Calcite precipitation during growth of *Synechococcus* sp. strain PCC 8806

Introduction

Calcium carbonate precipitation is a key process in the carbon cycle in marine and lacustrine environments (Merz-Preiß, 2000). A large number of studies have been published where the potential for cyanobacteria for calcite production is described (Lee et al., 2004). Two different cases are often cited as examples of massive, biologically-induced carbonate precipitation events, referred to as "whitings" in hard-lake and marine environments: the Fayetteville Green Lake (Thompson and Ferris, 1990, Thompson et al., 1997) and the Great Bahama Bank (Thompson, 2000; Morse et al., 2003). The whitings are composed of suspended fine-grained carbonate particles. In the Fayetteville Green Lake (FGL), blooms of cyanobacterial picoplankton are reported to be associated with the $CaCO_3$ precipitation events (Thompson et al., 1997). Furthermore isotopic evidences, mainly $\delta^{13}C$ enrichment of the calcite sediment suggested that the calcite precipitation is a biologically-mediated process (Thompson et al., 1997). The situation encountered in marine environments is more confused (Milliman et al., 1993; Morse et al., 2003). The withings form patches of milky waters that drift around for several days. Marine environments offer nutrient poor growth conditions for photothrophic organisms (lack of nitrate, phosphate...), and blooms appeared associated to upwelling events in which it is difficult to disentangle the various possible factors for calcite production. The origin of whitings in the Great Bahama Bank (GBB) has been hotly debated since many decades (Thompson, 2000; Broecker et al., 2001; Morse et al., 2003; Buros-Serrano, Marine Geology, 2009). Both biologically induced calcite precipitation phenomena (Robbins and Blackwelder, 1992; Robbins et al., 1997), as well as the idea that the GBB whitings may be caused by re-suspended sediments (Morse et al., 2003), have been proposed.

In fact the "biologically induced calcification process" (Thompson et al., 1997; Riding, 2006) illustrated two strongly associated phenomenon's: first, the generation of an alkaline microenvironment thought the uptake of inorganic carbon (through the carbonic anhydrase (CA) activity on bicarbonante; second, the role of the complex layers formed by the structure of the cell walls. The photosynthetic activity by the cyanobacteria, using bicarbonate as carbon source increases OH^- expulsion and favours carbonate oversaturation, changing the chemical micro-environment of the water layers adjacent to the cell. HCO_3^- is transported

through the membrane and dissociated within the cell, in the carboxysome, into CO_2 and OH^- by the carbonic anhydrase. CO_2 is used in the Kalvin cycle and the generated OH^- is excreted out of the cell through the cell membrane, where it reacts with HCO_3^- to form CO_3^{2-} (Miller and Colman, 1980; Thompson and Ferris, 1990; Kaplan et al., 1991; Merz, 1992; McConnaughey, 1994; Price et al., 1998; Kaplan and Reinhold, 1999; Badger and Price, 2003). The cytoplasmic membrane and the cell wall play probably an active role, increasing local cation concentrations (cation binding through molecules as polysaccharides) and acting as a diffusion barrier, slowing ion exchanges in this area (Schultze-Lam et al., 1992; Freytet and Verrecchia, 1998; Arp et al., 1999). According to these hypotheses, calcite nucleation takes place outside the cells close to the outer cytoplasmic membrane (in the S-layer). Nucleation contributes to lower the energy needed to make atom bonds in the solid form (Obst and Dittrich, 2005). Nevertheless demonstrations that cells or biological structures could play a role in the calcite precipitation by providing preferential nucleation sites are scarce. The macro-scale influence of cyanobacterial photosynthesis on pH inevitably complicates the laboratory demonstration of nucleation initiation. Indeed, in a carbonate system, it is impossible to vary a single component (of the CO_2 /HCO^{3-} /CO_3^{2-} system), without modifying the two other component(s) or altering the pH. Thus, this strong interdependency in the carbonate system should be considered. Obst et al. (2009) applying an experimental approach, keeping stable the carbonate system over sufficient timescales to study induction of nucleation over fixed saturation state suggested a passive surface-induced mechanism for $CaCO_3$ nucleation by cyanobacteria.

In the present study, the $CaCO_3$ precipitation was examined in batch condition with bicarbonate as Ci source. Various initial bicarbonate concentrations were tested for a same calcium concentration and oppositely various calcium concentrations were tested with a same bicarbonate concentration. Some combinations of calcium and bicarbonate/carbonate have been done to mimic sea water composition (10.3mM for Ca and 1818 µM for HCO_3^-). We have tried to precise the precipitation conditions, and the influence of the growth kinetic on extent of respective precipitation events.

Material and methods

Cyanobacterial cultures and experimental setup

➢ *Cyanobacterial strain and culture conditions*

The marine strain of Cyanobacteria, *Synechococcus* sp. PCC 8806 was used. Cells were maintained on low (0.38 mM) $CaCl_2$ ASN-III growth medium (Rippka et al., 1979), with atmospheric CO_2 as carbon source. Every 4 weeks, 10 % (v/v) of the culture was transferred to fresh sterile medium.

Experiments were performed under sterile conditions in glass reactor (2.5 l) containing 1.5 l culture medium (171 mM NaCl, 17.65 mM $NaNO_3$, 0.30 mM $MgSO_4 \cdot 7H_2O$, 0.18 mM K_2HPO_4, 0.03 mM $FeCl_3 \cdot 6H_2O$, 1 ml Trace metal mix A5 + Co, and 20 µg vitamin B_{12}). Nitrate (as $NaNO_3$) was added in quantity enough to sustain biomass growth without limitation. The initial pH of the medium was adjusted to 8.0. $NaHCO_3$ and $CaCl_2$ were added as variables in the mineral medium. $CaCl_2$ was added during mineral medium preparation and $NaHCO_3$ just before inoculation (see below). The experiments were inoculated with the cyanobacterial cultures; the target cell number varying between 2.0×10^7 and 1.3×10^8 cells ml^{-1}. After inoculation, the reactors were immediately sealed with rubber septum and plastic screwed end-cap to avoid CO_2 exchange with the atmosphere. The reactors were incubated at a temperature of 25 °C, with a 12h/12h light/dark cycle and a 1.79 klux (35.8 µmol $m^{-2}s^{-1}$) photon irradiance. Cultures were mixed with Teflon-coated magnetic stirring bars at 100 rpm in Incubator/Shaker (Snijders, ECD01E).

➢ *Experiments with different combinations of $NaHCO_3$ and $CaCl_2$*

The experiments were performed with different combinations of $NaHCO_3$ and $CaCl_2$ concentrations. Calcium and bicarbonate were introduced into the culture medium in variable amounts ranging from 0.34 mM up to 10.3mM for Ca and from 2.5 mM to 30mM for HCO_3^- (see table 1). The CO_2 present in the headspace, e.g. less than 10 µmol, is negligible compared to the introduced amount of HCO_3^-, therefore the headspace was not purged. The oxygen in the headspace at the beginning of experiments was 21 % (v/v). The pH was not regulated. All the experiments were conducted in batch conditions.

Table 1: Assay conditions

Assays	$[Ca^{2+}]$ mM	DIC mM	Inoculums charge cells ml^{-1}
1	0.34	30	2.0E+07
2	3.4	30	2.0E+07
3	6.8	30	2.0E+07
4	10.3	30	2.0E+07
5	10.3	2.5	4.5E+07
6	10.3	5	4.5E+07
7	10.3	7.5	4.5E+07
8	10.3	10	4.5E+07
9	10.3	25	5.1E+07
10	10.3	25	5.1E+07
11	10.3	25	5.1E+07
12	10.3	25	5.1E+07
13	10.3	10	3.0E+07
14	10.3	20	3.0E+07
15	10.3	30	3.0E+07
16	10.3	7.5	1.3E+08
17*	10.3	7.5	1.3E+08

* Addition of $CaCO_3$ (1g l^{-1}) at start of the culture

Analyses

- ***Biomass quantification***

The optical density was determined by a photo-spectrometer (Shimadzu, UV 1601) at 600 nm. A relation between $C_{biomass}$ (µM) and absorbance at 600 nm (0 to 0.5) has been established (with $R^2 > 0.98$). A correlation was also established between dry biomass and cell density. $C_{biomass}$ was determined by Rock Eval analysis. Good accordance was obtained between $C_{biomass}$ determined with the Rock Eval 6 and elementary analysis (CONHS) on cyanobacterial biomass (Li et al., 2010a).

- ***pH measurement***

The pH was continuously measured by a pH-metre, i.e. SevenMulti, METTLER TOLEDO equipped with software LabX direct pH (version 2.1) to record the data.

➢ ***Calcium quantification***

Calcium concentration (Ca^{2+}) was analyzed on culture medium samples filtered on a 0.2 µm cellulose acetate filter (Whatman). Filtrate samples were acidified (with 10 µl of a 2N HCl solution) and stored at + 4°C until analysis if not treated immediately. Calcium analysis was done by High Performance Liquid Chromatography (HPLC) on a DIONEX ICS 3000 equipped with eluent generator and a self regenerating suppressor (CSRS-ULTRA 4 mm). Cations were separated at 30°C on a DIONEX IonPac CS12 column (4 mm x 250 mm) with methasulfonic acid 20 mM at a flow rate of 1 ml/min and quantified with a conductivity detector. The injection volume was 25 µl (full-loop injection). Linear calibration curves (with R^2 >0.999) were obtained for calcium (0-400 µM). Detection limit was 5 µM

➢ ***Bicarbonate, carbonate and OH^- quantification***

Bicarbonate, carbonate and OH^- ions were quantified by titration analysis with an automatic titrator (Titrimo 702 SM, METHROM). 10 ml filtrate was analyzed immediately after sampling. Sample was not diluted to avoid pH change and equilibration with atmosphere. Automatic titration was done with H_2SO_4. Normality of H_2SO_4 was 0.01 N or 0.001 N depending on bicarbonate and carbonate concentrations in the sample. Calibrated increment volume of H_2SO_4 was 100 µl (corresponding to 1 µmol H^+ equivalent for 0.01 N). The data were recorded by software Tiamo (METHROM) and transferred into Excel under the form pH = *f*(µmol H^+) to determined accurately he inflexion points corresponding to the transition carbonate/bicarbonate (referred as to the first inflexion point) and bicarbonate/CO_2 (referred as to the second inflexion point). Respective bicarbonate and carbonate quantities are calculated from the cumulated quantities of H^+ (µmol) added to reach the two inflexion points. When OH^- ions are present (i.e. HCO_3^- absent) the amount of H^+ (µmol) added at the first inflexion point represents the sum of OH^- and CO_3^{2-}. Detection limits were 5 µmol for both CO_3^{2-} and HCO_3^-.

➢ ***Scanning electron microscopes***

Cyanobacterial cells and $CaCO_3$ were imaged with a Scanning Electron Microscopy (EVO MA10, ZEISS, Germany). 100 µl of sampling medium was deposited on a polycarbonate filter (0.2 µm) in close-contact with a filter paper saturated with deionised water (MilliQ) and placed in a desiccator with water recipient to maintain water saturation for 24 hours.

This procedure allowed maximal elimination of ions from the growth medium by diffusion into the filter paper. This procedure avoided ion crystallisation during the drying procedure and consequent interference on the electron microscopy imaging. The filter paper was then dried in a desiccator at room temperature for at least 48 hours. The dry samples were sputter coated with 10 nm gold/platinum before SEM imaging. The metalized sample was then observed with a conventional SEM mode. 5 kV of acceleration voltages was applied.

Results

General requirements

During cyanobacterial growth bicarbonate was rapidly metabolized. In the absence of calcium the carbon source is only distributed into an organic carbon ($C_{biomass}$) and an inorganic carbon (C_{CO3}^{2-}) according to the proposed bicarbonate photosynthetic uptake mechanism (Merz, 1992; McConnaughey, 1994).

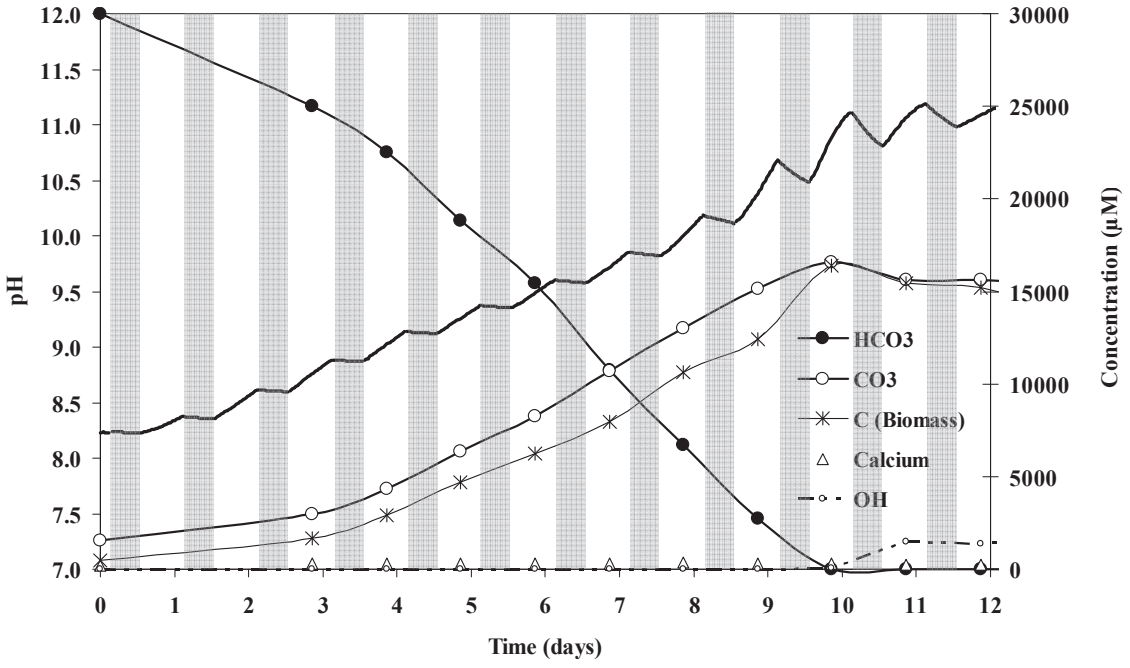

Figure 1: Cyanobacterial growth (assay 1 with 0.34mM Ca^{2+} and 30mM HCO_3^-) during 14 hours of light cycles (blank area) and 10 hours of dark cycles (gray area). Evolutions of pH (black line), HCO_3^- (filled circle), CO_3^{2-} (blank circle), $C_{biomass}$ (star), Ca^{2+} (blank triangle) and OH^- (blank circle with broken line).

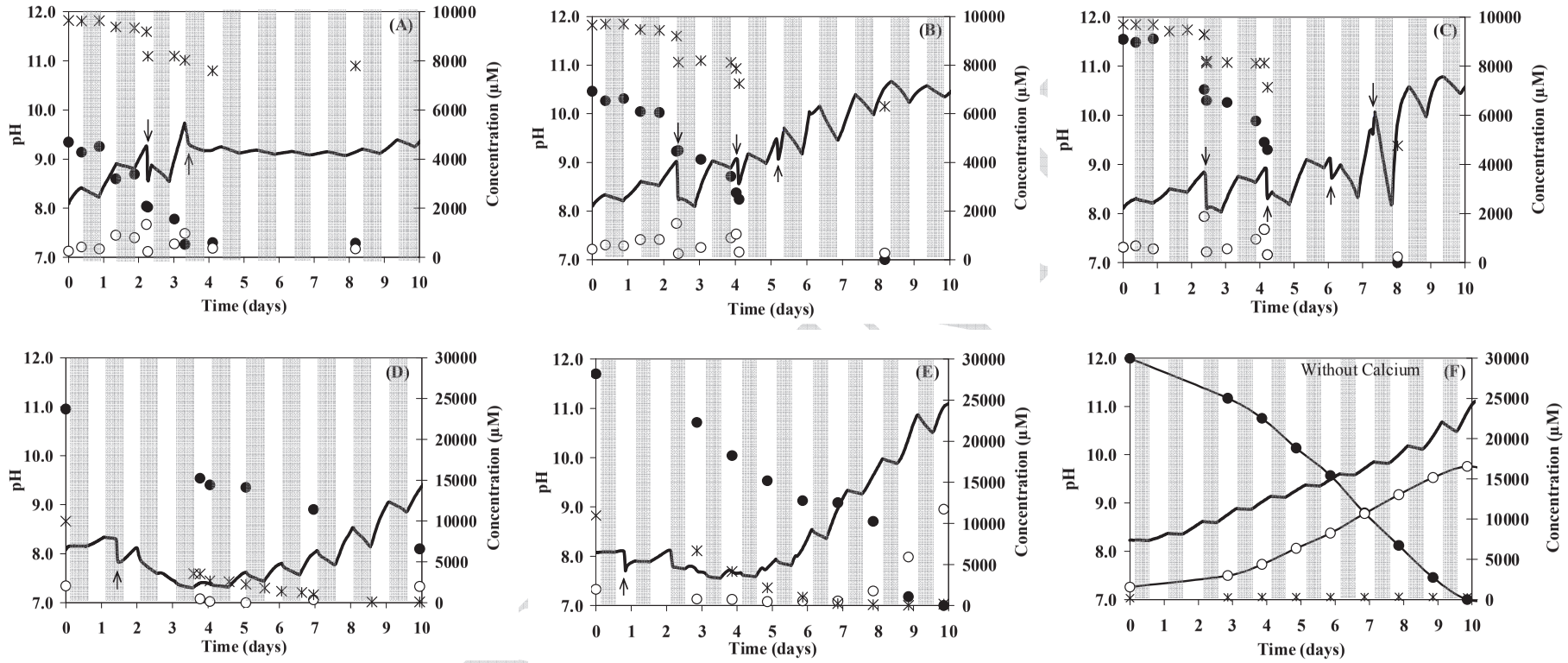

Figure 2: Cyanobacterial growth during light cycles (blank column) and night cycles (gray column). (A): assay 6 with 10.3mM Ca^{2+} and 5mM HCO_3^- (12 hours light cycle and 12 hours dark cycle); (B): assay 7 with 10.3mM Ca^{2+} and 7.5mM HCO_3^- (12 hours light cycle and 12 hours dark cycle); (C): assay 8 with 10.3mM Ca^{2+} and 10mM HCO_3^- (12 hours light cycle and 12 hours dark cycle); (D): assay 9 with 10.3mM Ca^{2+} and 25mM HCO_3^- (12 hours light cycle and 12 hours dark cycle); (E): assay 4 with 10.3mM Ca^{2+} and 30mM HCO_3^- (14 hours light cycle and 10 hours night cycle); (F): assay 1 with 0.34mM Ca^{2+} and 30mM HCO_3^- (14 hours light cycle and 10 hours dark cycle). Evolutions of pH (black line), HCO_3^- (filled circle), CO_3^{2-} (blank circle) and Ca^{2+} (star). Arrowheads indicate precipitation events.

The figure 1 and figure 2f presented an example of cyanobacterial growth on bicarbonate (30 mM) with a very low initial calcium concentration (i.e. 0.34 mM, assay 1), which illustrated the respective increase and decrease alternations of pH, corresponding to successive light (bicarbonate photosynthetic assimilation) and dark (dark respiration) phases. Nevertheless, the global pH increased during time course due to continuous change in the bicarbonate/carbonate ratio. A carbonate production of 14,987 µM was obtained for an initial bicarbonate concentration of 29,971 µM, reflecting an approximate 1:1 partitioning ratio between an organic carbon ($C_{biomass}$) and an inorganic carbon (C_{CO3}^{2-}). Indeed the CO_2 released during dark respiration may be re-used through photosynthesis. No calcite precipitation was detected despite the increase of the carbonate concentration and the calcium concentration measured was stable all along the time course (280±40 µM).

CaCO3 precipitations with various bicarbonate/calcium

- ***Constant calcium concentration (10.3mM)***

The marine cyanobacterial strain *Synechococcus* sp. PCC 8806 was grown on bicarbonate, varying its concentrations from 2.5 to 30 mM and with 10.3 mM $CaCl_2$ (concentration of calcium in the sea water). The continuous carbonate concentration increase during the bicarbonate photosynthetic assimilation is conducive to calcite precipitation. The experiment conditions are presented in the table 1. The evolution of pH was monitored until bicarbonate was completely consumed. The bicarbonate, carbonate, calcium, hydroxyl were measured on the filtrated samples.

For the experiments conducted with 5, 7.5 and 10 mM bicarbonate (assays 6 to 8 inoculated with a similar cyanobacterial charge), pH drops were easily identified on the pH profiles. The number of pH drop events increased when increasing bicarbonate concentrations from 5 to 10 mM. The distinct pH drops are marked by arrows in the figures 2a, 2b and 2c. The first pH drops were observed after ~2.2 days, during the light cycle for one experiment and at the transition between light/dark cycles for the two others experiments. Their durations were approximately 1 hour. The second precipitation event occurred at ~ 4.1 days, only for the assays with 7.5 and 10 mM bicarbonate. The duration of this second pH drop was quite similar to the first one. These pH drops (acidification) reflected sudden eliminations of carbonate ions from the carbonate system.

Calcium and carbonate measurements at the beginning and at the end of the two first pH drop events confirmed that calcium and carbonate concentration decreases were strictly

time-correlated to pH drop. Moreover good correlations were obtained between calcium and carbonate concentration decreases (approximately 1 for 1). The first precipitation event occurred at ca. 1,400 µM carbonate and ca. 9,800 µM calcium concentrations (table 2). The extent of $CaCO_3$ precipitated was approximately 1,050 µM. The residual carbonate concentration after the first precipitation event was low (around 250/400 µM) but not equal to zero, despite a situation of calcium excess. The second precipitation event (only for assays with 7.5 and 10 mM bicarbonate) occurred at ~ 4.1 days at ca. 1,100 µM carbonate and ca. 8,000 µM calcium. The $CaCO_3$ precipitated extent was estimated to ca. 800 µM. The residual carbonate concentration after this second precipitation event was around 300 µM, close to the value obtained after the first pH drop event.

For these three experiments conducted with 5, 7.5 and 10 mM bicarbonate, the results accredit that the precipitation of calcium carbonate during the cyanobacterial growth proceeds roughly on a discontinuous manner. Such a drop of pH occurring during the photosynthetic phase means that, over this short period (< 1 hour), the carbonate elimination rate was greater than the production one according to photosynthetic activity.

Table 2: Precipitation events: calcium and carbonate concentration at the beginning and the end of the pH drop. Assays with 10.3mM calcium and variable HCO_3^- concentrations (5, 7.5 and 30mM).

Assay		$[CO_3^{2-}]_{T0}$	Precipitation event numbering	onset pH	Start of pH drop		End of pH drop			
					$[Ca^{2+}]$	$[CO_3^{2-}]$	$[Ca^{2+}]$	$[CO_3^{2-}]$	Ca^{2+} decrease	CO_3^{2-} decrease
		µM			µM	µM	µM	µM	µM	µM
6	**HCO_3^- = 5 mM**	252	1	9.25	9 188	1336	8 184	233	1003	1103
7	**HCO_3^- = 7.5 mM**	428	1	9.02	9 196	1486	8 125	247	1071	1238
8	**HCO_3^- = 10 mM**	624	1	8.80	9 284	1873	8 174	437	1110	1436
13	**HCO_3^- = 10 mM**	452	1	8.66	10428	1359	nd	nd	nd	nd
6	**HCO_3^- = 5 mM**	252	2	9.72	8 023	970	nd	nd	nd	nd
7	**HCO_3^- = 7.5 mM**	428	2	9.08	7863	1052	7238	313	625	739
8	**HCO_3^- = 10 mM**	624	2	8.92	8 131	1351	7 139	325	992	1026
13	**HCO_3^- = 10 mM**	452	3	8.93	8881	1195	nd	nd	nd	nd

$[CO_3^{2-}]_{T0}$: Initial carbonate concentration

Others experiments have been operated always with the same calcium concentration (10.3 mM) but with higher bicarbonate concentrations, up to 30 mM.

The assays with 10, 20 and 30 mM bicarbonate (assays 13, 14 and 15) have been prepared with the same cyanobacterial charge. For these assays, the pH has been recorded during four days before inoculation. Despite the fact that the culture medium turned slightly

turbid, the pH did not change during this period and the respective bicarbonate and carbonate concentrations remained stable. It is evident that the carbonate quantity involved in the formation of $CaCO_3$ during this phase before inoculation is very low. According to the pH profiles, respectively 3 marked pH drops have been identified for these three assays (respectively 3, 3 and 2 pH drops, a third onset was not clearly obtained for 30 mM bicarbonate). Five occurred at the transition of light/dark cycles. In a general manner, considering the first drop for these three assays, it can be ruled that the higher the initial bicarbonate concentration the quicker the onset of the pH drop. Indeed for the experiments conducted with 10, 20 and 30 mM bicarbonate the first onset appeared at 3.5, 2.3 and 1.6 days; the second at 5.2, 4.8 and 2.7 days and the third at 7.2 and 5.7 days, respectively . This shortening time was clearly correlated with the initial carbonate concentration in the growth medium, according to the distribution of the two inorganic carbon species (bicarbonate and carbonate). For pH~8 the carbonate species represented about 6% of the total mineral carbon introduced.

For the assay with 30 mM bicarbonate (figure 2e), the theoretical carbonate quantity produced by photosynthetic bicarbonate assimilation was in large excess compared to the calcium quantity available, so the final time course pH profile for this assay is similar to the one with bicarbonate deprived calcium medium. A rapid alkalinisation occurred due to bicarbonate disappearance and progressive carbonate concentration increase. Moreover, hydroxyl ions are observed when bicarbonate was completely assimilated.

For other experiments with 25 (assays 9 to 12) or 30 mM bicarbonate (assays 4 and 15) the pH profiles presented only a first marked pH drop. It appeared very quickly after 0.8 to 1.7 days probably in relation with a higher cyanobacterial inoculum charge. After the first precipitation event, during the following 3.5 days, the pH profiles presented a continuous decline (the second pH drop cannot anymore be considered as an isolate event, because it is followed by a marked continuous pH decrease during the night cycle). Furthermore, the alkalinisation during the light period was almost completely masked and the pH remained stable at values ranging between 8.3 and 7.4. The pH profiles obtained for these cultures with high initial bicarbonate concentrations suggested that after the first pH drop, the calcite precipitation could occur continuously

For the assays with initial concentrations of 25 mM bicarbonate (assays 9 to 12), calcium, bicarbonate and carbonate concentrations were measured every 12 hours (at each light cycle transition), after the period of continuous pH decline mentioned above. In these

assays the calcium concentrations decreased continuously during the light period as well as during the dark period (figure 2d). Although pH profiles showed again increasing and decreasing alternation, the carbonate concentration remained very low (around 50/200 µM) during this period. These results reinforced the idea that for high photosynthetic activities and an initial 10.3 mM calcium concentration the precipitation phenomenon could become continuous.

For all the assays with varying initial bicarbonate concentrations (from 5 to 30 mM) and constant calcium concentration (10.3 mM), the table 3 assembled the calcium and carbonate concentrations at the beginning of each pH drop event. For each experiment the successive pH drop events have been numbered. It can be rule that the two first precipitation events are distinct precipitation events. Consequently the value of $\Omega = [Ca^{2+}]*[CO_3^{2-}]/K_{sp}$ has been calculated. K_{sp} has been taken as 4.3E-07 $mol^2(kg\ sol)^{-2}$ for salinity S = 35 according to Mucci (1983). The ions activity coefficient was not taken into account for this estimation.

For the highest bicarbonate concentrations tested (20 to 30 mM), the drop events became less and less clearly marked. In these situations, where continuous precipitation was suspected, the Ω was probably not significant.

Scanning Electron Microscopy (SEM) imaging of culture samples have been made for three assays with 10.3 mM calcium and variable HCO_3^- concentration (5, 7.5 and 10 mM). For each sample, many fields of 0.84 mm×1.139 mm have been systematically observed. Images representative of each culture stage are presented in the figure 3. At early stage of the cyanobacterial growth (0.2 day), cells sticking together were observed (figure 3A). A few hours before the first pH drop and at the beginning of this pH drop, calcite in a scattered form (non-structured) and some in cubic form was found in close vicinity of the cells. The size of the crystal was around 1 to 5 µm (figure 3B and 3C). In the middle of the pH drop event (after 40 min), cubic form calcite was predominant. The size of the calcite agglomerates was greater than 20 µm. Traces of cells trapped in the agglomerate could be observed (figure 3D). At the end of the precipitation event (45 at 50 min), agglomerate size had grown (some are as big as 50 µm) and cubic as well as non-structured forms were easily observed. Traces of cells embedded in the agglomerates are also observed (figure 3E). At 4.1 days of culture, larger calcite crystals were characteristic of later stages of crystallization (between 50 to 100 µm) (figure 3F).

Table 3: Calcium and Carbonate concentrations at the beginning of different precipitation events. Assays with 10.3mM calcium and variable HCO_3^- concentrations (5-30mM).

Assay		Precipitation event numbering	$[CO_3^{2+}]_{T0}$ µM	Start of pH drop: onset pH	Start of pH drop: $[Ca^{2+}]$ µM	Start of pH drop: $[CO_3^{2-}]$ µM	Start of pH drop: Ω $\Omega = [Ca^{2+}]*[CO_3^{2-}]/Ksp$
6	**HCO_3^- = 5 mM**	1	252	9.25	9 188	1336	29
7	**HCO_3^- = 7.5 mM**	1	428	9.02	9 196	1486	32
8	**HCO_3^- = 10 mM**	1	624	8.80	9 284	1873	41
13	**HCO_3^- = 10 mM**	1	452	8.66	10 428	1359	33
	Mean value				**9524**	**1513**	**34**
6	**HCO_3^- = 5 mM**	2	252	9.72	8 023	970	18
7	**HCO_3^- = 7.5 mM**	2	428	9.08	7863	1052	19
8	**HCO_3^- = 10 mM**	2	624	8.92	8 131	1351	26
	Mean value				**8 006**	**1 124**	**21**
13	**HCO_3^- = 10 mM**	3	452	8.93	**8 881**	**1195**	**25**
9	**HCO_3^- = 25 mM**	4	2052	7.40	3 512	466	NS
10	**HCO_3^- = 25 mM**	4	2052	7.38	3 409	505	NS
11	**HCO_3^- = 25 mM**	4	2052	7.53	3 906	474	NS
12	**HCO_3^- = 25 mM**	4	2052	7.36	3 293	457	NS
14	**HCO_3^- = 20 mM**	4	860	7.41	4 689	200	NS
	Mean value				**3 762**	**420**	
10	**HCO_3^- = 25 mM**	5	2052	7.57	2 291	442	NS
11	**HCO_3^- = 25 mM**	5	2052	7.68	2 167	424	NS
14	**HCO_3^- = 20 mM**	5	807	7.34	3 392	207	NS
	Mean value				**2 616**	**358**	
9	**HCO_3^- = 25 mM**	7	2052	8.01	969	352	NS
10	**HCO_3^- = 25 mM**	7	2052	7.91	1 084	384	NS
11	**HCO_3^- = 25 mM**	7	2052	7.99	887	336	NS
12	**HCO_3^- = 25 mM**	7	2052	7.99	861	372	NS
	Mean value				**950**	**361**	

$[CO_3^{2-}]_{T0}$: Initial carbonate concentration

Ksp: 4.3E-07 (mol^2 $(kg\ soln)^{-2}$) for the sea water salinity

NS: No signification

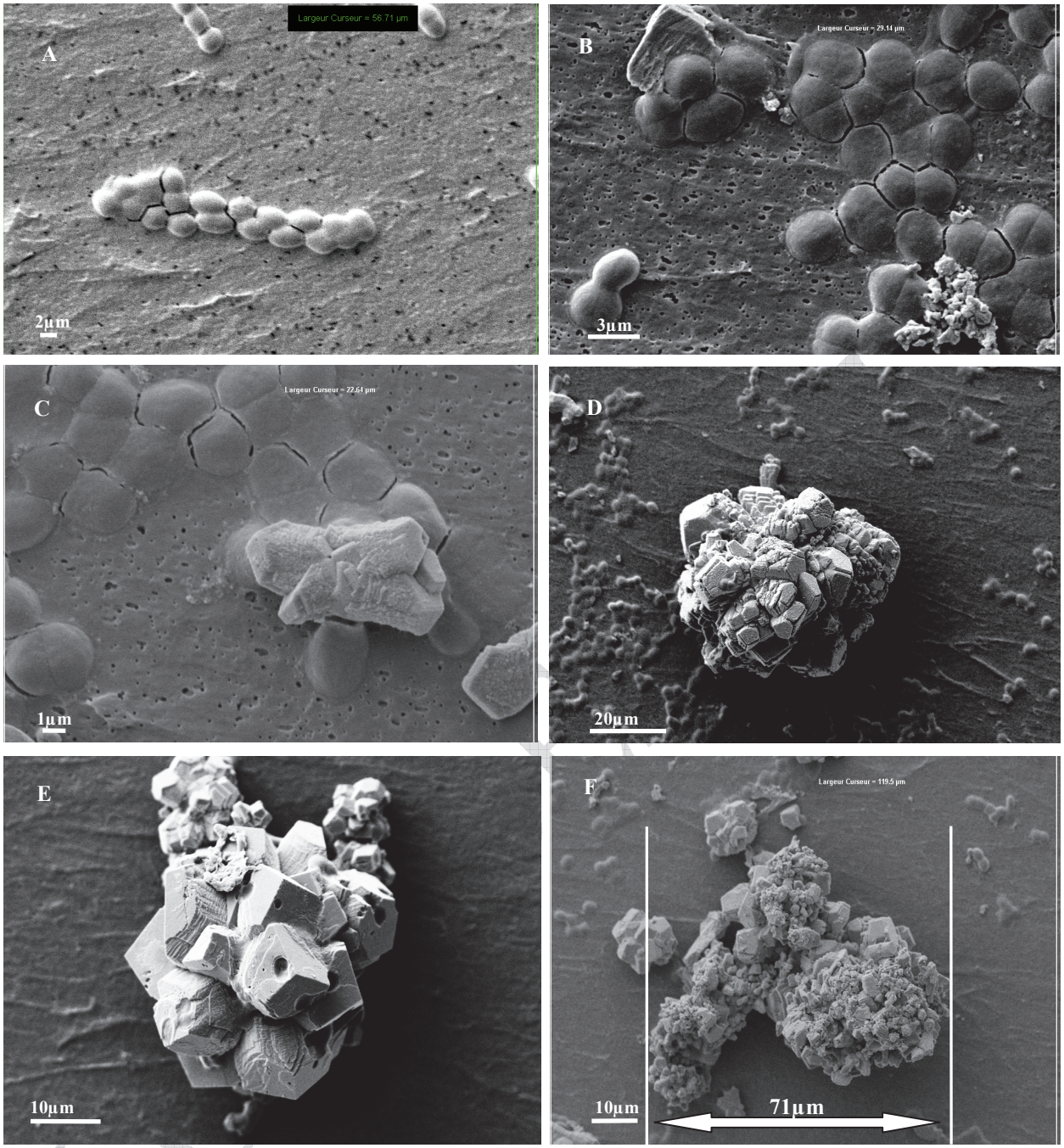

Figure 3: Scanning electron microscopy images at the different stages of crystal calcite formation. (A): at 0.2 days. (B) and (C): 2 hours before and at the beginning of the first pH drop (2.2 days). (D): during the pH drop (2.25 days). (E): at the end of the pH drop (2.26 days). (F): at a later stage (4.1 days).

➢ ***Varying calcium concentration (from 0.3 to 10.3mM)***

A series of assays with increasing initial $CaCl_2$ concentrations (ranging from 0.34 to 10.3 mM) has been performed while keeping the initial bicarbonate concentration constant at 30 mM. The different assays are presented in the table 1. The experiment performed with 0.34 mM calcium has been presented in the general requirement.

For the three other experiments conducted with 3.4, 6.8 and 10.3 mM initial calcium concentrations (assays 2 to 4), the first pH drop was clearly marked at 2.3, 1.5 and 0.8 days respectively.

For the assay with 3.4 mM calcium, only one pH drop occurred at the transition of the light/dark cycle. A pH increase was observed in the following photosynthesis cycle after the first pH drop. For the other two assays with 6.8 and 10 mM calcium, after the first precipitation event, the pH profile presented very weak or no increase during the four following photosynthesis cycles. Thus, continuous $CaCO_3$ precipitation after the first pH drop, as in situations discussed above (see assays 9 to 12, and assays 4 and 15) could be suspected.

Influence of calcite particles on $CaCO_3$ precipitation

Two experiments were performed to investigate the effects of $CaCO_3$ grain addition on the precipitation process. The initial concentrations of Ca^{2+} and HCO_3^- were respectively 10.3 mM and 7.5 mM. For one assay, 1g l^{-1} of fine powdered (grain size ca. 2~40 µm) $CaCO_3$ was added into the medium at the start of the culture. Before inoculation, the pH did not change and the respective bicarbonate and carbonate concentrations remained stable during a period of 4 days. Then after, these two assays have been inoculated with the same cyanobacterial charge.

Four marked pH drop events e.g. calcite precipitation could be identified for each assay (figure 4). For the experiments without $CaCO_3$ addition, the first pH drop event was clearly marked at 0.3 day. This first pH drop appeared earlier than in the assay without initial calcite addition (0.8 days). For this experiment with calcite addition, when the first precipitation event occurred, the carbonate concentration was ca. 930 µM. This value is a little lower than the lowest value obtained for the same calcium concentration, ca. 1100 to 1400 µM.

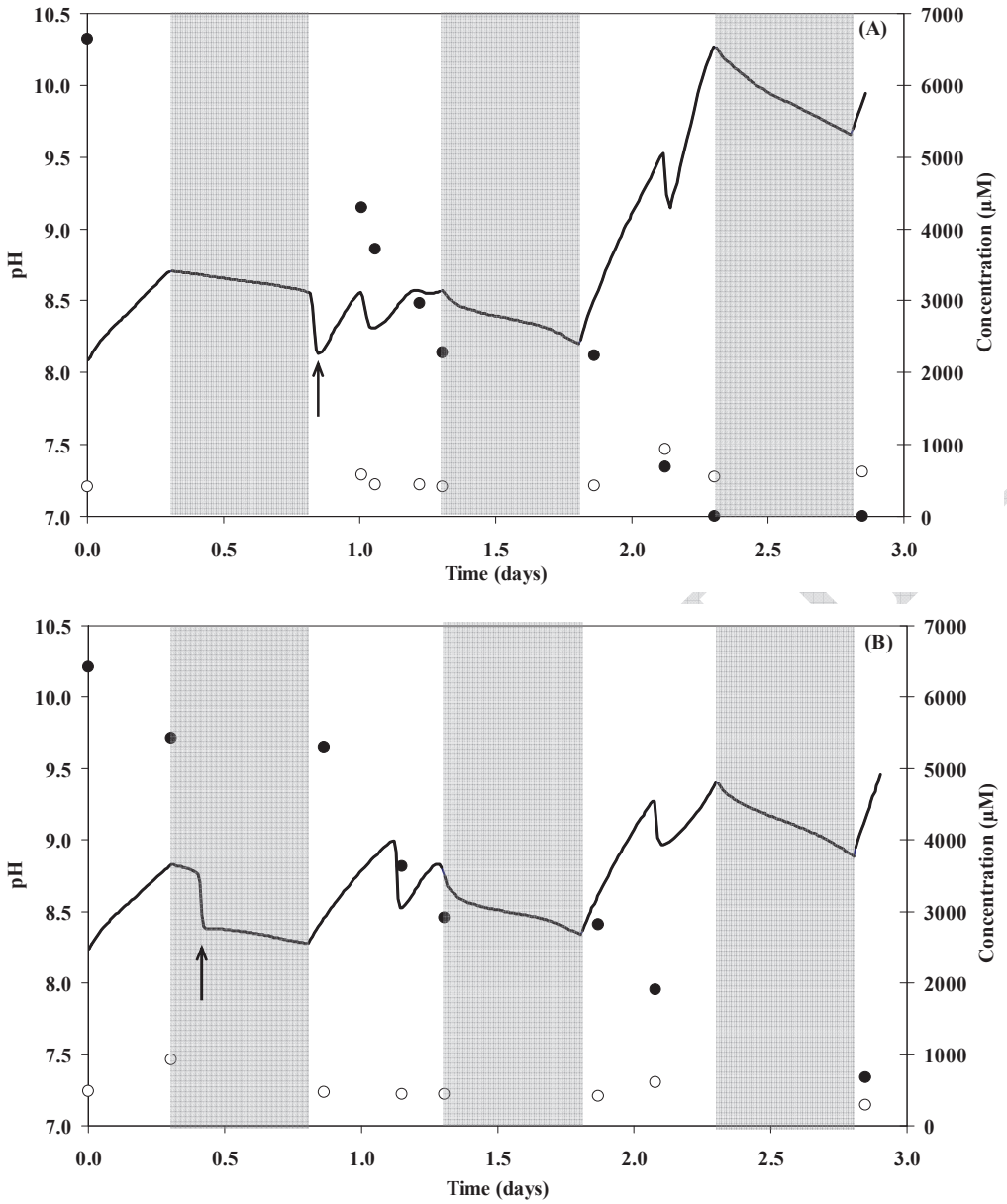

Figure 4: Cyanobacterial growth during light cycles (blank column) and dark cycles (gray column). (A): assay 16 (10.3mM Ca^{2+}; 7.5mM HCO_3^-; with 12 hours light cycle and 12 hours dark cycle) without $CaCO_3$ in the medium at the beginning of growth; (B): assay 17 (10.3mM Ca^{2+}; 7.5mM HCO_3^-; with 12 hours light cycle and 12 hours dark cycle) with $CaCO_3$ in the medium at the beginning of growth. Evolutions of pH (black line), HCO_3^- (filled circle) and CO_3^{2-} (blank circle). Arrowheads indicate the first precipitation events.

Conclusions

In order to determine the influence of the cyanobacterial photosynthesis processes on calcite precipitation, we grow the marine cyanobacterial strain *Synechococcus* sp. PCC 8806 on a bicarbonate medium, varying its concentration from 2.5 to 30 mM with 10.3 mM $CaCl_2$ (concentration of sea water). Control with a very low $CaCl_2$ content (0.34 mM) presented a typical progressive pH increase profile for cyanobacterial growth on batch condition which was due to the counterbalance between bicarbonate assimilated and carbonate produced. The alternation of light and dark cycles punctuated primarily the pH profile; the night period corresponding to the dark-respiration presented a systematic decrease of the pH related to the reaction between carbonate and CO_2 release from the cells, producing bicarbonate.

In experiments undertaken with calcium (>0.34mM), pH drops are systematically observed. These pH drop events are very short events (approximately one hour), clearly marked (pH decreases between 0.5 and 1 unit) and appeared during the light period. They can also appear at the end of the light period. Both calcium and carbonate decreases were strongly associated with the pH drop. Scanning electron microscope (SEM) images taken at different moments of the first pH drop event comforted the calcite precipitation with the pH drop. The precipitation was initiated in the cell vicinity as small grains (around 1 to 5 µm) and the major crystal growth was observed at the end of the pH drop. Moreover, slightly lower values for both calcium and carbonate concentrations have been measured for the second pH drop. This could perhaps accredit the role of cell structures or calcium carbonate grains as nucleation sites, favoring calcite crystallization according to the Ω calculated values. Experiments with higher initial bicarbonate concentrations contributed to higher carbonate productions and resulted in "continuous" calcite precipitation events succeeding to distinct precipitation events. Similar results could probably be obtained in the assay with high bicarbonate concentrations (30 mM) and a lower calcium concentration (6.8 mM). This "continuous" precipitation could be explained by both increases of biomass concentration and calcite precipitation contributing to favor the precipitation observed. This continuous precipitation mechanism occurred at neutral pH values, corresponding to very low carbonate concentration (around 50/200 µM), suggesting a possible limitation of $CaCO_3$ precipitation rate due to limited carbonate availability.

As cyanobacteria are ubiquitous and abundant in oceans and lakes, their influence on the calcite precipitation might present a significant impact on global carbon cycling. The

facilitated $CaCO_3$ precipitation due the presence of nucleation sites occur at lower Ω values and may contribute in natural environments to the carbon sink to the sediment. Moreover, the link of this precipitation mechanism with the cyanobacteria growth where the carbonate could be sequestered in an insoluble mineral form may be considered as a new future for reduction atmospheric CO_2 concentration and / or as an alternative path for capturing highly concentrated CO_2 gas fluxes.

The precipitation of $CaCO_3$ is mainly governed by the calcite saturation Ω, depending on the product of calcium and carbonate concentrations. Spontaneous nucleation need high oversaturation values ($\Omega = [Ca^{2+}]*[CO_3^{2-}]/K_{sp} > 100$, (with K_{sp} for 25°C), that are never encountered at large scales in natural environments (Dittrich and Obst, 2004).

In open environmental system as large lake or marine environments, the concentrations of carbonate and calcium in the bulk solution cannot be moved. Oppositely in batch condition, as in our experiments, photosynthetic assimilation of bicarbonate was responsible for increase of carbonate concentration in the bulk solution, contributing to favors oversaturation carbonate conditions. The pH drops are obtained with active photosynthetic activity (growth of the cells) and presence of calcium (from 3.4 to 10.3 mM) and accredited the rapid increase of the product $[Ca^{2+}]*[CO_3^{2-}]$. These results suggested that precipitation in our batch conditions is mainly governed by the concentration of carbonate and calcium in the bulk solution, according to the saturation curve for CaCO3 precipitation. Nevertheless cell materials or cell membranes could play an active role for the furniture of nucleation sites as mentioned by Obst et al (2009). Scanning electron microscope (SEM) imaging of environmental samples often showed cyanobacterial cells embedded in calcite or calcite crystal surfaces showing cell marks. This SEM imaging sustains a nucleation on the EPS matrix (or S-layers), perhaps followed by growth phase of the calcite crystal outside the cell, perhaps on EPS matrix that shed from the cells.

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