonate phases reported in the Visual MINTEQ database as well as for
192 ACC using a Ks of 2.32×10^{-8} (Kellermeier *et al.*, 2014).

193

194 **Scanning transmission electron microscopy (STEM) and energy dispersive x-ray 195 spectrometry (EDXS) analyses**

196 For microscopy analyses, cell pellets obtained by centrifugation were washed three times with
197 milli-Q water before resuspension in 500 μL of milli-Q water and deposition of 3 μL on carbon-
198 coated 200-mesh copper grids. Washing was necessary to avoid precipitation of salts upon
199 drying. This sample preparation procedure was repeatedly used and tested in past studies on

200 cyanobacteria forming intracellular carbonates (Benzerara *et al.*, 2014; Li *et al.*, 2016).
201 Although it may induce some alterations of the morphology of the cells (e.g., collapse), it allows
202 the preservation of intracellular CaCO₃ inclusions on the contrary to procedures using chemical
203 fixatives (Li *et al.*, 2016). The same procedure was also successfully used to study extracellular
204 Ca-phosphate precipitates formed by bacterial cells (e.g., Cosmidis *et al.*, 2015). In the present
205 study, only STEM analyses of CaCO₃ inclusions are discussed, not cell morphological features.
206 STEM analyses were performed in the High Angle Annular Dark Field (HAADF) mode using
207 a JEOL 2100F operating at 200 kV and equipped with a field emission gun, a high-resolution
208 UHR pole piece, and a JEOL EDXS detector with an ultrathin window allowing detection of
209 light elements. Semi-quantitative analyses of EDXS spectra was done using the JEOL Analysis
210 Station software following the procedure by (Li *et al.*, 2016). This was based on the use of K-
211 factors. After subtracting out the background noise in the EDXS spectrum, the software
212 performed a Gaussian fit of selected elemental peaks and calculated the area under the peaks.
213 From this, the atomic percentage of selected element was assessed: Ca, Mg in the carbonates;
214 Ca, Mg, K and P in polyphosphates.

215

216 **RESULTS**

217 **Cell growth and temporal changes of chemical parameters in growth medium**

218 All strains grew in BG-11 at different rates (Fig. 1 and Fig. S1). During the exponential growth,
219 generation times were 108 ± 2 h, 46 ± 1 h, 23 ± 2 h and 163 ± 19 h for *G. lithophora*,
220 *Cyanothece* sp., *T. elongatus*, and *Gloeocapsa* sp., respectively (Fig. S1). In parallel, pH
221 increased at different rates and reached different values for the different strains, starting from
222 7.5 up to 8.8, 9.2, 9.8 and 9.7 for *G. lithophora*, *Cyanothece* sp., *T. elongatus* and
223 *Gloeocapsa* sp., respectively (Fig. 1). In contrast, the pH, optical density at 730 nm (OD) and

224 dissolved [Ca] remained constant over the duration of the experiment in a non-inoculated
225 control BG-11 medium (Fig. S2).

226 The time evolution of dissolved extracellular Ca was significantly different for strains forming
227 intracellular carbonates *vs.* *Gloeocapsa sp.*, which does not form intracellular Ca-carbonates.
228 Calcium concentration decreased dramatically from $\sim 250 \mu\text{M}$ down to 2.75 ± 0.54 , 5.9 ± 1.3
229 and $12.8 \pm 3.6 \mu\text{M}$ ($n=3$) for *G. lithophora*, *Cyanothece sp.* and *T. elongatus*, respectively
230 (Table S1). For *G. lithophora* and *T. elongatus*, the decrease of Ca concentration varied in the
231 opposite way to the OD and was linear with time at rates of 0.47 ± 0.06 and $0.79 \pm 0.07 \mu\text{M}$ of
232 Ca per hour. For *Cyanothece sp.*, the decrease of Ca concentration also varied oppositely to OD
233 but there was a short transient phase during which the concentration of dissolved Ca stopped
234 decreasing and OD stopped increasing. This transient phase was not systematically observed in
235 cultures of *Cyanothece sp.* Overall, when normalized to the number of cells, the Ca uptake rates
236 varied in time and between strains from 0.07 to $0.01 \text{ fmol}\cdot\text{h}^{-1}\cdot\text{cell}^{-1}$, 0.11 to $0.001 \text{ fmol}\cdot\text{h}^{-1}\cdot\text{cell}^{-1}$
237 and 0.42 to $0.01 \text{ fmol}\cdot\text{h}^{-1}\cdot\text{cell}^{-1}$ for *G. lithophora*, *Cyanothece sp.* and *T. elongatus*, respectively
238 (Fig. S3). In contrast to what was observed for strains forming intracellular carbonates,
239 dissolved Ca concentration remained relatively constant for *Gloeocapsa sp.* cultures with only
240 a slight decrease down to $183 \mu\text{M}$.

241 The concentration of extracellular dissolved orthophosphates (here mostly HPO_4^{2-}) was
242 measured in cultures where Ca concentration decreased significantly, i.e. cultures of strains
243 forming intracellular Ca-carbonates, in order to test some potential correlation between these
244 two parameters. There was some uptake of Ca and orthophosphates by the cells. Thereafter,
245 what was incorporated by the cells is called the “fraction removed from the solution”. From the
246 measurements of dissolved Ca and orthophosphate at different times, it was possible to calculate
247 the Ca/P of this fraction: it corresponds to the ratio between the amount of Ca and the amount
248 of P removed from the solution between two consecutive time points (Fig. 2). Different

249 temporal evolutions were observed depending on the strains. For *G. lithophora*, the
250 concentration of dissolved orthophosphates decreased with time but not in a constant ratio with
251 Ca (Fig. 2). Indeed, the Ca/P ratio of the fraction removed from the solution decreased
252 continuously from more than 2 down to around 0 when the concentration of dissolved Ca
253 leveled down at 2.75 μM . For *Cyanothece* sp., there was only a slight decrease of dissolved
254 orthophosphates from 170 down to 100 μM with a Ca/P of the fraction removed from the
255 solution varying between 4 and 8 (Fig. 2). Finally, there was almost no variation of dissolved
256 orthophosphates in *T. elongatus* cultures. Therefore, no correlation was observed overall
257 between the temporal evolutions of dissolved Ca^{2+} and HPO_4^{2-} concentrations.

258 In all cultures, alkalinity (mostly $[\text{HCO}_3^-]$) increased continuously up to 3204, 4093 and
259 3591 μM for *G. lithophora*, *Cyanothece* sp. and *T. elongatus*, respectively (Fig. S4). This was
260 due to the dissolution of atmospheric CO_2 which increased at increasing pH in BG11. Alkalinity
261 was almost equal to $[\text{HCO}_3^-]$ in *G. lithophora* cultures. Some differences between alkalinity
262 and $[\text{HCO}_3^-]$ (20 to 33 %, i.e., 0.8 to 1.2 mM) were observed in the late stages of the cultures
263 of *Cyanothece* sp. and *T. elongatus*, due mostly to elevated concentrations of OH^- and
264 CO_3^{2-} present in the solution (Fig. S4).

265 Moreover, Mg and S concentrations decreased while an increase of NO_2^- concentration was
266 observed (Table S2-4). The measurement of all these concentrations allowed to calculate the
267 speciation of elements in the extracellular solutions and their saturation indices (SI) with respect
268 to different mineral phases, including Ca-carbonate and Ca-phosphate phases (Fig. 3;
269 Table S5-7). For *G. lithophora*, the culture medium was constantly undersaturated with all
270 Ca-carbonate phases, including ACC (Fig. 3). In contrast, the solution was supersaturated with
271 hydroxyapatite at least until 650 h with SI values varying between 4.9 and 6.9 (Table S5). It
272 became undersaturated with this phase after 650 h. For *Cyanothece* sp., solutions were always
273 undersaturated with ACC and slightly supersaturated with calcite at two time points only (114

274 and 282 h; SI of around 1 and 0.2; Table S6). For *Thermosynechococcus elongatus*, solutions
275 were slightly supersaturated with ACC at 162 and 234 h (SI of around 0.4 and 0.25,
276 respectively; Table S7).

277

278 **Intracellular distribution of Ca**

279 The mass of incorporated Ca normalized to the total cell dry mass was estimated from bulk
280 measurements for *G. lithophora*, *Cyanothece* sp. and *T. elongatus* cells (Fig. S5). Two
281 processes impacted oppositely the time variation of this parameter: on the one hand, Ca uptake
282 increased the normalized Ca mass content; on the other hand, cell division decreased it. The
283 normalized mass of Ca at ~700 h amounted to 26 ± 3 , 13 ± 2 and 15 ± 3 mg/g for *G. lithophora*,
284 *Cyanothece* sp. and *T. elongatus*, respectively (Fig. S5). A slight decrease of the cellular mass
285 proportion of Ca was observed for each strain after the time when extracellular dissolved Ca
286 reached its minimum. This can be explained by the fact that cellular division was still
287 continuing, while there was only little Ca left in the solutions. Standard deviations were too
288 high to infer precisely the evolution of the cellular mass proportion of Ca in the first stages of
289 growth.

290 In parallel, STEM observations were performed on cells pelleted at different stages of the
291 culture (Fig. 4-6; Table S8-10). No extracellular Ca-containing precipitate was observed in the
292 pellets. In contrast, most of *G. lithophora* (Fig. 4), *Cyanothece* sp. (Fig. 5) and *T. elongatus*
293 cells (Fig. 6) contained intracellular granules with Ca. EDXS maps showed the presence of two
294 different types of Ca-containing granules: 1) Ca-carbonates and 2) polyphosphates containing
295 Mg and K and some Ca. Cells of the inoculum ($t=0$ h) appeared similar to cells observed by
296 TEM at other stages and contained Ca-carbonates and polyphosphates as well (Table S8-10).
297 *Gloeocapsa* sp. cells only contained polyphosphate granules with a little amount of Ca (Fig.
298 S6).

299 *Gloeomargarita lithophora* cells contained a relatively constant number and volume of Ca-
300 carbonates upon time, i.e., 7.8 ± 4.1 inclusions/cell and $0.058 \pm 0.045 \mu\text{m}^3/\text{cell}$, respectively
301 (Table S8). Averages were calculated based on the number of observed cells, excluding cells
302 which did not contain any intracellular granule. These cells with no intracellular granule
303 represented 6 to 22 % of the total number of cells. The $\text{Mg}/(\text{Ca}+\text{Mg})$ ratio in the intracellular
304 carbonates formed by *G. lithophora* was relatively constant between 9.8 and 14.8 % during
305 most of the culture with one exception at 954 h when $\text{Mg}/(\text{Ca}+\text{Mg})$ ratios of 39 ± 18 % were
306 measured. Considering the cellular mass proportion of Ca and a density of $2.18 \text{ g}\cdot\text{cm}^{-3}$ for ACC
307 (Fernandez-Martinez *et al.*, 2013), Ca contained in carbonate granules as observed by STEM
308 represented between few percents and up to half of the total Ca bioaccumulated by
309 *G. lithophora* cells. Since the measurements of cellular mass proportion of Ca by bulk analyses
310 (ICP-AES) and STEM were independent and each was affected by relatively high uncertainties,
311 the proportion of Ca contained by carbonates has to be considered as a very rough first order
312 estimate.

313 *Cyanothece* sp. cells contained a relatively lower number of inclusions per cell (4.8 ± 2.8) but
314 with larger diameters, representing a larger volume on average (Table S9). Between 8 and 36 %
315 of the observed cells did not contain any inclusions in *Cyanothece* sp. cultures. $\text{Mg}/(\text{Ca}+\text{Mg})$
316 ratios of the carbonate granules were constant at around 4 %. Overall, Ca contained in
317 carbonates represented between 16 and 76 % of total Ca in the cells.

318 Finally, *T. elongatus* cells contained a more variable number of inclusions, between 4 and 23,
319 per cell (Table S10). The proportion of empty cells varied between 2 % and 21 %. Similarly to
320 *Cyanothece* sp. cells, $\text{Mg}/(\text{Ca}+\text{Mg})$ ratios were constant at a low value (~ 4 %) except at 525 h,
321 when they were slightly higher. Ca contained in carbonates represented a lower proportion
322 (1.5-29 %) than what was estimated on average for *G. lithophora* and *Cyanothece* sp.

323 Overall, the different strains forming intracellular carbonates accumulated Ca with high affinity
324 down to concentrations of few μM in the solutions, whatever their growth temperature and
325 growth rate (Table S1). Moreover, it appears that the average number of CaCO_3 inclusions per
326 cell and their diameter did not vary much with incubation time in a given strain. Therefore, it
327 was not possible to infer the formation of CaCO_3 inclusions by observing single cells by TEM
328 only (Table S8-10). However, with incubation time, there was an increasing number of cells
329 with a constant number of inclusions per cell, i.e., an increasing total number of CaCO_3
330 inclusions in the cultures, attesting intracellular precipitation of ACC in cultures.

331

332 **DISCUSSION**

333

334 **Changes of dissolved Ca concentrations are due to high cellular uptakes by intracellularly** 335 **calcifying cyanobacteria**

336 The concentration of dissolved Ca decreased significantly in the culture media of intracellularly
337 calcifying cyanobacteria down to a few micromoles per liter. Several previous studies
338 interpreted a similar decrease as the result of cellular uptake, although they sometimes used Ca
339 concentrations of several millimolar, i.e., higher than in our study and therefore corresponding
340 to a high supersaturation with respect to Ca-containing phases (Singh & Mishra, 2014).
341 However, it has to be noticed that such a decrease of dissolved Ca concentrations may have two
342 different origins: 1) extracellular precipitation of a Ca-rich phase and/or 2) uptake by the cells
343 which includes sorption at their surface and accumulation within the cells. Here, culture media
344 were supersaturated only with hydroxyapatite ($\text{SI} = 0.9$ to 10.1) at several time points for
345 different strains. Therefore, Ca-phosphate precipitation may theoretically have occurred.
346 However, several observations, when considered altogether, suggest that if Ca-phosphate

347 precipitation occurred, the decrease of dissolved Ca^{2+} was clearly not due to this process only
348 and cellular uptake and intracellular CaCO_3 precipitation were significant in all cases:

349 1) Sterile BG-11 was supersaturated with hydroxyapatite but no Ca decrease was observed
350 with time in non-inoculated controls. While this control is different in terms of pH and
351 the absence of possibly nucleating cell surfaces, it shows that supersaturation does not
352 imply effective precipitation.

353 2) Ca/P ratios of the fractions removed from the solutions were not constant over time, and
354 were very different between the cultures of different strains (Fig. 2). Therefore, the
355 fractions removed from the solution could not be a Ca-phosphate phase only, which
356 would have been characterized by a relatively constant Ca/P ratio. Moreover, Ca/P ratios
357 of the fractions removed from the solutions were most of the time not equal to the Ca/P
358 ratio of usual Ca-phosphates, which are comprised between 0.5 and 2 (Cosmidis *et al.*,
359 2015).

360 3) No extracellular Ca-phosphate precipitate was detected by STEM observations in all the
361 cultures.

362 4) Although extracellular solutions were supersaturated with hydroxyapatite at several
363 time points for *T. elongatus* cultures with a SI level up to 10.1 (the highest SI value
364 measured in all cultures), no significant decrease of dissolved P was observed. This
365 suggests that no significant precipitation of hydroxyapatite occurs at the SI values
366 measured in all these cultures. Under these conditions, despite the presence of cell
367 surfaces and high supersaturation, no Ca-phosphate precipitation occurred.

368 5) For *G. lithophora* cultures, extracellular solutions became undersaturated with
369 hydroxyapatite and other Ca-phosphate phases after 450 h, indicating that Ca-
370 phosphates did not control the solubility of Ca in these cultures, hence the concentration
371 of extracellular dissolved Ca, in these experiments. Ca-phosphate phases may have in

372 turn precipitated within cells similarly to what was observed for amorphous CaCO₃ but
373 such precipitates were not observed by TEM.

374 In contrast, the formation of polyphosphates, which were observed by STEM within cells of
375 *Cyanothece sp.* and *G. lithophora* but very rarely in *T. elongatus* cells may better explain the
376 observed decrease of dissolved P concentration in the cultures of *G. lithophora* and
377 *Cyanothece sp.*

378 It is known that cyanobacterial surfaces sorb Ca²⁺, at values of ~0.8 to 1.2 mg of calcium per g
379 of dry matter for the cyanobacterium *Gloeocapsa sp.* (Bundeleva *et al.*, 2014) and this
380 sometimes results in the precipitation of Ca-carbonates when extracellular solutions are
381 supersaturated (Schultze-Lam *et al.*, 1992; Dittrich & Sibling, 2006; Obst *et al.*, 2009). It is also
382 known that some Ca can also be complexed by intracellular proteins in bacteria (Gilabert, 2012;
383 Domínguez *et al.*, 2015). Here, analyses by transmission electron microscopy on whole cells
384 could not discriminate between these two pools of Ca. However, discriminating between these
385 two pools is not crucial here since under the conditions used in the present study, most of the
386 Ca was sequestered by polyphosphate and carbonate granules in cyanobacteria forming
387 intracellular carbonates. This was shown by STEM analyses and the fact that amounts of Ca
388 sorbed by *Gloeocapsa sp.* cells were small compared to total amounts of Ca accumulated by
389 intracellularly calcifying strains. The mass of Ca in CaCO₃ inclusions was roughly assessed by
390 TEM measurements, by counting the number of CaCO₃ inclusions per cell, measuring their
391 volume, assessing their Mg/(Mg+Ca) ratios and taking into account the number of empty cells
392 (Table S8-10). The comparison with the total Ca content in cells as measured by ICP-AES,
393 suggested that Ca in CaCO₃ amounted 19-31 % in *G. lithophora*, 16-76 % in *Cyanothece sp.*
394 and 1.5-29 % in *T. elongatus*. Considering all the uncertainties associated with these
395 measurements and the difficulty to compare robustly TEM with ICP-AES measurements, we

396 consider that these numbers are consistent with the idea that ACC are important Ca reservoirs
397 in these cells.

398 The three strains of cyanobacteria forming intracellular carbonates that were studied here
399 accumulated calcium up to 20-40 mg per g of dry matter (2-4 % in mass). In comparison, *E.*
400 *coli* cells accumulate a total of 0.19 mg of calcium per g of dry matter independently of the
401 extracellular calcium concentration (Gangola and Rosen, 1987). Bundeleva *et al.* (2014)
402 determined a maximum uptake of 0.8-1.2 mg of calcium per g of dry matter for the
403 cyanobacterium *Gloeocapsa sp.* Spores of *Bacillus cereus* and *Bacillus megaterium*, which are
404 notorious for being highly enriched in Ca, accumulate 14-23 mg/g of Ca (Foerster & Foster,
405 1966; Shibata *et al.*, 1992). Overall, this suggests that strains of intracellular carbonate-forming
406 cyanobacteria tend to accumulate Ca to a larger extent than other strains, although this should
407 be measured systematically for many other cyanobacterial strains. As a consequence, they also
408 tend to buffer the extracellular dissolved calcium to a low concentration between 3 to 13 μM in
409 batch cultures.

410 One implication of this high Ca uptake capability by cyanobacteria forming intracellular
411 carbonates can be tentatively discussed. The high affinity of these cyanobacteria for Ca may
412 decrease at least locally the concentration of dissolved Ca in the extracellular environment to
413 low values, especially in cases when the local dissolved Ca pool is not replenished as fast as it
414 is consumed by cells. In our batch experiments, where there is no replenishment of dissolved
415 Ca, this decrease lowered saturation of the solutions with respect to Ca-containing phases, such
416 as calcite or hydroxyapatite and therefore inhibited the precipitation of these mineral phases. In
417 nature, if this uptake remains significant, even in supersaturated solutions, this may also inhibit
418 or decrease the kinetics of CaCO_3 precipitation due to the lowering of the saturation index, at
419 least locally around the cells. The formation of intracellular carbonates may therefore decrease
420 the risk of cell encrustation by minerals, which is lethal in many cases (Couradeau *et al.*, 2013;

421 Miot *et al.*, 2015). Interestingly, different strategies have been developed by diverse
422 microorganisms, all allowing to avoid cell encrustation. This is the case for 1) some iron-
423 oxidizing bacteria, which induce a local decrease of pH (Hegler *et al.*, 2010) and/or produce
424 templating extracellular polymers (Chan *et al.*, 2011) and 2) some cyanobacteria forming
425 extracellular carbonates on a proteinaceous template (S layers) which can be shed from time to
426 time (Schultze-Lam *et al.*, 1992). It is also interesting to note that such a lowering of solution
427 saturation by active uptake of Ca inducing Ca-carbonate dissolution has been shown for some
428 cyanobacterial euendoliths (Ramirez-Reinat and Garcia-Pichel, 2012).

429

430 **Intracellular biomineralization is an active process: evidence and origin of the energy cost**

431 Formation of intracellular ACC granules in the extracellular solutions undersaturated with Ca-
432 carbonate suggests that at least locally around the granules, the intracellular solution was
433 supersaturated with ACC, i.e., cellular activity maintained an intracellular chemical
434 composition allowing precipitation of ACC and therefore different from that prevailing in the
435 extracellular solution. Therefore, intracellular CaCO₃ biomineralization is an active process,
436 i.e., it involves some energy cost to maintain a supersaturated environment in a globally
437 undersaturated solution.

438 The origin of this energy cost can be discussed. Parameters controlling CaCO₃ precipitation are
439 the activities of Ca²⁺ and CO₃²⁻. The latter depends on the activity of HCO₃⁻ and pH. Many
440 cyanobacteria actively import HCO₃⁻ using CO₂ concentrating mechanisms (CCM). This results
441 in high intracellular HCO₃⁻ concentrations up to 30 mM as measured in *Synechococcus* sp.
442 Nageli (strain RRIMP N1) and in *Chlorogloeopsis* sp. (strain ATCC 27193) (Badger &
443 Andrews, 1982; Skleryk *et al.*, 1997). Active uptake of bicarbonates may therefore account, at
444 least partly, for the energy cost necessary to intracellular CaCO₃ biomineralization. It would be
445 interesting to measure the δ¹³C composition of intracellular ACC in the future as a way to better

446 assess the potential source of C for these precipitates. However, while many cyanobacteria
447 show CCM capabilities, many do not form intracellular ACC (Benzerara *et al.*, 2014). The
448 specificity of cyanobacteria forming intracellular CaCO₃ may therefore rely on another process.
449 The intracellular pH in cyanobacteria is regulated, between 6.8 and 7.9 based on measurements
450 performed on strains *Arthrospira platensis* and *Synechocystis* sp. PCC 6803 (Belkin &
451 Boussiba, 1991; Jiang *et al.*, 2013). This is however less than the extracellular pH measured
452 here and therefore intracellular pH regulation does not favor intracellular CaCO₃
453 biomineralization. Only regulation of pH within vesicles at a value higher than in the
454 extracellular solution may favor intracellular CaCO₃ granule biomineralization with a cost of
455 energy. Finally, the intracellular concentration of free calcium has been shown to be regulated
456 within cells at very low values, around 100-200 nM with some possible increase up to 2.6 μM
457 in *Anabaena* sp. PCC 7120 (Torrecilla *et al.*, 2000; Barrán-Berdón *et al.*, 2011). This strain
458 does not form intracellular CaCO₃ granules (Benzerara *et al.*, 2014). The maintenance of a low
459 Ca concentration also costs energy but does not favor CaCO₃ biomineralization. In contrast, in
460 cyanobacteria forming intracellular CaCO₃, it is possible that the high Ca uptake that we
461 observed accounts for some of the energy cost. The pH and/or inorganic carbon and Ca
462 concentrations required for CaCO₃ precipitation can be calculated, considering the
463 approximation that the volume in which biomineralization occurs is filled with water containing
464 Ca²⁺ and inorganic carbon only. At a Ca²⁺ concentration of 2.6 μM (i.e., maximum intracellular
465 Ca concentration reported in the literature), this solution would be undersaturated with ACC
466 even at a pH of 13 and 450 mM of inorganic carbon (SI=-1). Therefore, Ca²⁺ concentration is
467 most likely higher at least locally where CaCO₃ granules form. The pH of the solution where
468 ACC forms, possibly within submicrometer-scale compartments, might be locally high. This
469 will be important to determine whether such intracellular pH heterogeneities exist in future
470 studies despite the challenge of measuring such local variations in cells. Considering a pH of

471 7.9, and an inorganic carbon concentration of 30 mM, which are the maxima reported in the
472 literature for the cytoplasm of cyanobacteria, the solution would be saturated with ACC for a
473 Ca^{2+} concentration higher than 441 μM , i.e., higher than the initial Ca concentration in BG-11
474 and much higher than the extracellular dissolved Ca concentrations down to which *G.*
475 *lithophora* ($[\text{Ca}]_{\text{min}}=2.75 \mu\text{M} \pm 0.54$), *T. elongatus* ($[\text{Ca}]_{\text{min}}=12.8 \mu\text{M} \pm 3.6$) and *Cyanothece*
476 *sp.* ($[\text{Ca}]_{\text{min}}=5.9 \mu\text{M} \pm 1.3$) accumulate Ca. Even if intracellular solutions are not pure water
477 and solubility of ACC might be modified by the presence of organics (Giuffre *et al.*, 2013), this
478 suggests that some active concentration of Ca may operate in intracellularly calcifying
479 cyanobacteria involving some energy cost. Accordingly, cyanobacteria forming intracellular
480 carbonates seem to accumulate Ca up to high levels as discussed above. This may appear
481 surprising considering that such high cytoplasmic concentrations of Ca have been suggested to
482 be toxic (e.g., Verhratsky and Parpura, 2014). It will be useful that future studies manage to
483 measure intracellular free Ca^{2+} in cyanobacteria forming intracellular CaCO_3 and compare these
484 concentrations with those measured in cyanobacteria not forming intracellular CaCO_3 .
485 However, one possibility is that intracellularly calcifying cyanobacteria regulate the
486 cytoplasmic concentration of Ca at a low value around 100-200 nM as observed in all other
487 bacteria and form intracellular compartments with a higher Ca concentration, in which Ca-
488 carbonates precipitate (Fig. 7). This would require an active import of Ca from the cytosol to
489 this compartment. The measured Ca uptake rates per cell, which vary with culture age and
490 between species from 0.001 up to 0.42 $\text{fmol}\cdot\text{h}^{-1}\cdot\text{cell}^{-1}$, may depend on the physiological state of
491 the cells as well as the efficiency of the transport systems of the different species. Despite
492 relatively large error bars, uptake rates normalized by cell numbers and measured in the present
493 study under these specific conditions will be interesting to compare with rates measured under
494 varying conditions and other strains in future studies. The presence of a compartment would
495 also mean that intracellular CaCO_3 formation by cyanobacteria is a controlled biomineralization

496 process involving specific cellular structures. Recent attempts to prepare ultramicrotomy thin
497 sections for TEM investigations, preserving ACC granules have been unsuccessful (Li et al.,
498 2016). Therefore, the existence of membranes delimitating potential compartments enclosing
499 ACC granules remains speculative. Future cryo-microscopy observations may help to test
500 further that hypothesis (Li *et al.*, 2016). The selective advantage provided by such an active
501 accumulation of Ca will also need clarification in the future, whether it may serve as a Ca-
502 detoxification or a storage mechanism. Based on the recognition that high Ca²⁺ concentrations
503 can be toxic (Degens and Ittekkot, 1986) and the observation of intracellular Ca-containing
504 amorphous granules in a phylogenetically broad range of animals (Simkiss, 1977), it has been
505 previously suggested that Ca-detoxification is a widespread process. Whatever the origin of the
506 energy cost for intracellular CaCO₃ biomineralization, the present observations have another
507 implication regarding the preservation of fossil traces of this biomineralization capability. Once
508 cells die, if the intracellular and extracellular solutions equilibrate and if the extracellular
509 solution is undersaturated with ACC, ACC granules may dissolve and therefore may not be
510 easily preserved as fossils. This might also explain the observation of cells with no ACC
511 granules here (up to 36 % in *Cyanothece* sp. cultures after 623 h), which may be dead or inactive
512 but this will require further analyses to determine the number of live/dead cells. In contrast,
513 how these ACC granules may possibly transform to crystalline granules or may remain
514 preserved in dead cells when the extracellular solution is supersaturated will be an interesting
515 issue to investigate in order to better assess the fossilization potential of these cyanobacteria in
516 the geological record.

517

518

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527

528 **Supplementary information is available at (journal name)'s website**

529

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677

678

679

680 **Figure legends**

681 **Fig. 1.** Time evolution of pH (open squares), optical density at 730 nm (closed circles) and
682 dissolved Ca (closed triangles). The pH and OD were measured in cultures of *G. lithophora*
683 (A), *Cyanothece* sp. (B), *Thermosynechococcus elongatus* (C) and *Gloeocapsa* sp. (D).
684 Dissolved Ca concentrations are shown separately for cultures of *G. lithophora* (E), *Cyanothece*
685 sp. (F), *Thermosynechococcus elongatus* (G) and *Gloeocapsa* sp. (H). Error bars were
686 calculated based on variations in triplicates and the precision of calcium concentration
687 measurements.

688

689 **Fig. 2.** Time evolution of the concentration of DIP ($[\text{HPO}_4^{2-}]$) and the Ca/P ratio of the fraction
690 incorporated by the cells. A, B and C correspond to DIP in cultures of *Gloeomargarita*
691 *lithophora* (A), *Cyanothece* sp. (B) and *Thermosynechococcus elongatus* (C). For all three
692 strains, error bars were calculated based on variations in triplicate cultures. When not visible,
693 error bars are smaller than the size of the symbols. Ca/P ratios of the fraction incorporated by
694 the cells are shown separately for cultures of *Gloeomargarita lithophora* (D), *Cyanothece* sp.
695 (E) and *Thermosynechococcus elongatus* (F). For these graphs, the three replicates are
696 represented by the different symbols (circle for replicate 1, triangle for replicate 2 and square
697 for replicate 3).

698

699 **Fig. 3.** Time evolution of the saturation index (SI) of solutions with calcite (filled circles) and
700 amorphous calcium carbonate (filled triangles) in cultures of *Gloeomargarita lithophora* (A),
701 *Cyanothece* sp. (B) and *Thermosynechococcus elongatus* (C). Error bars were calculated based
702 on variations in triplicates.

703

704 **Fig. 4.** STEM-EDXS analyses of *Gloeomargarita lithophora* cells collected after 186 h (A, B
705 and C), and 645 h (D, E and F). (A and D) STEM-HAADF images showing bright carbonates
706 (red circles) and light grey polyphosphate (green circles) granules. (B and E) Corresponding
707 EDXS maps of carbon (blue), phosphorus (green) and calcium (red). (C and F) EDXS spectra
708 of P-granules (green) and the Ca-granules (red) shown in A and D. Time points at which cells
709 were collected are reported on the growth curve.

710

711 **Fig. 5.** STEM-EDXS analyses of *Cyanothece* sp. cells collected after 114 h (A, B and C) and
712 624 h (D, E and F) of culture. (A and D) STEM-HAADF images showing bright carbonates
713 (red circles) and light grey polyphosphate (green circles) granules. (B and E) Corresponding
714 EDXS maps of carbon (blue), phosphorus (green) and calcium (red). (C and F) EDXS spectra
715 of P-granules (green), Ca-granules (red) and cell zone without granules shown in A and D.
716 Time points at which cells were collected are reported on the growth curve.

717

718 **Fig. 6.** STEM-EDXS analyses of *Thermosynechococcus elongatus* cells collected after 162 h
719 (A, C, D and E) and 525 h (B, F, G and H) of culture. (A, B, C and F) STEM-HAADF images
720 showing bright carbonates (red circles) and light grey polyphosphate (green circles) granules.
721 (D and G) Corresponding EDXS maps of carbon (blue), phosphorus (green) and calcium (red).
722 (E and H) EDXS spectra of P-granules (green) and Ca-granules (red) shown in C and F. Time
723 points at which cells were collected are reported on the growth curve.

724

725 **Fig. 7.** Scheme showing one hypothesis for the formation of intracellular carbonate inclusions.
726 In this scenario, calcium exchanges between the cytoplasm and the extracellular medium follow
727 the classical scheme as described in the literature, i.e., calcium is passively transported inwards
728 and actively exported outwards. Moreover, Ca is actively transported from the cytoplasm to a

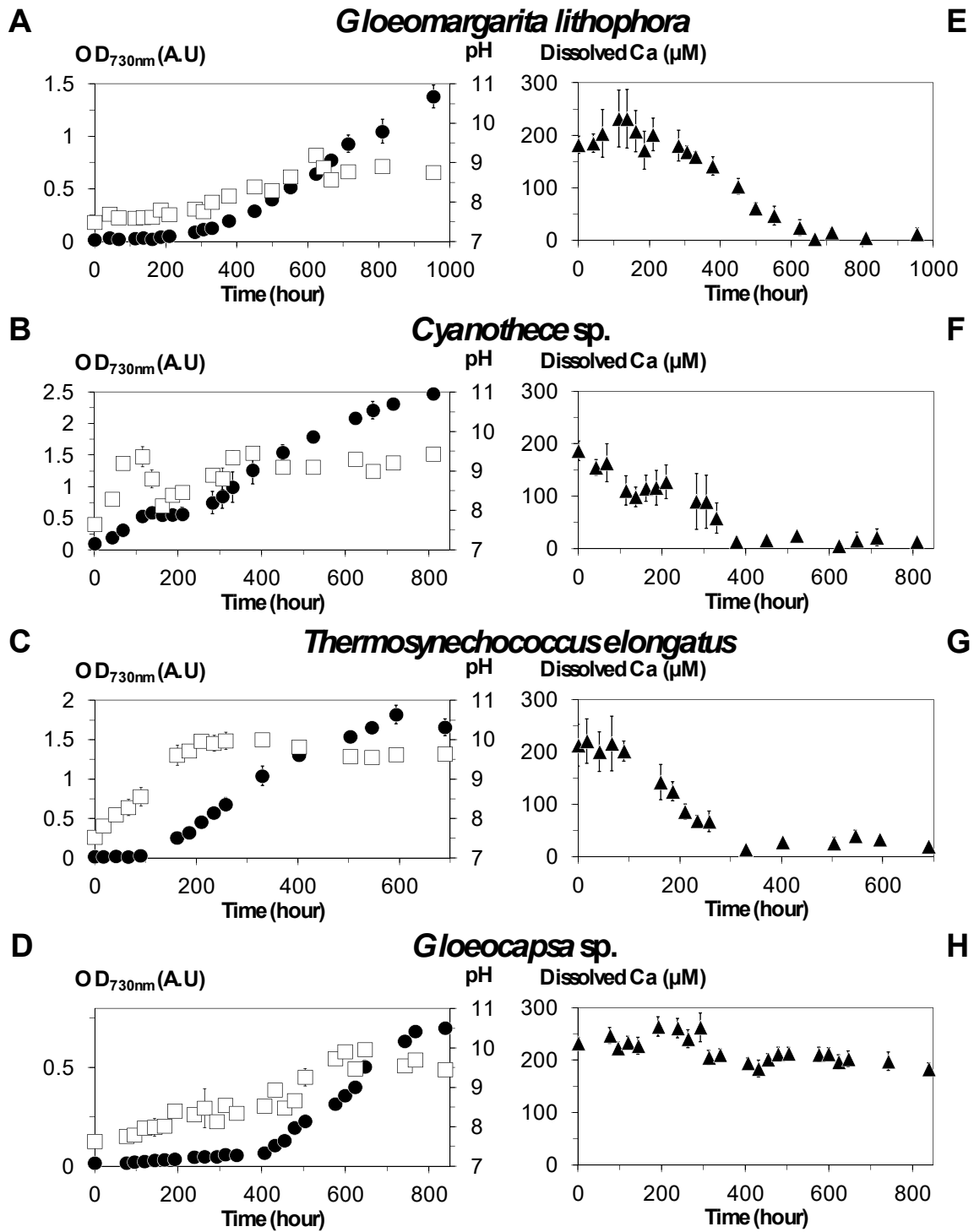
729 putative intracellular compartment where Ca-carbonate granules form. The thin and thick
730 arrows correspond to passive and active transport of calcium, respectively.

731

732

733

Figure 1



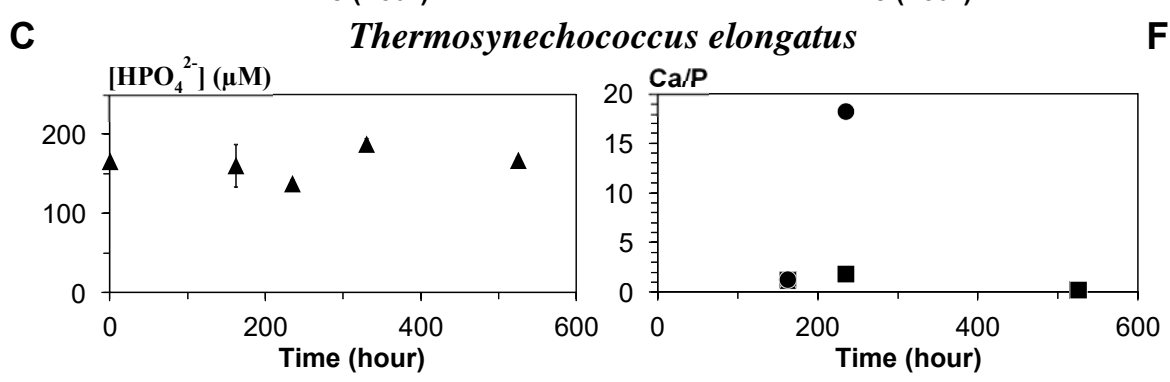
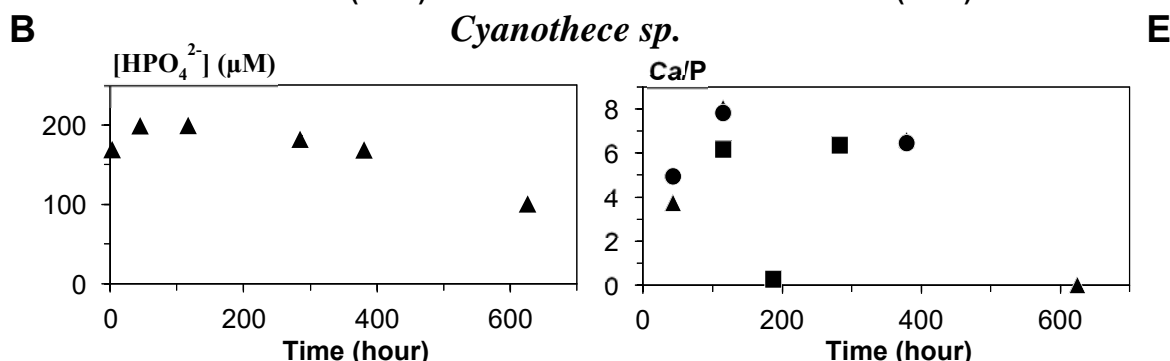
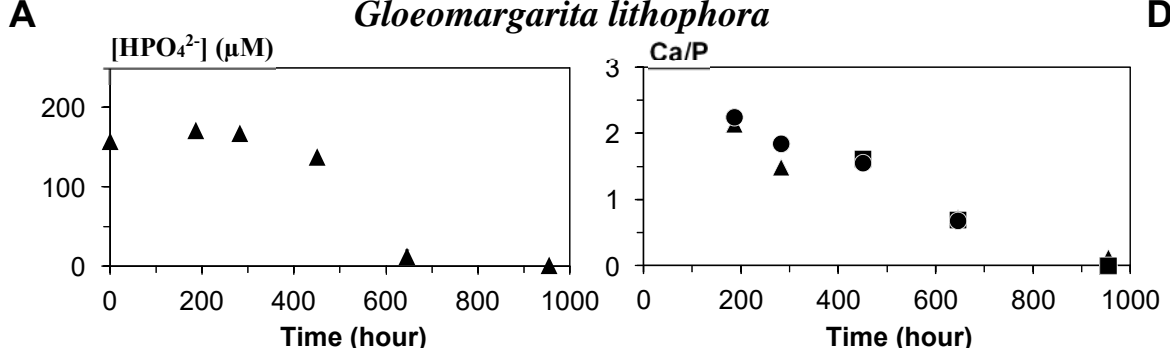


Figure 3

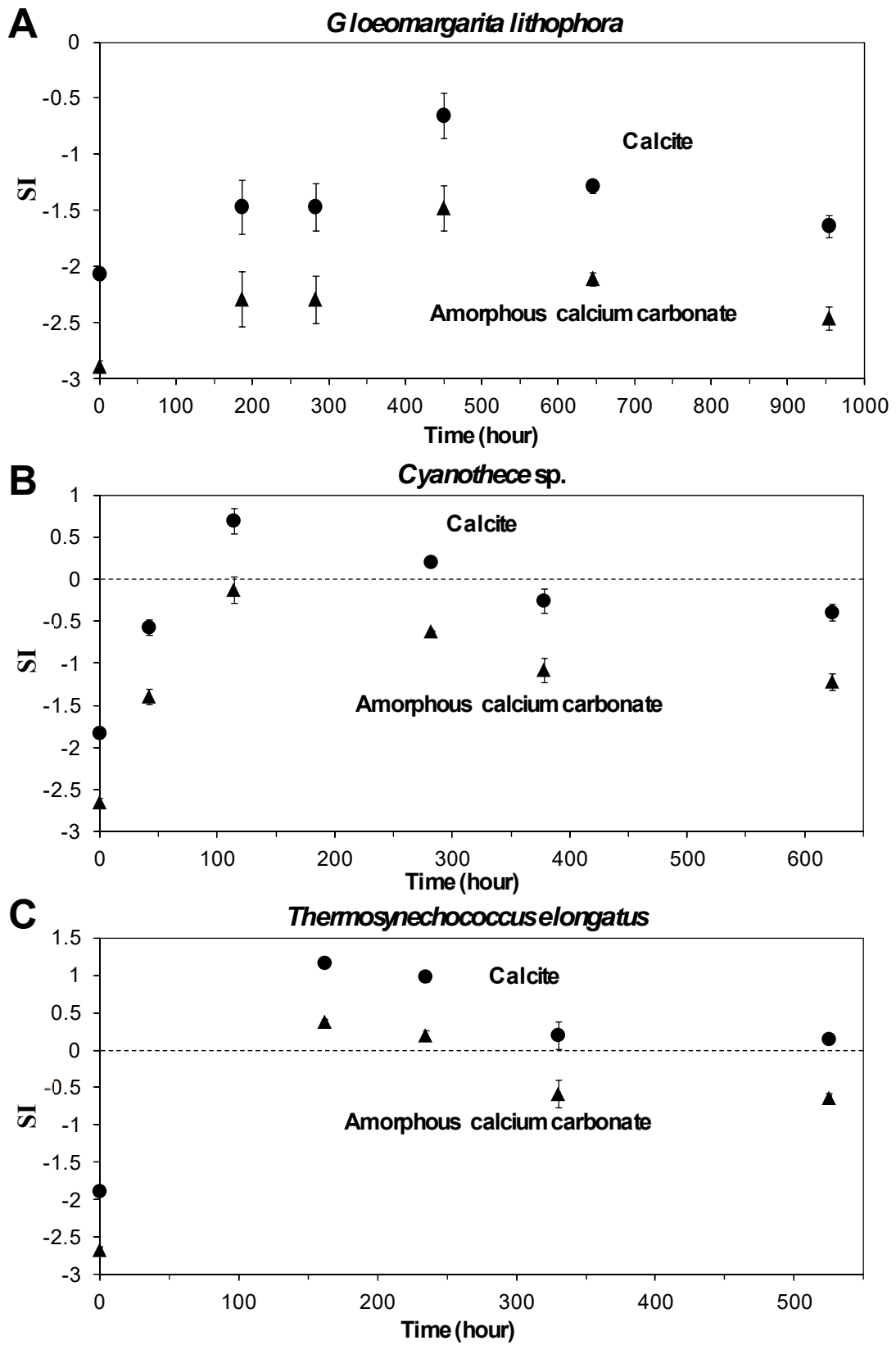


Figure 4

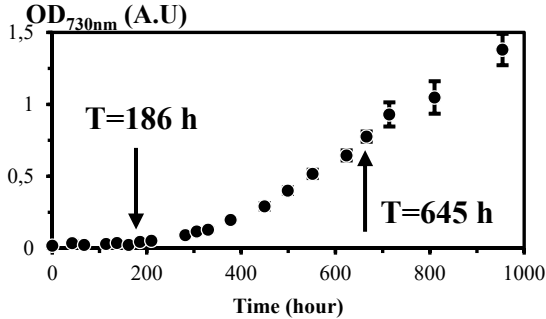
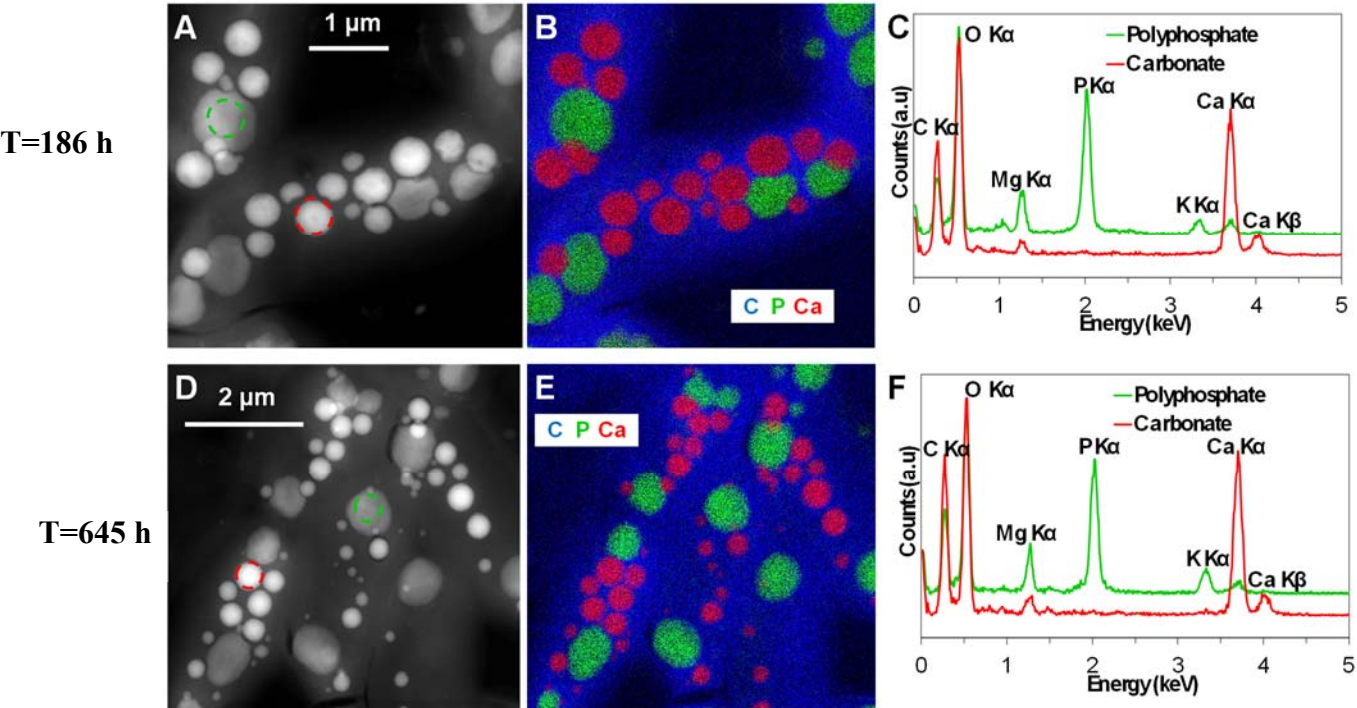
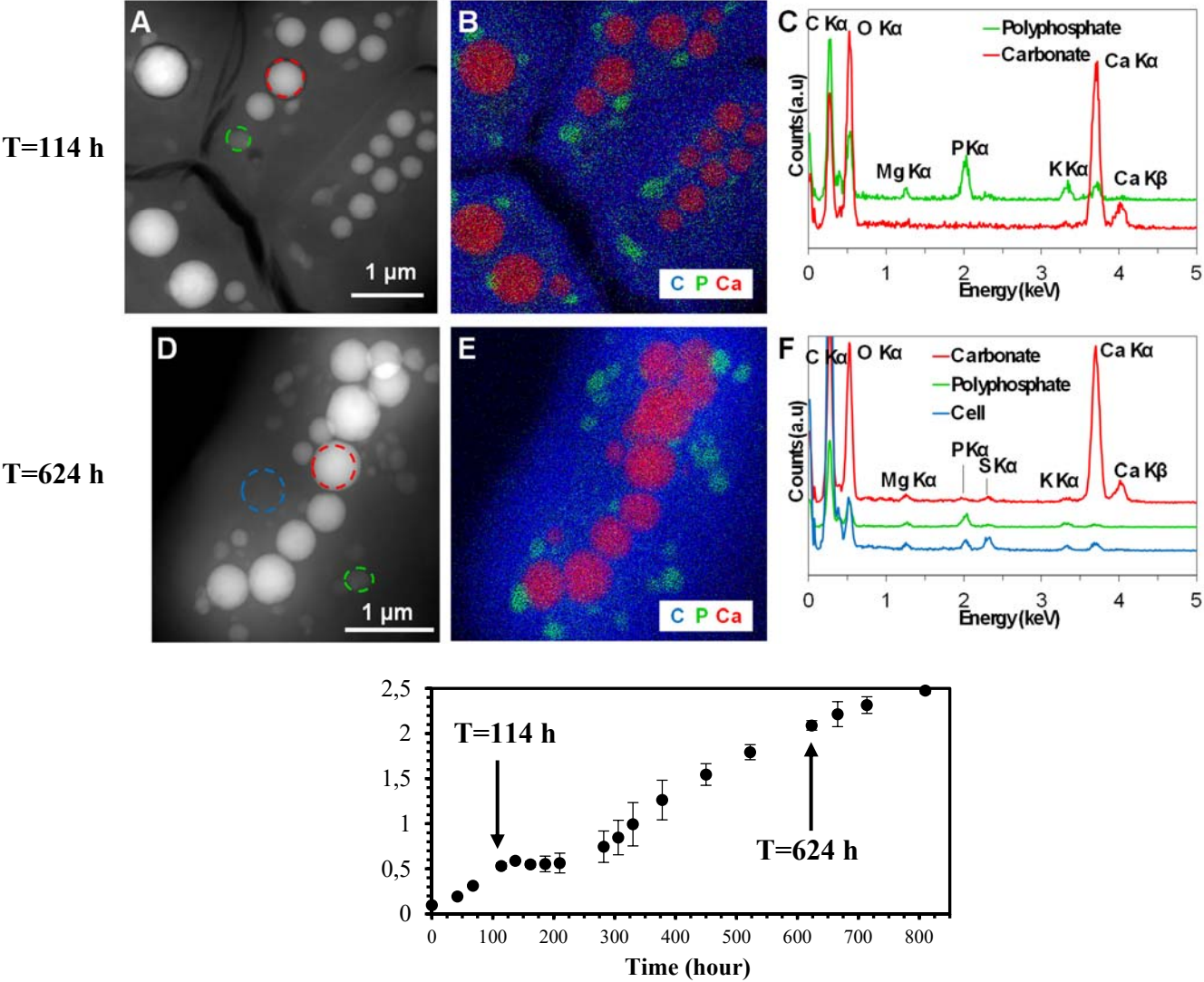


Figure 5



1 Figure 6

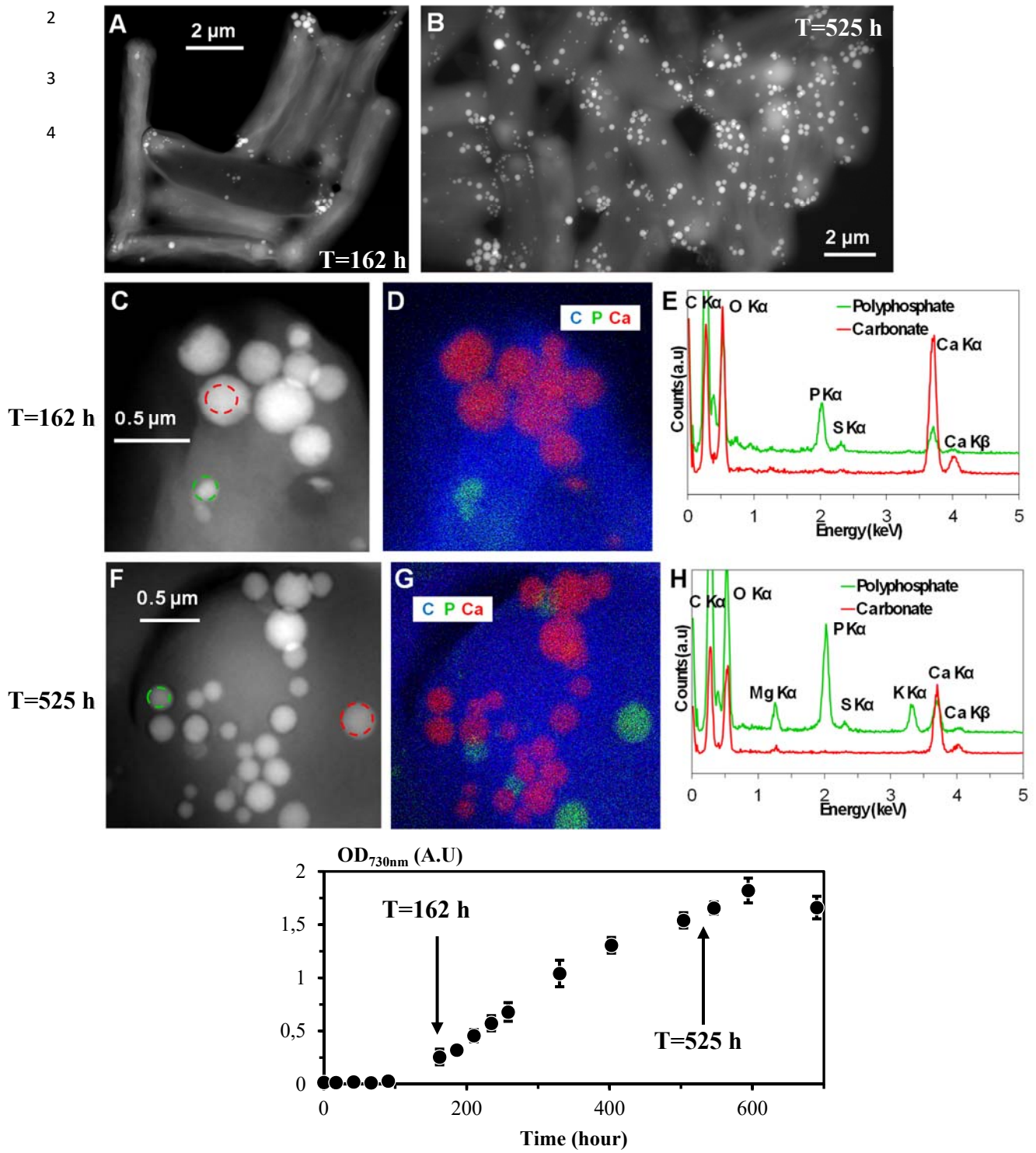
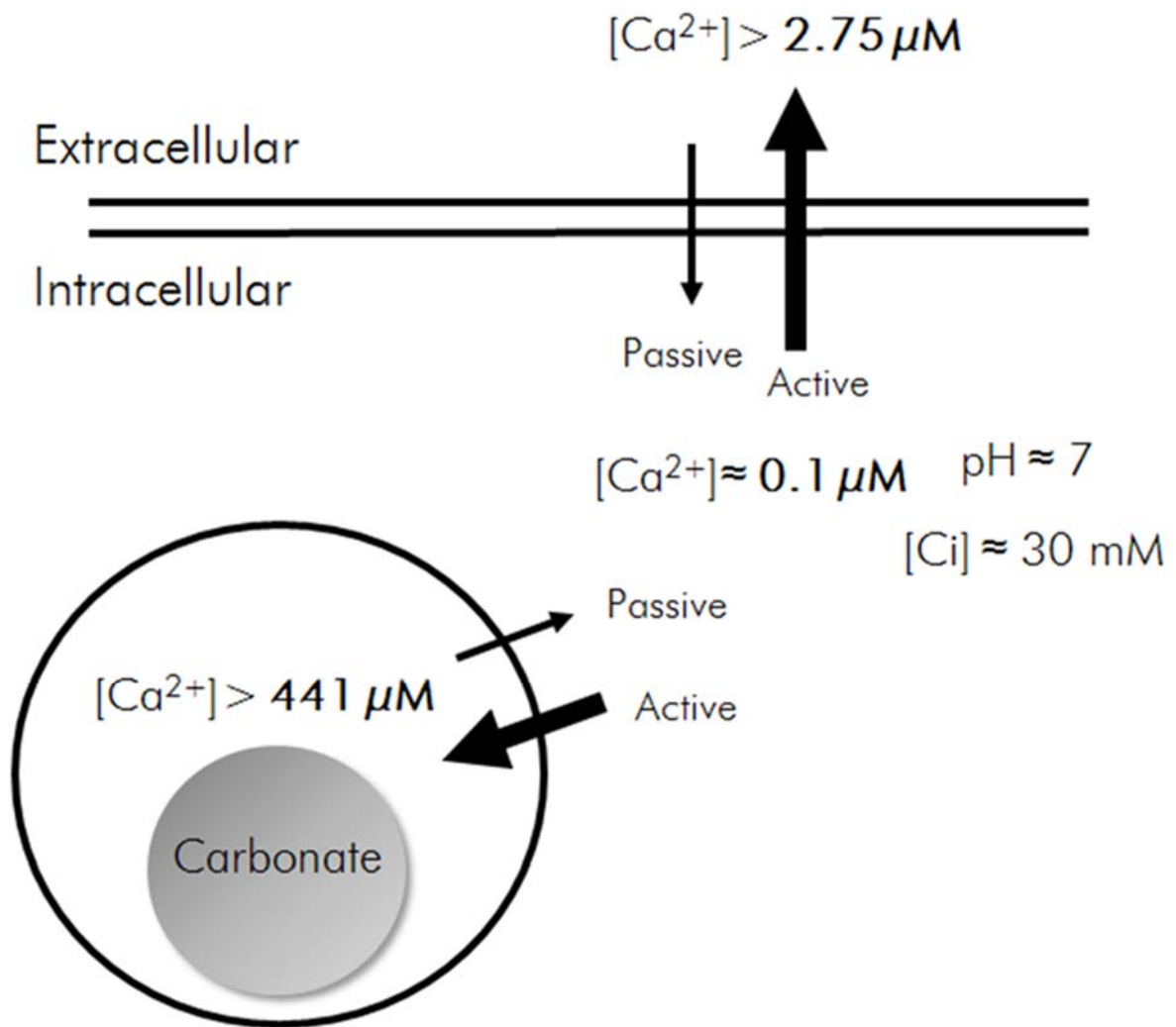


Figure 7



Supplementary material includes 6 figures and 10 tables

Figure S1

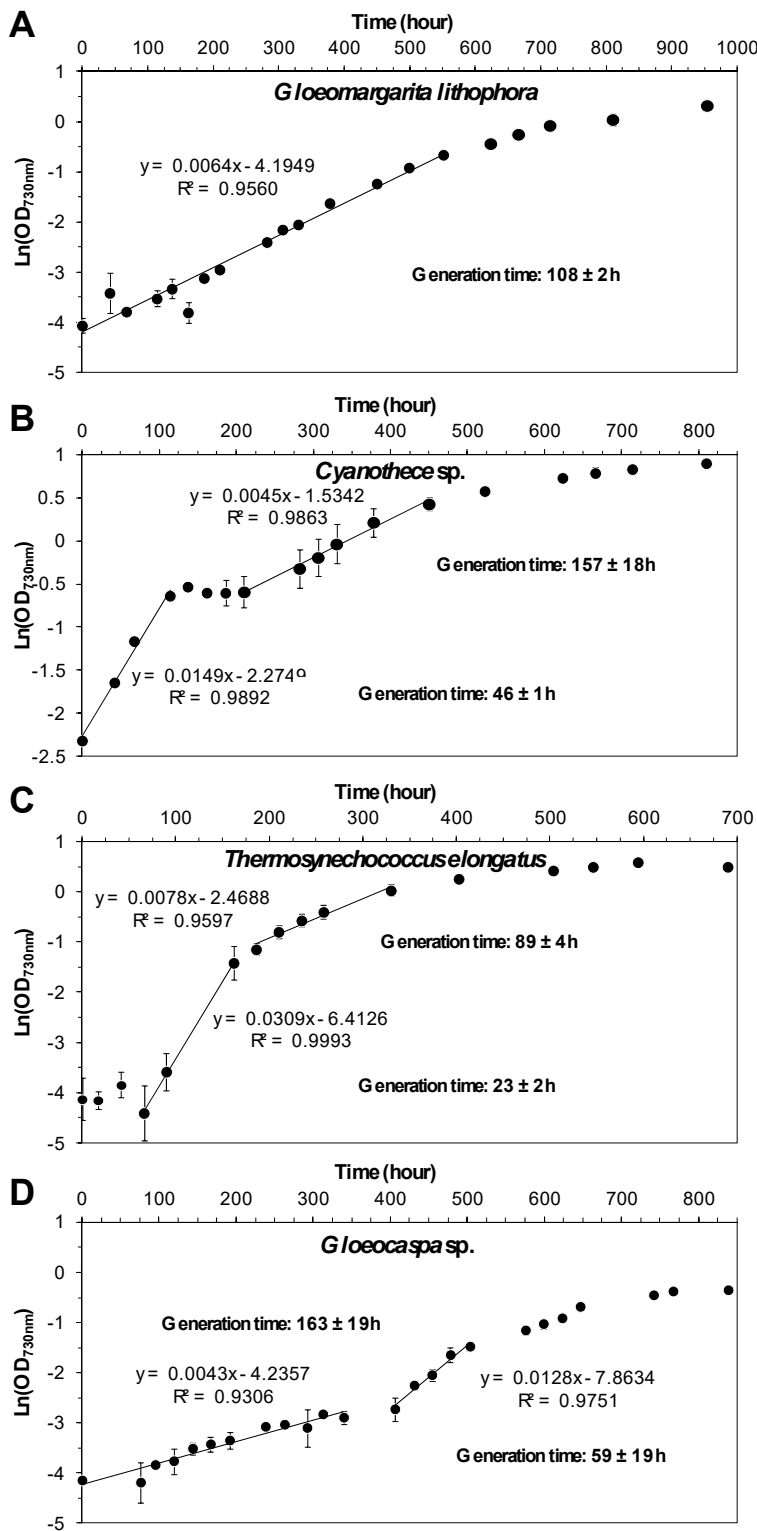


Figure S1: Growth of cultures of *Gloeomargarita lithophora* (A), *Cyanothece* sp. (B), *Thermosynechococcus elongatus* (C) and *Gloeocapsa* sp. (D) inoculated in BG-11. Several phases are discriminated in these graphs, marked by different generation times, i.e., slopes. Error bars were calculated based on variations in triplicates.

Figure S2

Non-inoculated BG-11

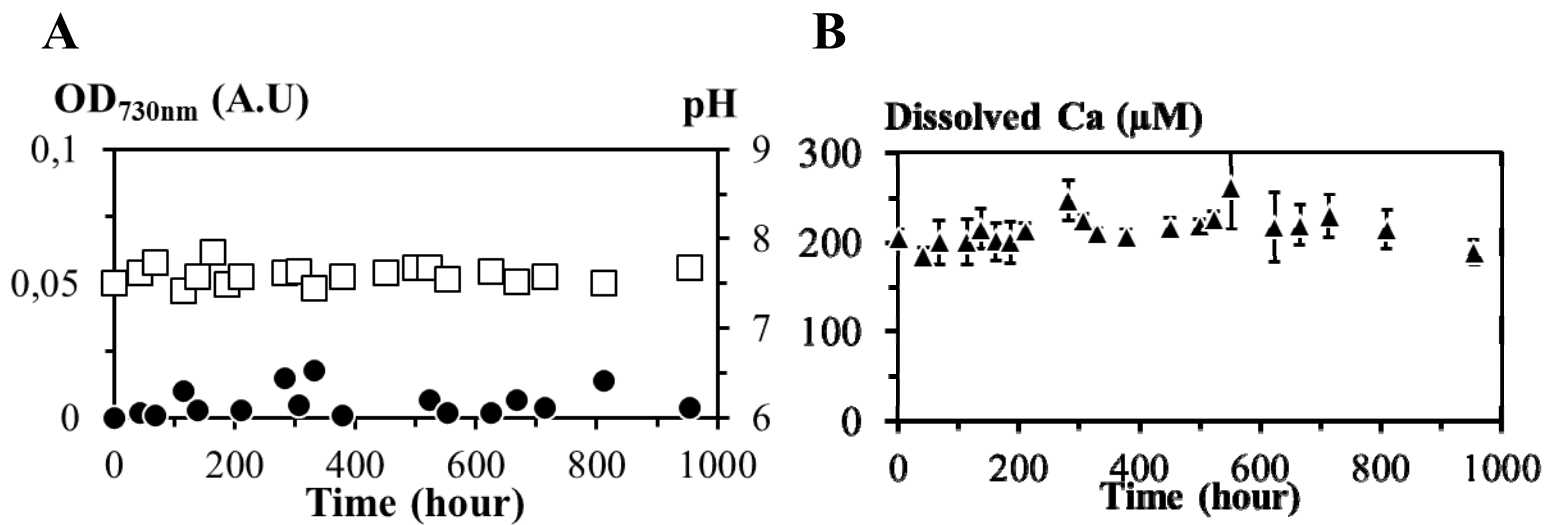
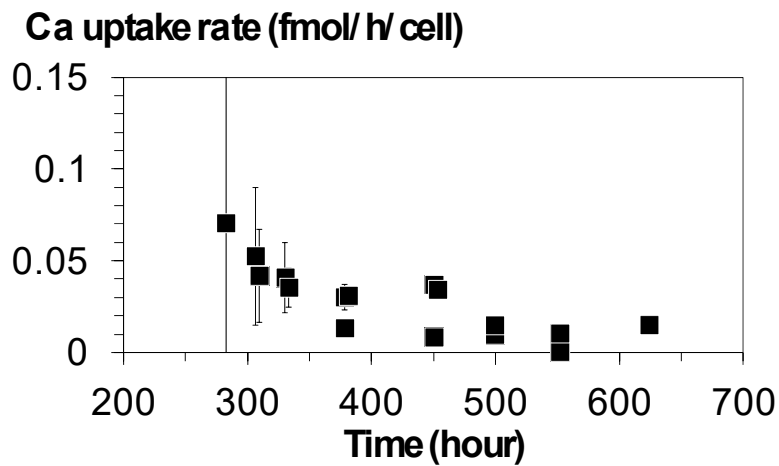


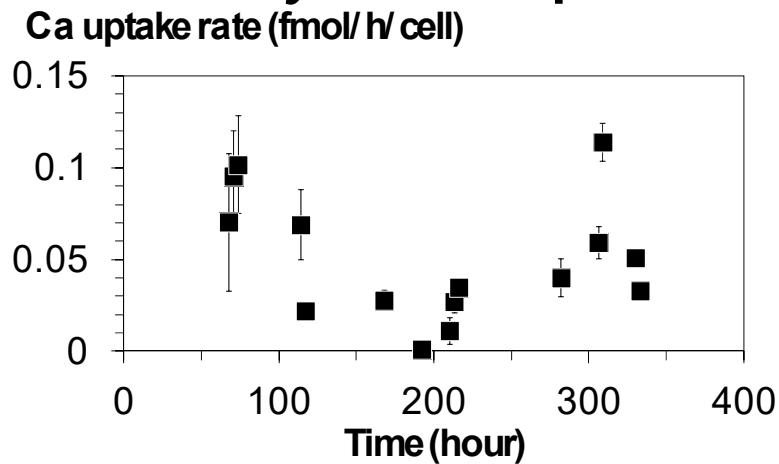
Figure S2: Time evolution of pH (open squares) and OD (closed circles) in (A) and dissolved Ca (closed triangles) in (B) in non-inoculated sterile BG-11 incubated at 30 °C under continuous light.

Figure S3

A *Gloeomargarita lithophora*



B *Cyanothece* sp.



C *Thermosynechococcus elongatus*

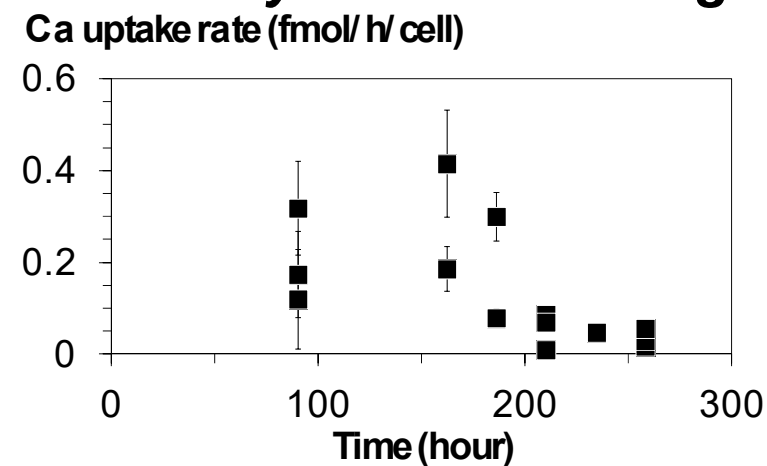


Figure S3: Uptake rate of Ca per cell versus time during the Ca uptake phase in cultures of *Gloeomargarita lithophora* (A), *Cyanothece* sp. (B) and *Thermosynechococcus elongatus* (C). Error bars were calculated based on instrumental precision. Values for the different replicates are reported in the graphs.

Figure S4

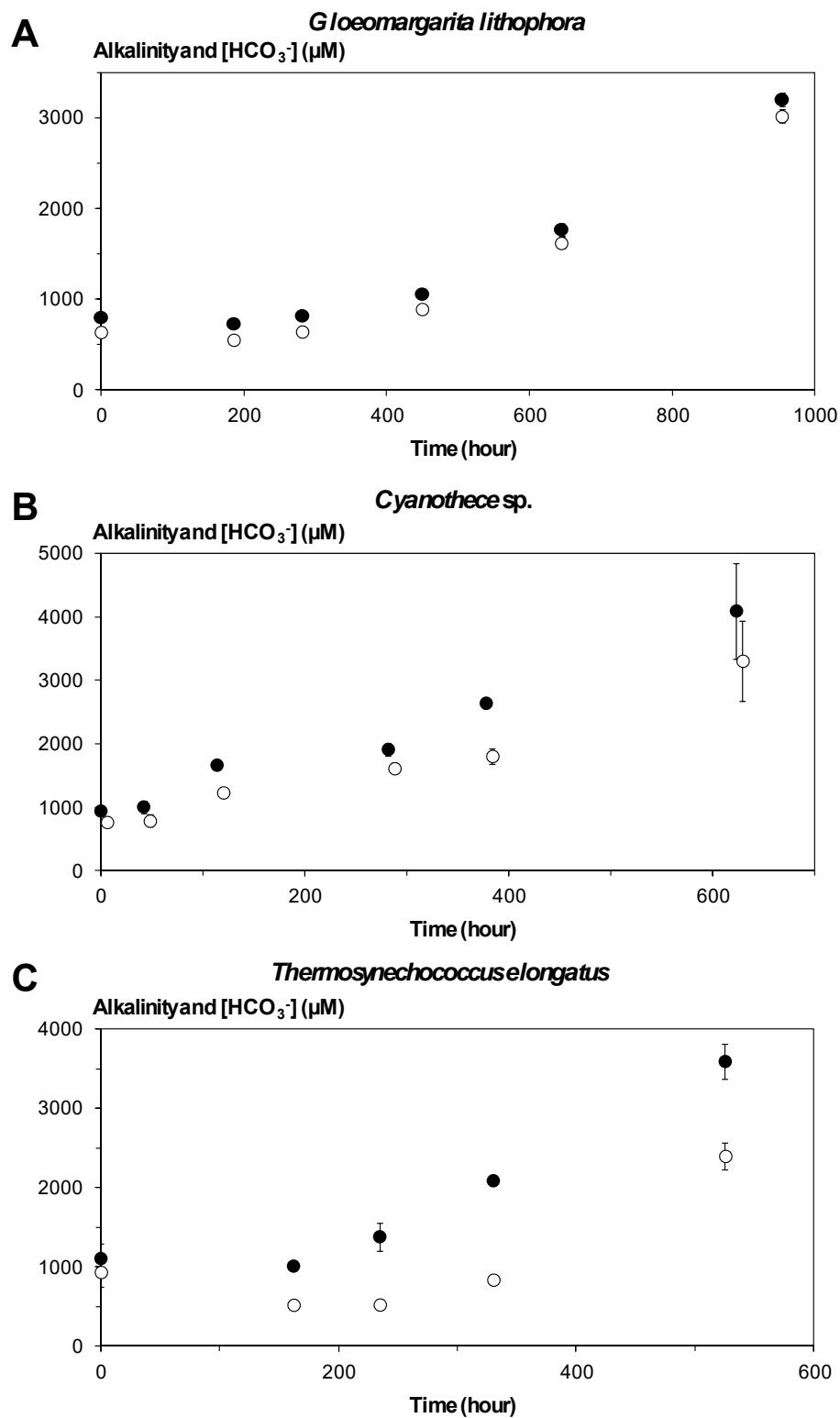


Figure S4: Time evolution of alkalinity (filled circles) and HCO_3^- (open circles) in extracellular solutions of *Gloeomargarita lithophora* (A), *Cyanothece* sp. (B) and *Thermosynechococcus elongatus* (C) cultures. Error bars were calculated based on variations in triplicates.

Figure S5

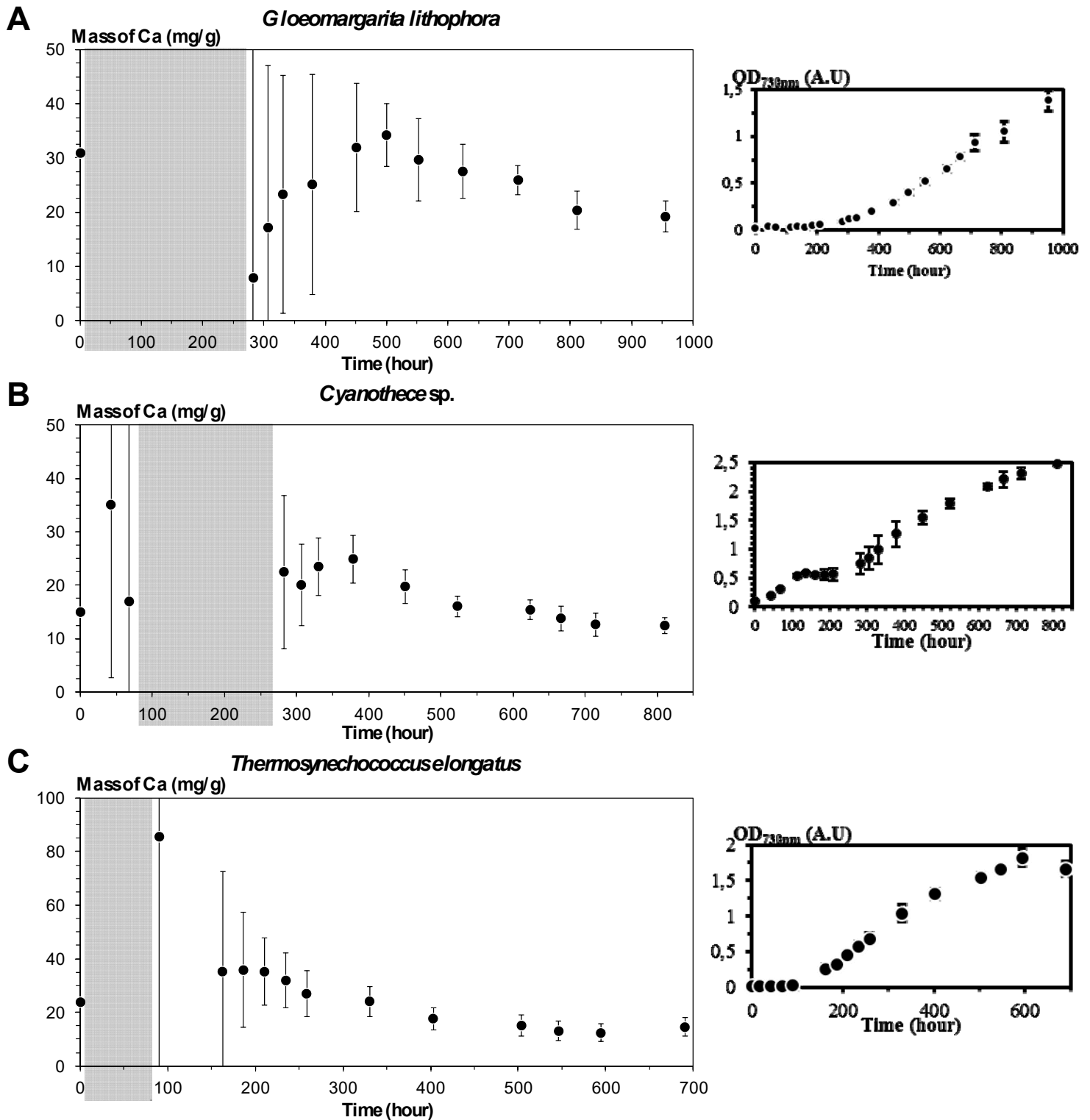


Fig. S5. Time evolution of the cellular mass proportion of Ca in cultures of *Gloeomargarita lithophora* (A), *Cyanothece sp.* (B) and *Thermosynechococcus elongatus* (C). The mass proportion of Ca was not calculated when no significant culture growth was observed. Standard deviations were calculated based on variations in triplicates and the precision of calcium concentration measurements. The grey areas correspond to lag phases and the transient phase with no growth for *Cyanothece sp.* culture. The same growth curves as in figure 1 are shown on the right.

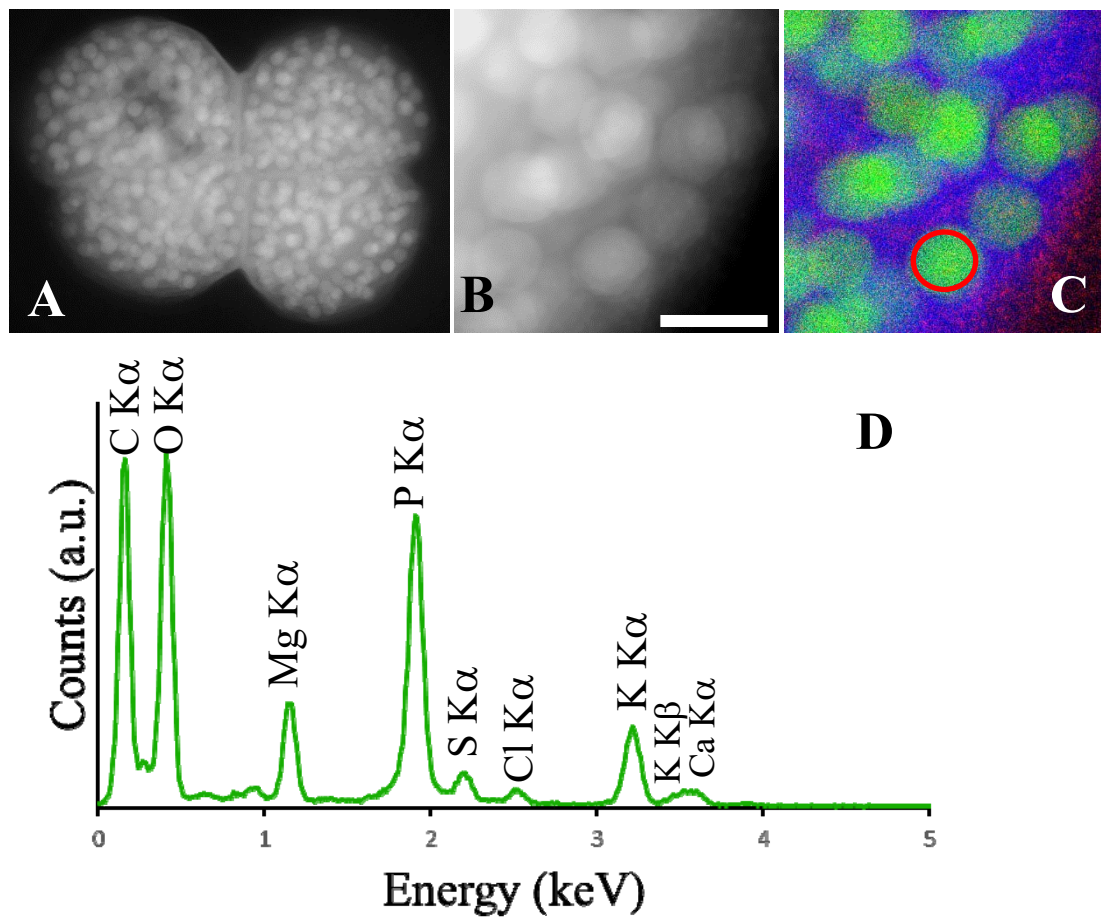


Figure S6: STEM-EDXS analyses of *Gloeocapsa sp.* cells. (A and B) STEM-HAADF images showing polyphosphate granules. (C) Corresponding EDXS maps of carbon (blue), phosphorus (green) and calcium (red). (D) EDXS spectrum of a P granule shown in C.

Table S1: Ca uptake rates, Ca affinities and cellular mass of Ca in cyanobacteria forming intracellular Ca-carbonates

Features	<i>Gloeomargarita lithophora</i>	<i>Cyanothece</i> sp.	<i>Thermosynechococcus elongatus</i>
Temperature (°C)	30	30	45
Final dissolved Ca (μM)	2.75 ± 0.54	5.9 ± 1.3	12.8 ± 3.6
Range of Ca uptake rate (fmol/cell/h)	0.01 - 0.07	0.001 - 0.11	0.01 - 0.42
Maximum measured cellular Ca mass ratio (mg/g of dry matter)	40	45	185

Time (h)		0	186	282	450	645	954
K	repl. 1	439	432	462	434	392	370
	repl. 2	395	437	445	426	402	380
	repl. 3	458	441	441	422	424	372
Mg	repl. 1	323	293	288	263	193	106
	repl. 2	287	292	284	260	199	105
	repl. 3	317	293	278	269	182	101
Na	repl. 1	18827	19788	19587	19018	19362	19165
	repl. 2	16813	19445	19519	18974	19819	19437
	repl. 3	18493	19652	19610	19706	19713	19261
N (NH ₄ ⁺ and NH ₃)	repl. 1	20	14	23	15	23	36
	repl. 2	17	15	13	13	14	37
	repl. 3	26	10	23	20	20	33
N (NO ₂ ⁻)	repl. 1	4	14	15	31	157	838
	repl. 2	4	12	16	37	134	845
	repl. 3	4	13	16	34	199	988
N (NO ₃ ⁻)	repl. 1	20181	19955	19895	23132	22926	20902
	repl. 2	20522	18887	19707	23492	23325	20603
	repl. 3	20486	19813	19846	23685	23037	20272
Cl	repl. 1	507	529	533	542	515	523
	repl. 2	507	546	533	588	513	536
	repl. 3	508	531	537	543	513	518
S	repl. 1	377	380	384	374	318	262
	repl. 2	378	394	382	403	320	262
	repl. 3	379	383	384	375	313	256

Table S2: Chemical analyses of K, Mg, Na, S, Cl and N species as a function of time in *Gloeomargarita lithophora* cultures. Measurements were performed on triplicate cultures (3 lines per element). Concentrations are in $\mu\text{mol/L}$.

Table S3

Time (h)		0	42	114	186	282	378	623
K	repl. 1	438	451	452	-	445	420	412
	repl. 2	466	457	453	-	446	373	444
	repl. 3	449	464	441	425	424	-	421
Mg	repl. 1	314	295	281	-	279	258	234
	repl. 2	322	292	285	-	281	234	238
	repl. 3	320	290	282	281	267	-	238
Na	repl. 1	18675	19453	19436	-	19527	19469	19754
	repl. 2	18875	19683	19623	-	19762	17700	19984
	repl. 3	18833	19585	19538	19841	20076	-	19886
N (NH ₄ ⁺ and NH ₃)	repl. 1	27	10	14	-	15	12	16
	repl. 2	31	10	16	-	23	10	20
	repl. 3	24	-	21	7	15	-	24
N (NO ₂ ⁻)	repl. 1	13	20	29	-	58	78	361
	repl. 2	16	21	36	-	65	88	354
	repl. 3	13	-	29	50	61	-	279
N(NO ₃ ⁻)	repl. 1	19685	19749	19194	-	18830	17570	19032
	repl. 2	19800	19801	19141	-	18905	17520	19033
	repl. 3	19805	-	19128	19146	17955	-	19344
Cl	repl. 1	512	519	527	-	535	542	527
	repl. 2	524	526	533	-	541	579	535
	repl. 3	515	525	537	533	540	-	534
S	repl. 1	375	369	356	-	333	290	208
	repl. 2	382	368	355	-	331	307	197
	repl. 3	375	371	358	337	310	-	190

Table S3: Chemical analyses of K, Mg, Na, S, Cl and N species as a function of time in *Cyanothece* sp. cultures. Measurements were performed on triplicate cultures. Concentrations are in $\mu\text{mol/L}$.

Table S4

Time (h)		0	162	234	330	525
K	repl. 1	454	463	-	436	424
	repl. 2	458	474	369	432	427
	repl. 3	464	455	430	426	428
Mg	repl. 1	297	269	-	164	109
	repl. 2	298	265	225	232	167
	repl. 3	305	281	266	250	195
Na	repl. 1	19625	20495	-	20553	19934
	repl. 2	19961	20527	16338	20226	19864
	repl. 3	19777	19673	18712	19946	19028
N (NH ₄ ⁺ and NH ₃)	repl. 1	50	35	-	33	45
	repl. 2	48	33	30	34	33
	repl. 3	49	33	35	38	35
N (NO ₂ ⁻)	repl. 1	3	31	-	62	105
	repl. 2	3	32	47	69	143
	repl. 3	3	20	37	59	114
N(NO ₃ ⁻)	repl. 1	20052	19794	-	21996	20699
	repl. 2	19590	19460	18872	21706	20906
	repl. 3	20466	19423	18814	21683	20156
Cl	repl. 1	523	542	560	562	541
	repl. 2	520	542	564	550	527
	repl. 3	522	529	535	550	515
S	repl. 1	434	416	399	346	246
	repl. 2	432	414	402	348	255
	repl. 3	439	418	397	364	258

Table S4: Chemical analyses of K, Mg, Na, S, Cl and N species as a function of time in *Thermosynechococcus elongatus* cultures. Measurements were performed on triplicate cultures. Concentrations are in $\mu\text{mol/L}$.

Table S5

Time (h)	0	186	282	450	645	954
ACC	-2.906	-1.946	-2.466	-1.387	-2.095	-2.325
	-2.906	-1.946	-2.466	-1.387	-2.095	-2.325
	-2.95	-2.444	-1.99	-1.291	-2.185	-2.492
Aragonite	-2.223	-1.262	-1.783	-0.704	-1.411	-1.642
	-2.223	-1.262	-1.783	-0.704	-1.411	-1.642
	-2.266	-1.76	-1.306	-0.608	-1.501	-1.809
Calcite	-2.082	-1.122	-1.642	-0.564	-1.271	-1.502
	-2.082	-1.122	-1.642	-0.564	-1.271	-1.502
	-2.126	-1.62	-1.166	-0.468	-1.361	-1.668
Vaterite	-2.636	-1.675	-2.196	-1.117	-1.825	-2.055
	-2.636	-1.675	-2.196	-1.117	-1.825	-2.055
	-2.679	-2.174	-1.719	-1.021	-1.914	-2.222
Dolomite	-4.341	-2.417	-3.431	-0.959	-1.036	-1.74
	-4.341	-2.417	-3.431	-0.959	-1.036	-1.74
	-4.44	-3.415	-2.468	-0.847	-1.059	-2.02
Hydroxyapatite	4.901	6.835	5.631	6.304	-2.02	-6.793
	4.901	6.835	5.631	6.304	-2.02	-6.793
	4.927	5.86	6.571	6.885	-6.836	-7.302
Ca ₃ (PO ₄) ₂ (am1)	-4.173	-3.203	-3.833	-3.744	-9.057	-12.162
	-4.173	-3.203	-3.833	-3.744	-9.057	-12.162
	-4.141	-3.687	-3.364	-3.388	-12.238	-12.446
Ca ₃ (PO ₄) ₂ (am2)	-1.443	-0.473	-1.103	-1.014	-6.328	-9.432
	-1.443	-0.473	-1.103	-1.014	-6.328	-9.432
	-1.411	-0.957	-0.635	-0.658	-9.508	-9.716
Ca ₃ (PO ₄) ₂ (beta)	-1.18	-0.211	-0.84	-0.751	-6.065	-9.17
	-1.18	-0.211	-0.84	-0.751	-6.065	-9.17
	-1.149	-0.695	-0.372	-0.396	-9.245	-9.454
Ca ₄ H(PO ₄) ₃ ·3H ₂ O(s)	-2.148	-1.174	-1.857	-2.263	-9.88	-14.422
	-2.148	-1.174	-1.857	-2.263	-9.88	-14.422
	-2.078	-1.65	-1.393	-1.778	-14.606	-14.765
CaCO ₃ ·xH ₂ O(s)	-3.405	-2.445	-2.965	-1.887	-2.594	-2.825
	-3.405	-2.445	-2.965	-1.887	-2.594	-2.825
	-3.449	-2.943	-2.489	-1.791	-2.684	-2.991
CaHPO ₄ (s)	-1.271	-1.267	-1.321	-1.816	-4.119	-5.556
	-1.271	-1.267	-1.321	-1.816	-4.119	-5.556
	-1.234	-1.259	-1.325	-1.686	-5.664	-5.615
CaHPO ₄ ·2H ₂ O(s)	-1.528	-1.524	-1.578	-2.073	-4.376	-5.813
	-1.528	-1.524	-1.578	-2.073	-4.376	-5.813
	-1.49	-1.516	-1.582	-1.943	-5.921	-5.872

Table S5: Saturation indices as a function of time of the extracellular solution of *Gloeomargarita lithophora* cultures with diverse carbonate and Ca-phosphate phases. Calculations were performed for triplicate cultures (3 lines per mineral phase).

Table S6

Time (h)	0	42	114	186	282	378	623
ACC	-2.698	-1.483	-0.279		-0.627	-0.936	-1.31
	-2.604	-1.303	0.027		-0.61	-1.218	-1.122
	-2.43		0.202	-1.336	-1.49		-1.133
Aragonite	-2.015	-0.799	0.404		0.057	-0.252	-0.627
	-1.92	-0.62	0.711		0.074	-0.534	-0.439
	-1.746		0.885	-0.653	-0.806		-0.449
Calcite	-1.874	-0.659	0.545		0.197	-0.112	-0.487
	-1.78	-0.48	0.851		0.214	-0.394	-0.298
	-1.606		1.026	-0.512	-0.666		-0.309
Vaterite	-2.428	-1.212	-0.009		-0.356	-0.665	-1.04
	-2.333	-1.033	0.297		-0.339	-0.947	-0.852
	-2.159		0.472	-1.066	-1.219		-0.862
Dolomite	-3.913	-1.437	1.269		0.459	1.255	0.527
	-3.728	-1.066	2.042		0.551	0.834	0.737
	-3.378		2.487	-0.79	0.079		0.699
Hydroxyapatite	5.429	7.739	8.852		8.595	1.916	1.374
	5.68	8.042	8.397		8.411	1.242	2.105
	6.049		7.218	6.711	2.455		2.401
Ca ₃ (PO ₄) ₂ (am1)	-3.89	-2.755	-2.414		-2.47	-6.819	-7.056
	-3.754	-2.613	-2.82		-2.598	-7.175	-6.631
	-3.566		-3.664	-3.49	-6.275		-6.431
Ca ₃ (PO ₄) ₂ (am2)	-1.16	-0.025	0.316		0.26	-4.09	-4.326
	-1.024	0.117	-0.09		0.132	-4.445	-3.901
	-0.837		-0.934	-0.76	-3.545		-3.701
Ca ₃ (PO ₄) ₂ (beta)	-0.897	0.237	0.578		0.523	-3.827	-4.064
	-0.762	0.379	0.173		0.394	-4.182	-3.639
	-0.574		-0.671	-0.497	-3.283		-3.438
Ca ₄ H(PO ₄) ₃ :3H ₂ O(s)	-1.827	-0.733	-0.823		-0.733	-7.103	-7.27
	-1.671	-0.609	-1.584		-0.933	-7.495	-6.727
	-1.476		-2.938	-1.908	-6.01		-6.421
CaCO ₃ xH ₂ O(s)	-3.197	-1.982	-0.778		-1.126	-1.435	-1.81
	-3.103	-1.802	-0.472		-1.109	-1.717	-1.621
	-2.929		-0.297	-1.835	-1.989		-1.632
CaHPO ₄ (s)	-1.234	-1.274	-1.705		-1.559	-3.58	-3.511
	-1.213	-1.293	-2.061		-1.632	-3.616	-3.392
	-1.206		-2.571	-1.715	-3.031		-3.287
CaHPO ₄ :2H ₂ O(s)	-1.491	-1.531	-1.962		-1.816	-3.837	-3.767
	-1.47	-1.55	-2.318		-1.889	-3.873	-3.649
	-1.463		-2.828	-1.971	-3.288		-3.544

Table S6: Saturation indices as a function of time of the extracellular solution of *Cyanotheca* sp. cultures with diverse carbonate and Ca-phosphate phases. Calculations were performed for triplicate cultures.

Table S7

Time (h)	0	162	234	330	525
ACC	-2.663	0.393		-0.819	-0.722
	-2.624	0.406	0.126	-0.562	-0.617
	-2.73	0.34	0.268	-0.375	-0.577
Aragonite	-2.006	1.05		-0.162	-0.065
	-1.967	1.063	0.783	0.096	0.04
	-2.073	0.997	0.925	0.282	0.08
Calcite	-1.876	1.18		-0.031	0.065
	-1.837	1.193	0.913	0.226	0.171
	-1.942	1.128	1.055	0.412	0.21
Vaterite	-2.392	0.663		-0.548	-0.452
	-2.354	0.676	0.396	-0.291	-0.346
	-2.459	0.611	0.539	-0.105	-0.306
Dolomite	-3.806	2.823		1.539	1.224
	-3.745	2.905	2.659	1.914	1.549
	-3.941	2.409	2.736	2.132	1.581
Hydroxyapatite	5.549	7.261		-1.103	2.721
	5.675	6.02	2.491	0.884	3.049
	5.423	10.149	5.989	1.742	3.914
Ca ₃ (PO ₄) ₂ (am1)	-3.014	-2.891		-8.064	-5.546
	-2.943	-3.723	-5.983	-6.825	-5.363
	-3.076	-0.949	-3.698	-6.314	-4.799
Ca ₃ (PO ₄) ₂ (am2)	-0.341	-0.219		-5.391	-2.873
	-0.27	-1.05	-3.31	-4.152	-2.69
	-0.403	1.724	-1.025	-3.642	-2.126
Ca ₃ (PO ₄) ₂ (beta)	-1.224	-1.101		-6.274	-3.756
	-1.153	-1.933	-4.193	-5.035	-3.573
	-1.286	0.841	-1.908	-4.524	-3.009
Ca ₄ H(PO ₄) ₃ :3H ₂ O(s)	-0.757	-2.102		-9.254	-5.526
	-0.671	-3.355	-6.605	-7.524	-5.304
	-0.817	0.839	-3.249	-6.852	-4.479
CaCO ₃ xH ₂ O(s)	-3.126	-0.07		-1.282	-1.185
	-3.087	-0.057	-0.337	-1.024	-1.08
	-3.193	-0.123	-0.195	-0.838	-1.04
CaHPO ₄ (s)	-1.381	-2.848		-4.828	-3.618
	-1.365	-3.27	-4.26	-4.337	-3.579
	-1.379	-1.85	-3.188	-4.175	-3.317
CaHPO ₄ :2H ₂ O(s)	-1.573	-3.04		-5.02	-3.809
	-1.557	-3.462	-4.452	-4.529	-3.771
	-1.571	-2.042	-3.38	-4.367	-3.509

Table

Table S7: Saturation indices as a function of time of the extracellular solution of *Thermosynechococcus elongatus* cultures with respect to diverse carbonate and Ca-phosphate phases. Calculations were performed for triplicate cultures.

Gloeomargarita lithophora

Time (h)	Number of analyzed		STEM (HAADF)			Volume of CaCO ₃ per cell		
	cells (empty cells)	carbonates granules	CaCO ₃ granules per cell		CaCO ₃ granules diameter (nm)			
0	31 (2)	219	7.6	± 3.0	218	± 82	0.059	± 0.033
186	54 (12)	369	8.8	± 5.9	221	± 89	0.076	± 0.071
282	116 (23)	765	8.2	± 4.3	201	± 86	0.055	± 0.046
450	83 (16)	520	7.8	± 4.7	211	± 80	0.056	± 0.040
645	86 (8)	508	6.5	± 2.8	204	± 82	0.045	± 0.037
954	-	411	-		197	± 68	-	

Time (h)	EDXS		STEM-EDXS		ICP-AES		Part of cellular Ca in CaCO ₃ granules	
	Mg ratio (Mg/Mg+Ca)		Mass of Ca in CaCO ₃ granules (mg/g of dry matter)		Total Ca in cells (mg/g of dry matter)		(%)	
0	10.4	± 1.9	9.6	± 5.6	31		31	± 18
186	9.8	± 2.0	12	± 12	45	± 29	27	± 32
282	10.1	± 2.1	9.0	± 7.7	45	± 16	20	± 19
450	11.6	± 2.6	9.0	± 6.7	46.7	± 2.5	19	± 14
645	14.8	± 5.1	7.0	± 6.2	33.3	± 0.7	21	± 19
954	39	± 18	-		17.3	± 2.1	-	

Table S8. Time evolution of intracellular carbonates in *Gloeomargarita lithophora*. The total number of analyzed cells (with and without granules) ~~The number of cells containing carbonate granules~~ is indicated. Empty cells (i.e., with no detected carbonate granule) are indicated in brackets. Numbers of analyzed carbonate granules, number of carbonate granule per cell, their diameters and their volume were estimated from on STEM-HAADF measurements. The Mg/(Mg+Ca) ratios in CaCO₃ granules were determined by EDXS analyses. The mass of Ca in CaCO₃ granules was determined by multiplying the volume of CaCO₃ per cell by the density of ACC (2.18 from Fernandez-Martinez *et al.*, 2013) taking into account the mass proportion of Ca per CaCO₃ granule. The mass of total Ca in cells was the difference between the initial concentration of Ca in BG-11 minus the concentration of dissolved Ca at a given time, divided by the number of cells at the same time. The percent of Ca in CaCO₃ granules was the mass of Ca in CaCO₃ granules by the total mass of Ca in cells.

Cyanothece sp.

Time (h)	Number of analyzed		STEM (HAADF)			Volume of CaCO ₃ per cell		
	cells (empty cells)	carbonates granules	CaCO ₃ granules per cell	CaCO ₃ granules diameter (nm)				(μm ³)
0	16 (4)	82	6.8 ± 3.4	277 ± 126				0.128 ± 0.092
42	47 (7)	145	3.6 ± 2.1	279 ± 139				0.075 ± 0.055
114	132 (10)	555	4.5 ± 2.0	317 ± 122				0.113 ± 0.070
282	85 (18)	316	4.7 ± 3.6	282 ± 109				0.083 ± 0.074
623	97 (35)	280	4.5 ± 2.9	300 ± 151				0.117 ± 0.125
Time (h)	EDXS		STEM-EDXS	ICP-AES		Part of cellular Ca in CaCO ₃ granules		
	Mg ratio (Mg/Mg+Ca)		Mass of Ca in CaCO ₃ granules (mg/g of dry matter)	Total Ca in cells (mg/g of dry matter)		(%)		
0	4.0 ± 2.2		11 ± 11	15		76 ± 70		
42	4.4 ± 2.2		6.7 ± 5.9	42.3 ± 4.9		16 ± 14		
114	3.0 ± 1.7		10.2 ± 8.5	42.9 ± 1.5		24 ± 20		
282	4.8 ± 2.2		7.4 ± 7.4	30.1* ± 1.2*		25* ± 25*		
623	3.2 ± 0.9		11 ± 12	17.9* ± 0.1*		60* ± 66*		

Table S9. Time evolution of intracellular carbonates in *Cyanothece sp.* * indicates that measurements were performed on two replicates only, while all other measurements were obtained from three replicates.

Thermosynechococcus elongatus

Time (h)	Number of analyzed		STEM (HAADF)				Volume of CaCO ₃ per cell	
	cells (empty cells)	carbonate granules	CaCO ₃ granules per cell		CaCO ₃ granules diameter (nm)		(μm ³)	
0	44 (6)	152	4.0 ± 2.6		111 ± 66		0.006 ± 0.006	
234	85 (3)	596	7.3 ± 4.7		129 ± 62		0.015 ± 0.020	
330	103 (2)	565	5.6 ± 2.6		131 ± 72		0.014 ± 0.017	
525	92 (19)	1656	22.7 ± 9.6		172 ± 64		0.087 ± 0.045	
Time (h)	EDXS		STEM-EDXS		ICP-AES		Part of cellular Ca in CaCO ₃ granules	
	Mg ratio (Mg/Mg+Ca)		Mass of Ca in CaCO ₃ granules (mg/g of dry matter)		Total Ca in cells (mg/g of dry matter)		(%)	
0	4.1 ± 1.5		0.4 ± 0.4		24		1.5 ± 1.5	
234	3.7 ± 1.2		0.8 ± 1.1		35.6* ± 0.9*		2.3* ± 3.2*	
330	3.7 ± 1.8		0.8 ± 1.0		25.6 ± 2.6		3.1 ± 4.1	
525	7.6 ± 2.4		4.7 ± 2.8		16.4 ± 0.6		29 ± 17	

Table S10. Time evolution of intracellular carbonates in *Thermosynechococcus elongatus*.

* indicates that measurements were performed on two replicates only, while all other measurements were obtained from three replicates.