Conversion of bicarbonate into biomass and calcium carbonate by the Cyanobacteria strain Synechococcus sp. PCC 8806

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Abstract

Utilization of bicarbonate in cyanobacterial photosynthesis releases hydroxyl ions which react with bicarbonate to produce carbonate. In sea water this increase in carbonate concentration can lead to calcite precipitation in the presence of calcium. In natural environments, this could take place in conditions such as high density cell blooms or mats. Cyanobacteria considered as calcifiers obviously rely on this physiological mechanism to perform calcification and photosynthesis in a putative 1:1 molar ratio. Our study was designed to thoroughly investigate this photosynthesis/precipitation ratio. We used the Rock Eval® technology to determine the quantity of both carbon and calcium incorporated into the biomass and calcium carbonate. *Synechococcus* PCC 8806 was grown in mineral culture medium enriched with various bicarbonate and calcium concentrations in a closed reactor preventing atmospheric CO_2 exchange. Our findings show that cyanobacterial growth on bicarbonate results in a rise of carbonate ions and large pH change. During cells growth, C_{HCO3-} was distributed to $C_{biomass}$ and C_{CO32-} with a ratio of ≈ 0.5. The biomass produced was independent of the calcium concentration in the growth medium. The calcium carbonate precipitation depends mainly on both calcium and bicarbonate concentrations.

Introduction

Throughout Earth's history, particularly during the Precambrian period, microorganisms (including cyanobacteria) have contributed to stromatolite formations and calcimicrobial reefs (e.g. in the Neoproterozoic) over shelfal areas (Riding, 2000). The pelagic component has become significant only much more recently, i.e. during the Mesozoic (Boss and Wilkinson, 1991).

Cyanobacteria have been reported as biological contributors for high oversaturation in carbonate of their microenvironment (Thompson et al., 1997). All the proposed cyanobacterial precipitation mechanisms are commonly based on mineral carbonate precipitation reactions linked to cellular physiological processes, according to the *in vivo* cyanobacterial CO_2 assimilation model driven by CCM enhanced photosynthesis (Miller & Colman, 1980; Thompson & Ferris, 1990; Kaplan et al., 1991; Merz, 1992; McConnaughey, 1994; Price et al., 1998; Kaplan & Reinhold, 1999; Badger & Price, 2003). The microscopic observations of organisms embedded in non structured calcite crystals show that the growth of crystals probably happens in the microenvironment of cell outer membranes (Dittrich and Obst 2004).

Cyanobacterial blooms resulting from favourable growth conditions, especially the input of nutrients, are often associated with whiting events, e.g. brief and large $CaCO_3$ calcification deposition events. Several field studies have stimulated the question on biologically active precipitation with cyanobacteria (Morse et al., 2003). Whiting settings have been extensively studied: for instance the Fayetteville Green Lake (hard water lake) (Thompson and Ferris, 1990; Thompson 1997) and the Great Bahamas Bank (marine environment) (Thompson, 2000)... Sedimentation rates of carbonate precipitates have been estimated from field and experimental data. In Fayetteville Green Lake (FGL), Thomson et al. (1997), on site, measured calcite precipitations between 3.5-4.0 mg per litre in the open water column between the water surface and 2 m below the surface during the spring, and even greater concentrations during the summer with a depth of 8 m. These authors also measured a decrease of the Dissolved Inorganic Carbon (DIC) concentration and inversely an increase of the $\delta^{13}C$ DIC value in the water column over these periods of spring and summer. This $\delta^{13}C$ DIC increase was attributed to the selective uptake of $\delta^{12}C$ for photosynthetic CO_2 fixation by the cyanobacteria bloom, but a concomitant $\delta^{13}C$ enrichment of the calcium carbonates in the most recent bottom sediments was also observed. The same evolutions of the $^{12}C/^{13}C$ isotopes (as observed on FGL site) have successfully been reproduced in laboratory microcosms using

the water and the micro-florae of this site. The differences isotopic values obtained in the calcite (from sediments) and in the DIC (water column) may suggest that the precipitation occurred in chemical microenvironments that were not at equilibrium with the bulk of the lake water. These authors, despite interesting isotopic studies on FGL site, did not present information about carbon partition into biomass and calcite.

Moreover no studies present clearly reliable mass balance for carbon and calcium, the major elements involved in cyanobacterial calcification processes. Merz's pioneer work (1992) on microbial mats has reported an approximate 1:1 ratio of calcification to photosynthesis (around 1 to 1.2 mol carbon fixed in biomass for 1 mol precipitated carbonate) under field conditions. Nevertheless the biomass was not measured directly. Daily variations of alkalinity and calcium have been observed in the interstitial water of the mats in the reported experiments. These variations have been used to indirectly calculate the carbon incorporated in calcium carbonate precipitates. Yates and Robbins (1998) also calculated calcification from initial and post incubation alkalinity, pH, and calcium measurements to determine the ratio between calcification and photosynthesis. These authors mentioned that DIC calculation based on alkalinity and pH variations may not accurately reflect calcification or photosynthesis.

Literature also lacks in information concerning the onset and the extent of $CaCO_3$ precipitation during active cyanobacterial growth. In order to better understand $CaCO_3$ precipitation by cyanobacteria, it is crucial to have accurate mass balances of carbon and calcium during the growth. Mass balances are difficult to obtain because the calcified cyanobacteria are a mix of organic and inorganic carbon which is ill-suited for most analytical methods. The purpose of this study was to develop and validate an analytical strategy based on the use of the Rock-Eval 6 (RE6) technique to determine the carbon distribution in calcium carbonate and biomass during cyanobacterial growth on bicarbonate. RE6 is able to determine precisely the organic and inorganic carbon of rocks (Behar et al., 2001), but has never been applied to the quantitative and qualitative characterisation of biological samples thus far. In this study, the RE6 was calibrated with pure cyanobacterial biomass cultivated in calcium deprived medium and pure calcium carbonate. It was utilized for the monitoring of bicarbonate assimilation and $CaCO_3$ precipitation by the marine calcifying cyanobacterium *Synechococcus sp.* PCC 8806 on various bicarbonate/Ca^{2+} combination cultures.

Materials and Methods

Cyanobacterial Cultures and Experimental Setup

➢ *Bacterial Strain and Culture Conditions*

The marine calcifying cyanobacterial strain *Synechococcus sp.* PCC 8806 used in this study was maintained on low (0.38 mM) $CaCl_2$ ASNIII growth medium (Rippka et al., 1979) by transferring 10% (v/v) of the culture to fresh sterile medium every 4 weeks with atmospheric CO_2 as the carbon source.

Experiments were performed in 500 mL clear glass serum bottles equipped with sampling ports and filled with 200 mL of culture medium containing 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 $FeCl_3 \cdot 6H_2O$, and 1 mL Trace Metal Mix A5 + Co (Rippka et al., 1979). The pH of the medium was adjusted to 8.0. $NaHCO_3$ and $CaCl_2$ were introduced as variables in the mineral medium. When needed, $CaCl_2$ was added to the medium prior to autoclaving and $NaHCO_3$ was added from a sterile stock solution just prior to inoculation.

The culture flasks were inoculated with cyanobacteria to a target concentration of 1×10^7 cells mL^{-1} and immediately sealed with a rubber septum and plastic screwed end cap to avoid CO_2 exchange with the atmosphere.

All cultures were grown at 25°C in a plant growth chamber (Snijders, ECD01E) with a 12h/12h light/dark cycle, 1.79 klux photon irradiance, and an agitation rate at 60 rpm.

➢ *End-Point Experiments*

Calcium and bicarbonate were added to the culture medium in variable amounts ranging from 0.34 mM up to 6.8 mM for Ca and from 5 mM to 15 mM for HCO_3^- Each assay represented one bicarbonate and one calcium concentration and was performed in triplicate. The headspace composition was at atmospheric conditions; therefore the concentration of oxygen in the headspace at the beginning of each experiment was 21 % (v/v) while that of CO_2 was negligible (less than 10 µmol) compared to the introduced amounts of HCO_3^-. At the end of the incubation, the whole culture was processed to determine the overall mass balance (see below Sample Preparation).

➢ *Kinetic Experiments*

Kinetic experiments were set up as described for the end-point experiments, except that the initial culture (with inoculum added) was immediately split into sub-cultures. One subculture was sacrificed for analysis at each kinetic time point. Mass balance determination,

especially the analysis of the insoluble content of the culture required the entire content of a culture flask to be processed.

- ***Abiotic Precipitation Experiments***

Abiotic precipitation experiments were set up using the same concentrations of bicarbonate and calcium as for the experiments inoculated with cyanobacteria. Abiotic precipitation was performed in mineral medium and in distilled water. The calcite precipitation was initiated by increasing the pH value to 11 with NaOH. Bottles were closed immediately after NaOH addition and analyzed after 7 days exposure in the same way as described below.

- ***Sample Preparation***

At the end of each assay, the headspace was sampled before opening the bottle for CO_2 and O_2 quantification. The pH of the culture medium was measured (pH-meter, Mettler Toledo) and the medium was then filtered through a 0.45µm PTFE filter to separate the supernatant from the insoluble matter (cells + calcium carbonate). Filtrate was either analyzed immediately or stored prior to analysis in top-filled tubes at 4°C to avoid re-equilibration of the filtrate with atmospheric CO_2. The insoluble matter was not washed to avoid losses of calcium carbonate and lysis of cells. The bacterial and mineral cake was dried for 24 h at 100°C and stored at +4°C until further analysis.

Analyses

- ***Quantification of CO_2 and O_2***

Carbon dioxide and oxygen concentrations in the headspace were measured by gas chromatography (GC) with a thermal conductivity detector on a Varian 3800 chromatograph equipped with a Porapak Q (80/100 mesh, Chrompack) column (2 mm ID, 2 m long) for CO_2 and with a molecular sieve column (80/100 mesh, Altech; 2 mm ID, 2 m long) for O_2. Helium was used as the carrier gas with a flow rate of 30 mL/min. The operational temperatures for CO_2 analysis were 130°C, 130°C, and 100°C for the injector, detector, and column respectively, and for O_2 analysis were 130°C, 130°C, and 50°C. 250µL of headspace gas were injected. Concentration were calculated from the peak area according to calibration curves obtained with reference gases, e.g. CO_2 5%v/v ± 0.096 in N_2, (Air Liquide, France) for CO_2, and atmospheric gas (O_2 = 20.9 %v/v) for O_2. Detection limits were 100 nmol and 2 nmol for O_2 and CO_2 respectively.

- ***Quantification of Anions and Cations***

The concentrations of cations (Ca^{2+}, Mg^{2+}) and of anions (NO_3^-, NO_2^-) were determined by analysing the filtrate (culture medium samples after filtration on 0.45 µm PTFE filter). Ions were quantified by High Performance Liquid Chromatography (HPLC) on a DIONEX ICS 3000 equipped with an eluent generator and two self regenerating suppressors (CSRS-ULTRA 4 mm for cation and ASRS-ULTRA 4 mm for anion).

Cations and anions were separated at 30°C on either a DIONEX IonPac CS12 column (4 mm x 250 mm) with 20 mM methasulfonic acid at a flow rate of 1 mL/min (cations) and on an IonPac AS9-HC column (4 mm x 250 mm) with a gradient of KOH at a flow rate of 1 mL/min respectively (anions) and quantifyed with a conductivity detector. The volume analyzed was 25 µL (full-loop injection). Linear calibration curves (with $R^2 > 0.999$) were obtained for calcium (0-400 µM), magnesium (0-1000 µM), and nitrate (0-2.5 mM) and a polynomial curve was established for nitrite (0-2.5 mM) with $R^2 > 0.999$. Detection limits were respectively 5 µM, 5 µM, 20 µM, and 50 µM.

- ***Quantification of HCO_3^-, CO_3^{2-} and OH^-***

The concentration of bicarbonate, carbonate and OH^- ions were determined by titration with an automatic titrator (Titrimo 702 SM, METROHM). 40 mL of culture medium were analyzed immediately after filtration with a 0.45 µm PTFE filter. The samples were not diluted to avoid changing the ratio of bicarbonate/carbonate due to pH change and atmospheric CO_2 re-equilibration. Titration was performed with 100 µL step volumes of either a 0,01 N H_2SO_4 solution (corresponding to 1 µmol H^+) or a 0.001 N solution depending on the expected concentrations of bicarbonate and carbonate. The data were saved by software Tiamo (METROHM). The two inflexion points corresponding to the transitions between carbonate/bicarbonate and bicarbonate/CO_2 respectively were extracted by the second-derivative method. When OH^- ions are present in the sample, the first inflexion point corresponds to the transitions between carbonate and bicarbonate. In this situation the quantity of H^+ (µmol) added at the first inflexion point represented OH^- neutralization plus carbonate/bicarbonate transition. The detection limits were 5 µmol for CO_3^{2-}, HCO_3^- and OH^-.

- ***Quantification of Mineral Carbon (MinC) and Dissolved Organic Carbon (DOC)***

The concentration of Dissolved Organic Carbon (DOC) and Mineral Carbon (MinC) were determined on a TOC 5050 (Shimadzu, Japan). This apparatus uses two lines of analysis, the first line for total carbon (TC) quantification and the second one for mineral carbon (MinC) quantification. DOC was calculated by subtracting MinC from TC. A maximum of 100 µL of filtered growth culture samples prepared as described above, was injected in the TC line where it was completely decomposed to CO_2 and quantified by an infra-red detector. A

maximum of 250 µL of sample was injected in the MinC line where it reacted with a solution of 20% ortho-phosphoric acid (v/v), allowing conversion of both bicarbonate and carbonate to CO_2 which was then quantified using the same IR detector. The TC line was calibrated with a potassium hydrogenophtalate solution ($C_8H_5KO_4$) between 0 and 83 mM. The MinC line was calibrated with a carbonate/bicarbonate solution (50/50, w/w) between 0 to 41 mM. The ranges of detection were 50 ppb to 4,000 ppm and 50 ppb to 5,000 ppm for TC and MinC respectively.

- ***Elemental analysis of cyanobacterial cells***

The elemental composition of the cyanobacterial cells was determined according to the ASTM 5991 method. Biomass from calcium deprived cultures was harvested by centrifugation, and then carefully washed with a 9‰ NaCl solution to eliminate salts from the culture medium. Washed biomass was dried at 80°C and carefully crushed in a mortar. The C, N, H analysis, the O analysis and the S analysis needed each approximately 1-3 mg of sample.

- ***Rock Eval 6 Analysis***

The concentration of carbon present in the biomass and in the calcium carbonate was quantified by Rock Eval 6 analysis (RE 6, Vinci Technologies, France). The dryed insoluble matter (biomass plus calcium carbonate) was introduced in a crucible. This dry insoluble matter was first analyzed with the pyrolysis cycle and then with the oxidation cycle as described by Behar et al. (2001). The pyrolysis cycle was operated with nitrogen as carrier gas (in absence of oxygen). Oven temperature conditions were a steady 300°C for 3 min followed by an increase at a rate of 25°C min^{-1} to reach 650°C. The oxidation cycle was then operated on the residue from the pyrolysis cycle, in the presence of oxygen. Oven temperature conditions were a steady 300°C for 1 min, followed by an increase at a rate of 20°C min^{-1} to reach 850°C.

The biomass is quantitatively decomposed into volatile compounds (in a hydrocarbon type form) detected by the flame ionization detector (FID) during the pyrolysis cycle and into CO and CO_2 detected with the infrared detector during both the pyrolysis and oxidation cycles. The calcium carbonate is only decomposed into CO and CO_2 during the two RE6 analytical steps: pyrolysis and oxidation. Cyanobacterial biomass cultivated on medium without calcium (also used for elemental analysis) and calcium carbonate (purity > 98%, VWR) are taken as analytical standards to optimize the pyrolysis/oxidation cycles and to calibrate the respective $C_{Biomass}$ and C_{CaCO3} quantifications.

The post oxidation residue (mass weighted in the crucible after pyrolysis and oxidation cycles) was systematically measured.

➢ ***Scanning Electron Microscopy***

Cyanobacterial cells and $CaCO_3$ minerals were imaged with a Scanning Electron Microscopy (JEOL, Netherlands). The cells and calcium carbonate were filtered from the cyanobacterial cultures. The filter was suspended for 1 hour in a 2.5 % glutaraldehyde solution (v/v) for fixation. After fixation, the insoluble matter (biomass plus calcium carbonate) was recovered by centrifugation and washed 3 times in a phosphate buffer (pH 7.4) to remove the residual glutaraldehyde and the remaining salts and then filtered again with a 0.45 µm PTFE filter. The fixed insoluble matter was recovered from the filter and dried at 40°C for 24 hours. After that samples were sputter coated with 10 nm gold before SEM imaging.

Results and Discussions

Calibration of the Rock Eval 6 analysis

The ability of the Rock Eval 6 (RE 6) analysis to precisely quantify a mixture of organic carbon (OC) and inorganic carbon (IC) was tested by analyzing biomass (grown on culture medium without calcium), calcium carbonate (purity > 98%) and mixtures of biomass plus calcium carbonate in various proportions. The elemental composition of the biomass of *Synechococcus sp.* PCC 8806 grown on maintenance culture medium (0.38 mM calcium) was: 45.9% C, 6.8% H, 8.9% N, 31.2% O and 0.6% S ($C_4H_{7.13}O_{2.04}N_{0.66}S_{0.02}$).

The OC response occurred at 53 % C (w/w) in the pyrolysis cycle and 47 % C (w/w) in the oxidation cycle respectively (Figure 1). A linear correlation between biomass and organic carbon (OC) analyzed by RE 6 was established over a range of biomass from 0 to 20 mg:

$$OC = 33.8 \times biomass + 9.1 \quad (R^2 > 0.9999) \qquad \text{(equation 1)}$$

where OC is expressed in µmol of carbon and biomass in mg.

The IC response was observed essentially during the oxidation cycle (97%) (Figure 1). A linear correlation between calcium carbonate ($CaCO_3$) and inorganic carbon (IC) analyzed by RE6 was obtained over a range of calcium carbonate from 0 to 60 mg:

$$C = 10.92 \times CaCO_3 - 6.66 \quad (R^2 > 0.9999): \qquad \text{equation 2,}$$

where IC is expressed in µmol of carbon and $CaCO_3$ in mg.

Three mixtures of biomass and calcium carbonate were analyzed by RE 6, ranging from 7 mg to 15 mg for biomass and from 7 mg to 60 mg for $CaCO_3$. The IC ($\approx C_{CaCO3}$) and OC ($C_{Biomass}$) values (µmol C) obtained for these three mixtures are presented in Table 1, as

well as the respective quantities (mg) of biomass and $CaCO_3$, calculated from the data using the two equations 1 and 2. $CaCO_3$ calculated values were systematically slightly overestimated (≈ +2% for 30 mg $CaCO_3$ introduced), while biomass was systematically slightly underestimated (≈ -1% for 15 mg biomass introduced). Since the divergences remained low, we considered the RE 6 analysis as appropriate to accurately estimate the inorganic and organic carbon in the dry insoluble matter (biomass plus calcium carbonate).

Carbon and Calcium Mass Balances in Cyanobacterial Cultures

This analytical protocol was applied to obtain precise calcium and carbon mass balances in cyanobacterial cultures. Experiments were setup in triplicates with different initial concentrations of bicarbonate (sole carbon source) and calcium. These ranged from 5 to 15 mM and 0.34 to 6.8 mM respectively (Table 2). These cultures were analyzed after total disappearance of bicarbonate in the growth medium, which corresponded to the end of photosynthetic growth in our closed experimental reactor. Cultures were also analysed immediately following inoculation (on additional replicates).

The concentration of DOC, MinC, bicarbonate, carbonate, hydroxyl ions and soluble calcium were determined in the filtrate. For each assay, the totality of the insoluble matter recovered by filtration was analyzed by RE 6. The mass recovery for the RE 6 analysis was systematically verified: the sum of biomass (mg), calcium carbonate (mg) and residue (mg) obtained from the RE 6 data was compared with the weight of the dry insoluble matter introduced in the crucible. The post oxidation residue (residual mass of the crucible after pyrolysis and oxidation cycles) was systematically measured in all samples and corresponded mainly to mineral salts originated from the culture medium (including calcium). This residue was also observed in abiotic $CaCO_3$ precipitation controls performed on salt medium culture but not in distilled water. All samples analyzed presented a RE 6 mass recovery ranging between 99 and 109 % (w/w) (Table 2).

Carbon and calcium balances were determined by comparing the carbon and calcium present at the beginning and at the end of the experiment. The carbon balance was performed as following. The carbon initially present in the form of both dissolved organic carbon and biomass introduced by the inoculum and in the form of bicarbonate and carbonate present in the culture medium was compared to the carbon present at the end of the experiment and distributed between an insoluble organic and inorganic matter, measured by RE 6, and a soluble bicarbonate, carbonate and dissolved organic carbon in the filtrate. The calcium balance was performed as following. The calcium present at the beginning of the experiment

in form of soluble ion (Ca^{+}) was compared to the calcium present at the end of the experiment, while a part of it is present as soluble ion (Ca^{2+}) in the growth medium and a remaining part is present as $CaCO_3$ in the insoluble matter. Assuming that all the carbonate precipitated in the form of $CaCO_3$, the insoluble calcium fraction can be extrapolated from the inorganic carbon measured by RE 6, according to the $CaCO_3$ stoichiometry.

The cultures presented carbon mass balances ranging from 92 to 107% and calcium mass balances from 83 to 107%. The data presented in Table 2 clearly show carbon and calcium balances with a precision < 5% when combining the RE 6 with the other analytical methods. It is thus possible to monitor precisely the carbon partition during cyanobacterial growth and concomitant $CaCO_3$ precipitation.

Kinetic Parameter Evolutions During Growth on Bicarbonate

Three series of assays with various initial calcium concentrations, 0.34, 3.4 and 6.8 mM and bicarbonate concentrations, 2.5, 5 and 7.5 mM were performed. The evolution of pH, bicarbonate, carbonate, calcium, hydroxyl ion, DOC, biomass and calcium carbonate were monitored during the experiment. Figure 2a presents a representative example for a culture supplemented with 1,500 µmol HCO_3^- (7.5 mM) and 1,360 µmol $CaCl_2$ (6.8 mM) monitored over 15 days. As bicarbonate is rapidly used for cyanobacterial growth, the pH of the medium increased to reach ca. 11.3 after 5 days at which point it remained stable. Bicarbonate is completely consumed after 6 days. During these 6 days, a rapid decrease of Ca concentration due to $CaCO_3$ precipitation in the growth medium was observed. In all kinetic experiments, the bicarbonate was always completely utilized regardless of the quantity introduced (1,000 to 3,000 µmol). Thereafter, OH^- alkalinity was systematically measured, as already reported by Lee *et al.*, (2004).

The biomass production and calcium carbonate precipitation during bicarbonate assimilation are presented in Figure 2b. Both the production of biomass and precipitation of calcium carbonate finished on day 6. DOC was analysed immediately after inoculation and was found to be constant during the cyanobacterial growth, but increased after the complete disappearance of the bicarbonate. This suggests that cell lysis occurred rapidly at the end of the growth phase. The cell lysis could explain the variable initial DOC charge (around 100 to 150 µmol C) for the different cultures, which could depend on both the age and the volume of the inoculum used.

For 7.5 mM bicarbonate, the maximal optical density (at 600 nm) was ≈ 0.5 which corresponded to ≈ 100 mg/L of biomass (dry matter). For these absorbance values, photo-

limitation was very moderate (data not shown). The smallest generation time (G) was greater than 1 day and was obtained during the first days of culture.

Conversion Ratio of Bicarbonate into Biomass and Calcium Carbonate

Synechococcus biomass growth appeared independent of the calcium concentration in the growth medium (Figure 3a). Indeed the HCO_3^- assimilation and the carbonate production (expressed as the sum CO_3^- + $CaCO_3$) appeared independent of the calcium concentration in the growth medium (between 0.34 and 6.8 mM) (Figure 3b and 3c). In this kinetic study the carbon balance has been calculated without taking into account the DOC that strongly impacted the OC distribution due to biomass lysis in prolonged experiments (see data presented in Table 2). The C balances measured during the growth phase (expressed as the sum $[C_{biomass}+ C_{CO3-} + C_{CaCO3}]/ C_{HCO3-}$ consumed) are presented in the Figure 3d for the assays with 7.5 mM bicarbonate and various calcium concentrations (0.34; 3.4 and 6.8 mM). The carbon and calcium balances obtained were around 97/103 %, excepted for the first points (first day and some data for the second day). The relevance of the carbon balance values for these early points in this kinetic study may be questioned since very low biomass and carbonate are produced and low bicarbonate consumed.

The ratio between $C_{biomass\ synthesized}$ and $C_{carbonates\ produced}$ (expressed as the sum of CO_3^{2-} + $CaCO_3$) is presented in the Figure 3d. Since the production of *Synechococcus* biomass is independent of the calcium concentration, this ratio has been calculated by combining all the data from the experiments undertaken with the three different calcium concentrations (0.34; 3.4 and 6.8 mM). Just before the complete bicarbonate assimilation, this ratio increased from ≈ 0.46 to ≈ 0.52. This upward tendency at the end of the growth could reflect the indirect use of carbonate as carbon source for biomass production. In fact, in batch conditions, the reversible equilibrium between the two carbonate species regenerates bicarbonate which is incorporated into the biomass, despite the low bicarbonate concentration predicted by DIC, H^+ (pH), pK_1* and pK_2* values (* denotes stoichiometric equilibrium constant). In this condition, hydroxyl ions are detected, due to the chemical conversion of carbonate to bicarbonate (necessary for the charge balance) and the assimilation of bicarbonate. Nevertheless the lysis phenomenon observed systematically in batch conditions when the bicarbonate was completely consumed suggested a possible inhibition of the cyanobacterial growth at pH upon 11, limiting the carbonate fraction re-used. Theses conclusions highlighted the difficulties to precisely determine the ratio between biomass and carbonate (as free carbonate + $CaCO_3$) when using bicarbonate as sole carbon source. Nevertheless, to our

knowledge, this is the first time detailed experimental mass balance data on carbon assimilation are reported with bicarbonate as carbon source, in presence and in absence of calcium in the growth medium. This ratio is still fairly close to the predicted value of 0.5, reinforced cyanobacterial CO_2 assimilation model driven by CCM enhanced photosynthesis. One bicarbonate ion is incorporated into biomass generating a hydroxyl ion which reacts with a second bicarbonate to form carbonate as per equation 3:

$$2HCO_3^- \rightarrow [CO_{2biomass}] + H_2O + 1CO_3^{2-} \qquad \text{(equation 3)}$$

$CaCO_3$ Precipitation during Growth

The carbonate produced during the growth of *Synechococcus* can react with the dissolved calcium present in the growth medium and form $CaCO_3$. In the presence of high calcium concentrations (3.4 and 6.8 mM), the green colour of the cyanobacteria rapidly faded and the culture turned cloudy white due to the formation of fine grains of $CaCO_3$. A strong reduction of dissolved calcium concentration occurred simultaneously with the precipitation of calcium carbonate and was well correlated to the quantity of calcium present in the precipitated $CaCO_3$ (Figure 2). Scanning Electron Microscopy (SEM) imaging of the cells and precipitates show that the cyanobacterial cells were embedded in calcium carbonate aggregates of rhomboidal and irregular forms (Figures 4a, 4b). For the lowest calcium concentration tested (0.34 mM), the aged culture turned yellow and the calcium carbonate precipitation was not visible to the eye. For this $CaCl_2$ concentration, the very low quantity of $CaCO_3$ detected is within the range of the detection limit. In our experimental conditions, as mentioned before, the kinetic of biomass production was independent of the calcite precipitation, strongly suggesting that the $CaCO_3$ precipitation by the cyanobacteria is constrained by environmental, not physiological, factors. In fact for a same initial bicarbonate concentration (7.5 mM), the higher the $CaCl_2$ concentration, the quicker the $CaCO_3$ precipitation occurred. $CaCO_3$ precipitation appeared after 2/3 days and 3/4 days for $CaCl_2$ concentrations of 6.8 and 3.4 mM respectively (data not shown)

The $CaCO_3$ precipitation during cyanobacterial growth has been systematically quantified. According to stoichiometry the maximal $CaCO_3$ masses that could be obtained are constrained by the limiting specie present in the medium: calcium or carbonate. The carbonate production was estimated using a bicarbonate conversion ratio of 0.5 during photosynthetic bicarbonate assimilation. In all combinations of bicarbonate with $CaCl_2$, the quantities of $CaCO_3$ precipitated were systematically lower than the maximal possible values from the

stoichiometric point of view. In addition, soluble calcium and carbonate were systematically present at the end of the growth experiments.

For example, it has been shown that an initial bicarbonate concentration in the culture medium of 7.5 mM would generate at the end of the *Synechococcus sp.* PCC 8806 growth about 3.75 mM of carbonate. With initial calcium concentrations of 3.4 and 6.8 mM the culture conditions represent respectively a case where the calcium and carbonate concentrations are stoichiometrically in the same order of magnitude, and one case with an excess of calcium. Data shown in Figure 5 show that the $CaCO_3$ precipitated averaged ca. 331 and 572 µmol respectively, lower than the maximal possible values expected to be 620 and 697 µmol respectively.

Abiotic $CaCO_3$ precipitation controls were performed in approximately the same range of Ca^{2+} and CO_3^{2-} concentrations as used for the growth experiments. They were done in mineral growth medium and also in distilled water. For the experiments performed in distilled water, the amount of $CaCO_3$ precipitated was close to the expected values. For the abiotic controls performed with mineral growth medium, the amounts of precipitate were systematically lower than the theoretically expected values. Theses differences observed in the abiotic controls (in water or in mineral medium) can be attributed to ion activities. The shape of the crystals in the abiotic control experiments differs from that of those observed in the biotic assays, and are on the order of a few microns in the typical calcite rhombohedral form (Figure 4c).

The quantities of calcium carbonate precipitated in the abiotic and biotic experiments are presented in the Figure 5. For calcium concentrations of 6.1/6.6 mM, two abiotic and one biological experiments represented situations with calcium ions present in stoichiometric excess. The $CaCO_3$ quantities precipitated represented in all essays about 80 % of the maximal $CaCO_3$ quantity that can precipitate whatever the carbonate concentration (from 2.5 to 5.0 mM). The differences obtained could be attributed to the ion activity of the growth medium. For calcium concentrations of ≈ 3.2 mM, two abiotic and one biotic experiment represented situations with carbonate ions present in stoichiometric excess. In these conditions the $CaCO_3$ quantities precipitated increased progressively from 53 to 78% (of the maximal $CaCO_3$ quantities that can precipitate) with rising carbonate concentrations (from 2.5 to 7.5 mM). Theses differences, mainly for the two lowest carbonate concentrations could not be attributed only to the ion activity, but reflect also that the $CaCO_3$ precipitation is also governed by both calcium and carbonate concentrations. This reinforces that, in biological

experiments conducted in batch conditions, the calcium carbonate precipitated is mainly governed by calcium and carbonate concentrations, as in abiotic controls.

Conclusions

This study reports the use of the Rock Eval 6 analysis for identification and quantification of organic and inorganic carbon in cyanobacterial cultures of the strain *Synechococcus sp.* PCC 8806 using bicarbonate as the sole carbon source. The experiments have been undertaken with or without calcium. This technique has made it possible to obtain unprecedented mass balance analyses for the two major elements, e.g. carbon and calcium, involved in cyanobacterial photosynthesis and calcification. The recovery of each element ranged from ca. 90 to 100%. Accurate mass balances obtained experimentally confirm the proposed 1:1 partitioning ratio between $C_{biomass}$ and $C_{carbonate+CaCO3}$ produced during photosynthesis and precipitation on bicarbonate. Nevertheless, it is crucial to precisely describe the culture conditions in which this ratio is obtained, since chemical equilibriums of carbonate are greatly affected by these culture conditions. The biomass produced was independent of the calcium concentration in the growth medium and the calcification appeared as an indirect consequence of the cyanobacterial photosynthetic activity. In closed growth conditions, it could be shown that the quantities of $CaCO_3$ precipitated are systematically lower than maximal possible quantities according to $CaCO_3$ stoichiometry, regarding the most limiting species. Moreover it has been shown that the $CaCO_3$ precipitation occurred quicker with increasing initial calcium concentration suggesting strongly that the precipitation of $CaCO_3$ during cyanobacterial growth is constrained by chemical factors, mainly the respective concentrations of Ca^{2+} and CO_3^{2-}.

Ecological Implications

Favourable growth conditions can lead to cyanobacterial blooms which are often associated with whiting events which corresponds to an associated calcium carbonate precipitation. Fayetteville Green Lake (a hard water Lake) and the Great Bahamas Bank (a marine environment) are two whiting settings that have been extensively studied (Thomson et al., 1997). Moreover, in cyanobacterial mats, two factors have been found to drive biological calcification (Merz, 1992): bicarbonate uptake and suitability of the bacterial sheath as a nucleation site for calcium carbonate. The present study demonstrate that the assimilation of two bicarbonate ions during photosynthesis yields the production of one carbonate ion in the environment, as proposed previously (Paasche, 1964; Merz, 1992), reinforcing carbonate

oversaturation in the immediate vicinity of the outer membranes of the cyanobacterial cells. Biofilms dominated by cyanobacteria, as in mats, offer micro-environmental niches in which interstitial water composition may be dramatically altered during photosynthesis. This could result in water alkalization that is primarily driven by bicarbonate assimilation. The results of this paper obtained in closed cultures fully support this hypothesis.

Fayetteville Green Lake (FGL) (Thomson et al., 1997) may be considered as an intermediate situation between close and large open environmental systems. In this hard lake, higher DIC values than in marine waters are observed and vary during the spring and summer seasons. Moreover the proofs for precipitation of calcium carbonate appeared very convincing.

Oppositely, in marine whiting, the calcium carbonate oversaturation in the bulk solution is repeatedly questioned. In these large open systems, it is questionable if the pH values are modified because their values as well as the bicarbonate and carbonate concentrations are considered to be constant. In the case of the Great Bahama Bank, no pH differences were noted for the water, inside or in vicinity of the whitings (Morse et al., 2003), suggesting that whiting induced by cyanobacteria are probably much more complex and still not completely understood (Thompson, 2000). Perhaps different mechanisms rule the precipitation of calcium carbonate in vitro and in vivo. Nevertheless the precipitation events in natural whiting despite non-alkalization of the environment were probably regulated both by the calcite saturation state Ω and nucleation sites. Obst et al. (2009) have demonstrated the evidence of a preeminent role of bacterial surface in $CaCO_3$ nucleation. In natural environments, such nucleation surfaces may also originate in the form of small calcite crystals or organic particles (as EPS, …) during the upwelling events, which could prompt the cyanobacterial blooms (Morse et al., 2003; Morse and Mackenzie, 1990).

It would be interesting to re-examine the precipitation onset and extent with a calcium concentration close to that in seawater.

The basic data presented in this paper will find future application in three fields: (1) a better understanding of the carbon cycle and in particular for quantifying the role of cyanobacteria as carbon sink in relation to global warming; (2) a better understanding of the carbonated sedimentary records in marine and lacustrine environments; (3) the definition of industrial cyanobacterial cultures for concomitant biomass production and calcium carbonate precipitation as an industrial solution for renewable energy supply and long term mineral carbon storage.

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Table Captions

Table 1: Determination of Biomass and $CaCO_3$ added as mixtures by Rock Eval 6 analysis

Table 2: Carbon and Ca balances and distributions after complete bicarbonate assimilation. Experiments with various bicarbonate and calcium concentrations.

Figure Captions

Figure 1: Rock-Eval 6 analysis of Biomass, $CaCO_3$ and mixture of biomass plus $CaCO_3$: Pyrolysis and oxidation responses.

Figure 2: Cyanobacterial growth on bicarbonate (1500 µmol) with $CaCl_2$ (1360 µmol). (a) pH, bicarbonate, carbonate, calcium, hydroxyl; (b) $C_{Biomass}$, C_{CaCO3}, DOC.

Figure 3: Bicarbonate assimilation (a), biomass production (b) and carbonate + $CaCO_3$ production (c) during growth on bicarbonate. Experiments with 7.5 mM bicarbonate and variable calcium concentrations (0.34, 3.4 and 6.8 mM Ca. (d) ratio: $C_{Biomass}/(C_{Biomass}+C_{CO3}+C_{CaCO3})$; C balance: $(C_{Biomass}+C_{CO3}+C_{CaCO3})/C_{HCO3-}$.

Figure 4: SEM images of filtered insoluble matter; (a) and (b) $CaCO_3$ precipitates and cells in cyanobacterial growth cultures; (c) abiotic $CaCO_3$ precipitates.

Figure 5: Production of $CaCO_3$ with abiotic controls and biological assays. (Squares represented abiotic controls and circles biological assays).

Table 1: Determination of Biomass and $CaCO_3$ added as mixtures by Rock Eval 6 analysis

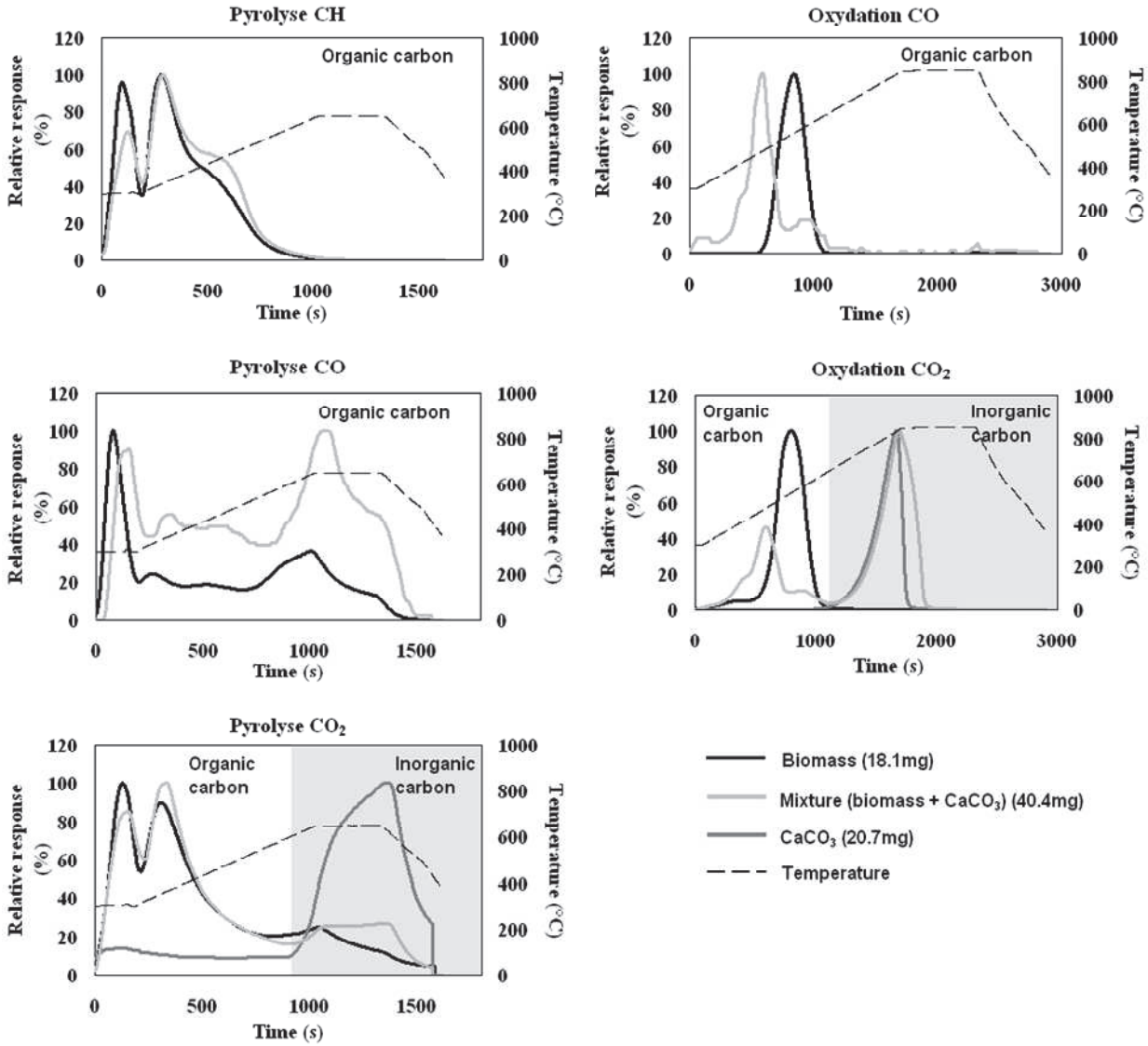
	$CaCO_3$ introduced	Biomass introduced	Mineral Carbon (RE data)	Organic Carbon (RE data)	$CaCO_3$ calculated*	Biomass calculated*
	mg	mg	µmol	µmol	mg	mg
1	7.0	7.0	72.1	238.6	7.2	6.8
2	30.2	10.2	329.5	347.4	30.8	10.0
3	59.7	15.2	651.0	515.4	60.2	15.0

* Calculated from RE data

Table 2**:** Carbon and calcium mass balances and distribution after complete bicarbonate assimilation. Experiments with various initial bicarbonate and calcium concentrations.

Conditions	Initial		Final				Balance			
	Bicarbonate + carbonate	Calcium	C Biomass (*)	$CaCO_3$	Carbonate	Calcium	Carbon balance	Calcium balance	RE Mass recovery	$CaCO_3$ / theoretical $CaCO_3$
	µmol	µmol	µmol	µmol	µmol	µmol	%	%	%	%
Calcium 0.34mM/HCO3- 7.5mM										
Mean value	1505	67	1032	12	580	49	107	91	102	17
SD	0	0	46	1	95	3	6	3	3	2
SD (% M value)	0	0	4	13	16	7	6	3	3	13
Calcium 0.34mM/HCO3- 15mM										
Mean value	2975	66	1567	14	1470	57	102	107	107	22
SD	0	0	55	10	17	2	1	17	3	16
SD (% M value)	0	0	3	72	1	4	1	15	3	72
Calcium 3.4mM/HCO3- 7.5mM										
Mean value	1495	590	993	339	93	234	96	97	105	58
SD	0	0	123	7	3	15	2	3	1	1
SD (% M value)	0	0	12	2	3	6	2	3	1	2
Calcium 6.8mM/HCO3- 5mM										
Mean value	1060	1243	657	296	75	922	97	98	104	54
SD	0	0	17	38	5	15	4	3	2	7
SD (% M value)	0	0	3	13	7	2	4	3	2	13
Calcium 6.8mM/HCO3- 7.5mM										
Mean value	1620	1231	862	550	103	657	94	98	105	66
SD	0	0	23	35	16	31	2	1	4	4
SD (% M value)	0	0	3	6	16	5	2	1	4	6
Calcium 6.8mM/HCO3- 7.5mM										
Mean value	1460	1243	867	527	62	650	100	95	109	69
SD	0	0	27	14	14	17	0.4	0.4	1	2
SD (% M value)	0	0	3	3	23	3	0.5	0.4	1	3

*C Biomass = CBiomass (RE) + DOC(final - initial)



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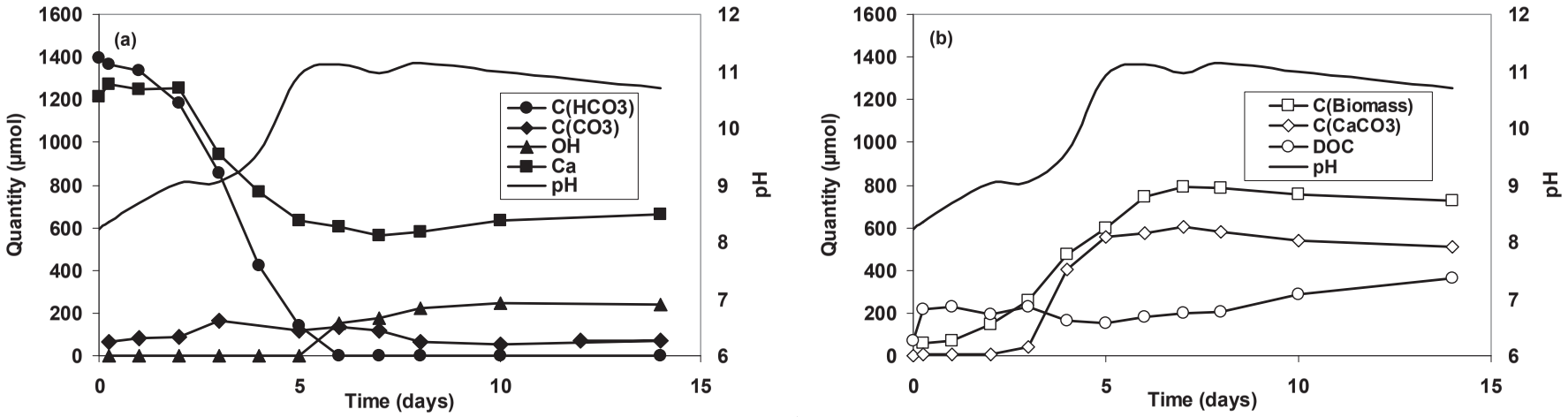

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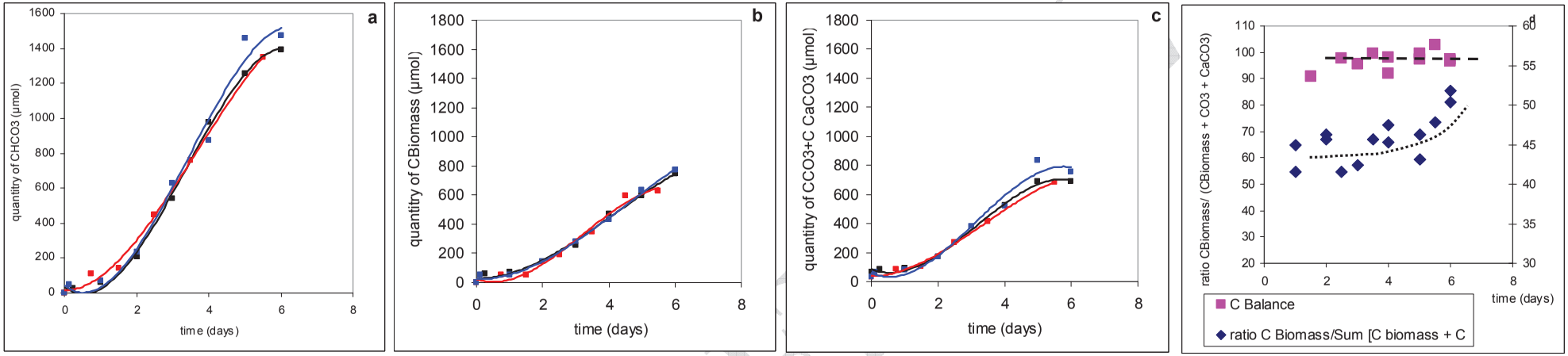

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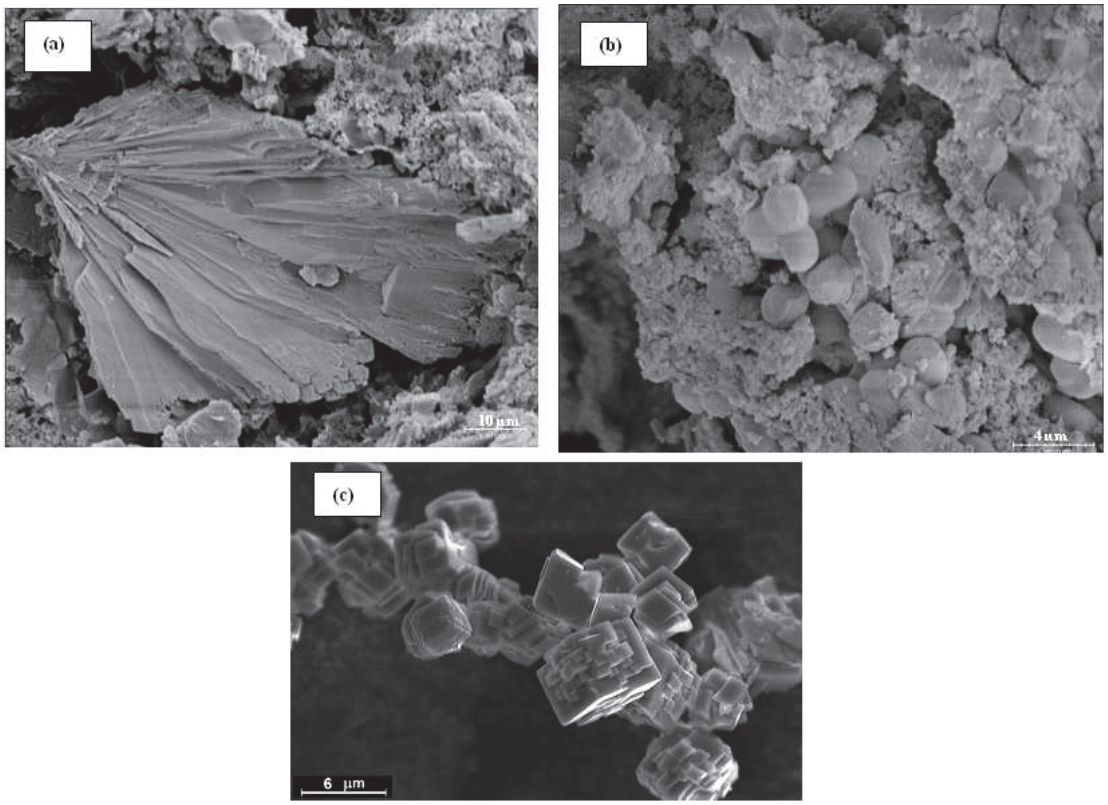

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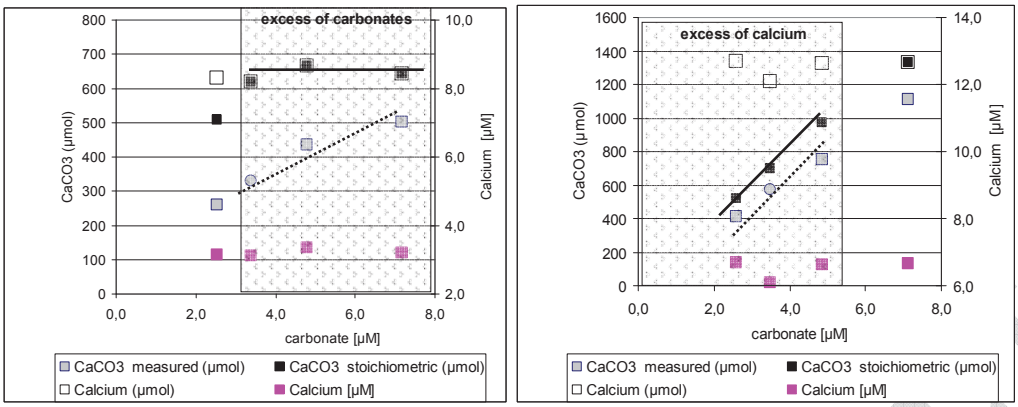

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