Cyanobacteria culture in bicarbonate system to optimize biomineralization

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Abstract

The well known calcifying potential of the cyanobacteria is questioned for capture and storage of CO_2 in the form of $CaCO_3$. However our understanding of the metabolism concerning favourable conditions for calcite precipitation, when growing under flue gas conditions, still may progress. Cyanobacteria are known to use bicarbonate and CO_2 as mineral carbon sources and a number of CO_2 and HCO_3^- transporters contribute to the accumulation of HCO_3^- into the cytosol. Nevertheless only CO_2 can serve as substrate for the Rubisco. CO_2 is obligatorily converted into HCO_3^- in the cyanobacterial cytosol using NADPH dehydrogenase complexes producing protons, which have consequence on the cell microenvironment, unfavourable to alkalinisation of the extracellular environment, oppositely to HCO_3^-. Conversely, cyanobacterial growth on bicarbonate instead of CO_2 favoured $CaCO_3$ precipitation through alkalinisation of the extracellular environment. CO_2 assimilation by cyanobacteria through a carbonate system in which injected CO_2 is converted to HCO_3^- could be considered as an option of CO_2 capture solution with $CaCO_3$ precipitation. In this study, non limiting CO_2 supply condition was performed by CO_2 injection through a pH regulation system into the carbonate system. The expected bicarbonate and carbonate concentrations increase during the time course, due to nitrate consumption, was used to favour the condition

of $CaCO_3$ precipitation. Performances were precise with regard to photo-limitation phenomenon.

Introduction

Biomineralization could be a potential for carbon capture and storage (CCS) to lower atmospheric concentrations of carbon dioxide (CO_2), particularly it could be applied to point-sources of flue gas emissions with elevated CO_2 levels, e.g. cement manufactures, refineries, coal fire power plants,... Biomineralization through calcium carbonate ($CaCO_3$) precipitation is a natural phenomenon in marine, freshwater, and terrestrial ecosystems. This phenomenon has been described all over the past geological history (Altermann et al., 2006; Riding, 2006) and present days.

The calcification phenomenon has been extensively studied for calcifying microorganisms, and seems to be dependant on seawater composition in marine environment (Stanley, 2006; Stanley et al., 2005) as well as on the morphology and physiology of these calcifying microorganisms (Lowenstam and Weiner, 1989). In modern cyanobacteria calcification, Merz (1992) and Merz-Preiß and Riding (1999) suggested two calcification styles, i.e. impregnation and encrustation, responding to environmental factors, and also restricting calcification to certain taxa, cyanbacteria could be distinguished as "calcifying and no calcifying strains". According to Lee et al. (2006), species of cyanobacteria *Synechococcus* are known to be responsible for $CaCO_3$ precipitation in the presence of calcium via the carbonate formation in the culture medium due to an alkaline environment development during cell growth. Different mechanisms have been proposed and classified into biologically induced and biologically controlled. Moreover, the situation is probably more complex, Obst et al., (2009) suggested a passive surface-induced mechanism for $CaCO_3$ nucleation by cyanobacteria, the cells membrane contributed to reduce the specific interfacial free energy of the calcite nuclei.

The main accepted fact is that cyanobacterial calcification is clearly related to photosynthetic carbon uptake (Altermann et al., 2006; Riding, 2006). The inference of a relationship between CO_2 concentrating mechanisms (CCM)-stimulated photosynthesis and calcification has been rapidly suggested. In cyanobacteria, whatever the mineral carbon source entering the cell, only bicarbonate is present in the cytosol. The CO_2 entering the cell is converted to HCO_3^- by two known CO_2 uptake systems located in the thylakoid membrane (Price et al, 2008; Price et al., 1998). Moreover the carbonic anhydrase (CA) that operates the cleavage of the cytoplasmic bicarbonate into CO_2 is confined next to the Rubisco in the

carboxysome and has not be found in the cytosol (Kaplan and Reinhold, 1999). The proposed mechanism of inorganic carbon (Ci) photosynthetic assimilation gave prominence to the production of hydroxyl ions reacting outside the cell with bicarbonate in bicarbonate-rich environments to form carbonate ions in the vicinity of cyanobacteria cell membrane, inducing $CaCO_3$ precipitation in the presence of calcium (Miller and Colman, 1980; Thompson and Ferris, 1990; Merz, 1992; McConnaughey, 1994; Price et al., 1998; Kaplan and Reinhold, 1999; Badger and Price, 2003). In many natural aquatic environments, Ci and/or micro-nutrients availability and their supply rates are often severely limited. In these natural environments with CO_2 limitation (<15 µM CO_2), cyanobacteria can activate both CO_2 and HCO_3^- inducible transport systems in order to stimulate carboxysome Rubisco (Price et al, 2008). Despite of low Ci transfer into the cells, precipitations events could occur in freshwater areas, for example in the Everglades, Florida (Merz, 1992), in oligotrophic hard water as Lake Michigan (Dittrich and Obst, 2004), or Fayetteville Green Lake (FGL) (Brunskill et al., 1969; Thompson et al., 1997). Lacustrine calcite precipitation can result in sedimentation rates of up to 1 mm per year, nevertheless achieving large carbonate deposits over geological time (Teranes et al., 1999).

Considering biomineralisation as an alternative for geological CO_2 sequestration means enhancing both biomass productivity and precipitation efficiency. For this objective, a high Ci transfer rate into the cells is necessary. In this condition, the nature of the inorganic carbon source is probably critical for precipitation. Under high CO_2 supply, the H^+ generated during CO_2 uptake (conversion of CO_2 to HCO_3^- using 1 OH^- per CO_2 hydrated) could be neutralized by the OH^- produced by the conversion of HCO_3^- to CO_2 in the carboxysome (Price et al., 2008; Jansson and Northen, 2010). Under these conditions, the CO_2 fixation tends probably to be globally pH-neutral. Consequently, the high CO_2 transfer will tend to lower the pH at the cell-surface microenvironment generating unfavourable conditions to calcification. Moreover microalgae growth (Chlorella for example) inhibition has been reported with 10% and 15% CO_2 concentrations in the flue gas stream (Chiu et al., 2008). In these conditions with 2 to 15 % CO_2 (v/v) in gaz flux, the average pH was 7.6 to 6.8. More surprisingly, weak Ci fixation into biomass (2% of the total CO_2 sequestered) by cyanobacteria *Aphanothece microscopica Nägeli* using 15% CO_2 air has been reported (Jacob-Lopes et al., 2009), suggesting the existence of other routes of CO_2 conversion.

When HCO_3^- is the predominant carbon source, the conversion of HCO_3^- to CO_2 in the carboxysome will tend to generate an alkaline microenvironment at the vicinity of the cell membrane. In batch condition with calcium excess (greater than half of initial bicarbonate

concentration), approximately 50% of the carbon substrate is converted into $CaCO_3$ during cyanobacterial growth on bicarbonate. Moreover, the cyanobacterial carbon assimilations were quite similar, regardless the initial calcium concentrations in the batch cultures (Li et al., submitted).

Developments are needed to evaluate more precisely the potentiality of cyanobacteria to capture CO_2 (via photosynthesis and $CaCO_3$ precipitation) and to optimize the process with CO_2 rich flue gas as main carbon source. In this study, we cultivated *Synechococcus sp.* PCC 8806 in an open-system with atmospheric CO_2 as carbon source (limiting CO_2 supply condition as in major natural environments). Non-limiting CO_2 supply condition was also operated through a pH regulation, using the carbonate system (bicarbonate/carbonate) to capture CO_2 (0.003 v/v in air) injected. In these two approaches, very low calcium concentration was used to examine the photosynthetic response to the culture conditions with no $CaCO_3$ precipitation interference.

Material and methods

Cyanobacterial cultures

The marine strain of cyanobacteria *Synechococcus* sp. PCC 8806 was used for the present study and was maintained on ASN-III growth medium (Rippka et al., 1979) with atmospheric CO_2 as carbon source. The culture was transferred to fresh medium with low $CaCl_2$ concentration (0.38 mM) every 4 weeks. All cultures were grown at 25°C in an Incubator/Shaker (Snijders, ECD01E) under an intermittent light regime (12/12h light/dark cycle) with a photon irradiance of 1.79 Klux (35.8µmol $m^{-2}s^{-1}$).

- ***Cyanobacterial growth under strong CO_2 limiting supply***

The culture was operated in batch condition in clear glass serum bottles (2.5 l), filled with 1 l culture medium containing 171 mM NaCl, 17.65 mM $NaNO_3$, 0.30 mM $MgSO_4 \cdot 7H_2O$, 0.125 mM $CaCl_2$, 0.18 mM K_2HPO_4, 0.03mM $FeCl_3 \cdot 6H_2O$, 1 ml Trace metal mix A5 + Co (Trace metal mix A5 + Co (g l^{-1}): H_3BO_3 2.86, $MnCl_2 \cdot 4H_2O$ 1.81, $ZnSO_4 \cdot 7H_2O$ 0.222, $Na_2MoO_4 \cdot 2H_2O$ 0.390, $CuSO_4 \cdot 5H_2O$ 0.079, $Co(NO_3)_2 \cdot 6H_2O$ 0.049), and 20 µg vitamin B_{12}. The initial DIC (carbonate plus bicarbonate) concentration was 0.38 mM. Culture pH was adjusted initially to 8.0. According to this pH value, bicarbonate is the main carbon species (≈ 95%). The calcium concentration added into the mineral medium was very low (0.125 mM)

so preventing calcium carbonate precipitation during the Ci assimilation. CO_2 supply in the culture medium was achieved using atmosphere as inorganic carbon source (CO_2), under a gentle agitation with a Teflon-coated magnetic stirring bar (100 rpm). The liquid-column's height was 5.1 cm for 1L volume, and the liquid/gas interface's diameter was 12 cm.

The bottles were inoculated with cyanobacterial maintenance cultures to a target concentration of 1×10^7 cells ml^{-1}. Glass bottles were sealed with a plastic screwed cap equipped with holes for pH measurement, sampling and atmosphere exchange. Glass bottles were incubated in the same conditions as for maintenance cultures.

➢ ***Cyanobacterial growth with unlimited CO_2 supply***

The cultures were performed on the medium (1l) used in the CO_2 limiting supply experiments, in a glass reactor (2.5 l). However, 2.1 mM inorganic carbon was added in the mineral medium in the form of sodium bicarbonate or sodium carbonate according to the regulation pH value. Culture pH was regulated by CO_2 injection in the culture medium (acidification of the culture medium). pH below the regulation value was not corrected.

The CO_2 was injected through a porous ceramic diffuser located at the centre of the reactor section upon the stirring magnet. The gas injected was an air mixture: 0.3% CO_2; 20% O_2 and 79.7% N_2 (Air Liquide®). The entrance pressure of the air/CO_2 mixture was 250 mbar. The pH was regulated using a multi-parameter transmitter M400 (METTLER TOLEDO). Two solenoid valves were positioned at the gas entrance and gas exit. The two valves were only opened for gas injection during pH regulation events (until pH value reached the target pH). CO_2 content from the exhaust gas was estimated by bubbling in a soda lime compartment. Nitrate was added to permit growth without limitation during a few cycles. The reactors were inoculated with cyanobacterial cultures to a target concentration of 1×10^7 cells ml^{-1}. They were incubated in the same conditions as for maintenance cultures for temperature, photon irradiance and intermittent light regime.

The cultures were grown at 25°C in an Incubator/Shaker (Snijders, ECD01E) under an intermittent light regime (12/12h light/dark cycle) with a photon irradiance of 1.79 Klux (35.8µmol $m^{-2}s^{-1}$).

Analyses

➢ ***Cell density determination***

The cell density was measured at 600 nm with a photospectrometer (Shimadzu, UV 1601). The cells were also counted with a *Malassez* haemocytometer under optic magnitude

(×1000). A relation between $C_{biomass}$ (µM) (determined by Rock Eval analysis) and absorbance at 600 nm (between 0 and 0.5) has been established, with $R^2 > 0.98$. The $C_{biomass}$ content in the cell was in good accordance with the CONHS determination for cyanobacteria cells (Li et al., submitted).

- ***pH measurement***

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

- ***Anions determination***

Nitrate and nitrite were quantified by High Performance Liquid Chromatography (HPLC). The apparatus used was a DIONEX ICS 3000 equipped with an eluent generator and a self-regenerating suppressor (ASRS-ULTRA 4 mm for anion). The separation of the anions was done at 30 °C on an IonPac AS9-HC column (4 mm x 250 mm) with a gradient of KOH at 1 ml /min of flow rate. The injection volume of the sample was 25 µl (full-loop injection). Anions were detected with a conductivity detector. Software "Chromeleon" was utilised to pilot the DIONEX ICS 3000 equipment and to save the acquisitions data. Linear calibration curve (with R^2 >0.999) was obtained for nitrate (0-2.5mM) and polynomial curve for nitrite (0-2.5mM) with R^2 >0.999. Anions were analyzed in culture medium filtrate. The filtration was done with a 0.45 µm PTFE filter. Samples were stored at +4°C in closed sampling tubes until analyze.

Bicarbonate, carbonate and OH^- ions were quantified by titration analysis with an automatic titrator (Titrimo 702 SM, METHROM). 30 ml filtrate was analyzed right after, without sample dilution to avoid pH changes and equilibration with atmosphere during sample handling. Automatic titration was done with 100 µl calibrated increment volume of H_2SO_4 (corresponding to 1 µmol H^+ equivalent for 0.01N). Normality of H_2SO_4 was 0.01N or 0.001N depending on bicarbonate and carbonate concentrations. The data were recorded by software Tiamo (METHROM) and transferred into Excel to determine precisely on the pH/H^+(µmol) graph the inflexion points corresponding to transitions from hydroxyl and carbonate to bicarbonate (referred to the first inflexion point) and from bicarbonate to CO_2 (referred to the second inflexion point). Bicarbonate and carbonate are calculated from the repective quantities of H^+ added (µmol) to reach the 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 CO_3^{2-}, HCO_3^- and OH^-.

- ***CO_2 determinations***

The CO_2 concentration in the headspace of the reactors was analyzed by a gas chromatography (GC). The equipment used was a gas chromatograph Varian 3800 equipped with a Porapak Q (80/100 mesh, Chrompack) column (2 mm ID, 2 m long). Helium was used as carrier gas at a flow rate of 30 ml/min. The operational temperatures were: 130, 130, and 100°C for injector, detector, and column respectively. The detection was done with a thermal conductivity detector. 250 µl of the headspace of the bottle was injected. The CO_2 response was calibrated with CO_2 (5%v/v ± 0.096) in N_2, certified by Air Liquide®.

Results

Evolution of bicarbonate, carbonate and hydroxyl ions during cyanobacterial growth under strong limited CO_2 supply

The values of pH, $C_{biomass}$ production, bicarbonate, carbonate and hydroxyl ions concentrations measured during the cyanobacterial growth experiments under strong limited CO_2 supply are presented in the **Figure 1**. Bicarbonate, carbonate and $C_{biomass}$ are expressed in µM of carbon. As suggested by the cross-cutting curves of **Figure 1,** the early growth phase was achieved consuming the initial bicarbonate stock (about 340 µmol). Utilization of HCO_3^- in photosynthesis releases CO_3^{2-} according to **equation 1**.

$$2HCO_3^- + \gamma \rightarrow [CO_2 biomass] + H_2O + 1CO_3^{2-}$$ (equation 1: photosynthesis in HCO_3^- excess)

The pH increase (up to around 10) during the first six days reflects the permanent change between bicarbonate consumed and carbonate produced. At this stage the $C_{biomass}$ production from the initial bicarbonate quantity provided could be approximately estimated to 170 µmol. After the complete removal of the initial bicarbonate, the biomass growth proceeded only on CO_2 transferred from the gas phase into the culture medium. The $C_{biomass}$ increased linearly during the first 70 days to reach a concentration of 5600 µM, with a constant CO_2 assimilation rate estimated around 65 µmol C/day (expressed as $C_{biomass}$). The results reflect a biological growth on mineral carbon source limited by the CO_2 supply. Over this period the respective concentrations of OH^- and carbonate increased slightly around 350/500 µM respectively, the bicarbonate being always under the detection limit. The pH profile, fairly stable around 10, reflected a carbonate system in which the bicarbonate specie was close to nil.

After 70 days, an increase in the CO_2 supply was operated by reduction of the culture volume (from 1000 ml to 750 ml). The agitation in the reactor was not changed. The CO_2 assimilation rate increased from 65 to 160 µmol $C_{biomass}$/day. Changes in bicarbonate,

carbonate and hydroxyl concentration were observed simultaneously with both bicarbonate and carbonate increases and OH^- ions concentration decrease. These subsequent evolutions due to change in CO_2 supply conditions reflected the CO_2 speciation in the culture medium according to the two following equations (equations 1 and 2), and are associated with an acidification of the culture medium (pH 8.8). These evolutions suggested a short transient period (between 80 and 90 days) where the net CO_2 supply in the culture medium was greater than the photosynthetic bicarbonate assimilation potential.

$1CO_2 + 1H_2O + 2OH^- \rightarrow 1CO_3^{2-} + 2H_2O$ (equation 2: acidification in presence of OH^-)

$1CO_2 + 1H_2O + 1CO_3^{2-} \rightarrow 2HCO_3^-$ (equation 3: acidification in presence of carbonate)

The biomass concentration increased rapidly due to the assimilation of bicarbonate resulting from CO_2 supply through the carbonate system (see **equations 2 and 3**). Then after, the instantaneous biological demand became again greater than the CO_2 supply. Again, the bicarbonate concentration decreased, and the carbonate concentration increased

A detailed observation of the pH evolution during the dark and light periods is presented in the **Figure 2**. During the dark phase, the photosynthesis ceased. The CO_2 entry in the carbonate system was responsible for the decrease of pH observed over the 12 hours of night period and was estimated by the evolution of hydroxyl, bicarbonate and carbonate concentrations (see **Table 1**) according to **equations 2 and 3**. CO_2 supply before and after 70 days have been respectively estimated to 42 and 90 µmol of CO_2 over a period of 12 h.

During the light period, the rapid increase of the pH value observed during the two first hours supported the assimilation of the night-rebuilt bicarbonate pool. For three photosynthesis cycles studied (non successive), the bicarbonate uptake has been estimated during these two first hours. The inorganic carbon consumption ways used during cyanobacterial photosynthesis (**equations 1** and **4)** depended on the initial bicarbonate concentration at the start of the light cycle. For low bicarbonate concentration (below 50/100 µM), the inorganic carbon uptake proceed mainly through **equation 4**, the reaction of OH^- with HCO_3^- becoming impossible. The two following equations (**1** and **4**) reflected photosynthesis in excess of HCO_3^- (equation 1) and in limitation of HCO_3^- (equation 4) (see **Table 2**). According to these successive mineral carbon assimilation equations, the variations of hydroxyl ions, bicarbonate and carbonate contents during these two hours are in good agreement with the two following equations (**1** and **4)**:

The CO_2 supply during the first two hours of the light period were estimated at 7 and 15 µmol of CO_2, according to the CO_2 supply calculated over the night period for the two situations, respectively before and after 70 days.

- for bicarbonate consumption:

$2HCO_3^- + \gamma \rightarrow [CO_2 biomass] + H_2O + 1CO_3^{2-}$ (equation 1: photosynthesis in HCO_3^- excess)

$HCO_3^- + \gamma \rightarrow [CO_2 biomass] + OH^-$ (equation 4: photosynthesis in limitation of HCO_3^-)

- for CO_2 supply, with the two following equations:

$1CO_2 + 1H_2O + 2OH^- \rightarrow 1CO_3^{2-} + 2H_2O$ (equation 2: acidification in presence of OH^-)

$1CO_2 + 1H_2O + 1CO_3^{2-} \rightarrow 2HCO_3^-$ (equation 3: acidification in presence of carbonate)

After the total bicarbonate consumption, the pH value was rather stable during the 10 following hours of the light period. A global suggested mechanism of Ci assimilation during this pH stable period (with bicarbonate concentration below detection limit) could be theoretically written as CO_2 reacting with OH^- and CO_3^{2-} (equations 2 and 3) to generate HCO_3^- which is immediately consumed according to the equation 4.

Cyanobacterial growth under unlimited CO_2 supply

In excess of bicarbonate in the bulk solution, the photosynthetic assimilation of bicarbonate conducted to a decrease of bicarbonate concentration and consequently to an increase of carbonate concentration according to the **equation 1**. The resulting alkalinisation of the medium was counteracted through pH regulation via synthetic air injection into the photo-bioreactor, supplying CO_2 in the culture medium. This pH regulation generated the re-establishment of the initial ratio between bicarbonate and carbonate according to **equation 3.** The configuration of the line-gas with two solenoid valves maintained the gas phase upon the culture medium out of contact with atmosphere during all the time. The CO_2 potentially present in this gas phase after the air/CO_2 injection pulse could be trapped into the carbonate medium during the interval time between two valve stimulation events. A general scheme of gaseous CO_2 supply, reaction with the carbonate system, and bicarbonate assimilation with regulated pH is shown in the **Figure 3**. The biological mineral carbon demand continuously increased over time with biomass increase concentration in batch conditions.

Cell density, nitrate, nitrite, carbonate, bicarbonate and gas phase composition were monitored during this experiment. The pH was continuously recorded.

The specific growth rate was studied at different pH, respectively 8.2, 9.0, 9.5, and 10.2 corresponding to HCO_3^-/CO_3^{2-} ratios of respectively 85/15, 50/50, 15/85 and 0/100). Care was taken to prevent nitrogen limitation during growth rate measurements, and the mineral composition (micro-nutrients) was not limiting over the number of cell divisions considered. The specific growth rates were calculated only after the pH reached the target value. At the beginning of each light period, cyanobacterial growth proceeded without CO_2 injection until the pH reached the target value (the pH decreases during the night period due to dark respiration). The specific growth rate was determined on successive light periods using biomass data (absorbance at 600 nm). The estimated specific growth rate (0.04 h^{-1}) appeared to be maximal for 9.5 pH value. Nevertheless, for all the pH tested, the growth rates measured on successive light cycles decreased continously and may be due to photo-limitation. The absorbance values ranged on the interval 0 to 0.8. Miller et al., 1984 suggested also a similar growth rate of 0.07 h^{-1} for the cyanobacterium *Synechococcus leopoliensis* in HCO_3^- limited chemostat. This value was obtained in a carbon-sufficient chemostat culture at pH 9.5 with a [DIC] of 1700 µM in the culture reactor.

During time course, the concentrations of the two carbonate species (bicarbonate and carbonate) increased whatever the regulation pH values. This drift of the two species concentrations was due to the nitrate assimilation by cyanobacteria during growth. Brewer and Goldman's studies (1976; 1980) have demonstrated that uptake of one mole of nitrate (NO_3^-) by algae was responsible to alkalinity increase by one mole H^+ uptake or OH^- release. No change in alkalinity happens when urea is assimilated. According to this nitrate assimilation uptake, the drift of bicarbonate and carbonate concentration could be calculated. .The nitrate consumption (expressed as *x*) contributed to alter the ratio *r* which depends of the pH regulation value (= bicarbonate/carbonate), according to:

$$xNO_3^- \rightarrow xOH^-$$ (assimilation of nitrate) eq. 5

$$xHCO_3^- + xOH^- \rightarrow xCO_3^{2-}$$ (neutralisation of OH^- ion in presence of HCO_3^-) eq. 6

The CO_2 injection (expressed as *y*) by pH regulation contributed to restore the *r* value for a given pH (see equation 3), increasing the respective bicarbonate and carbonate concentrations.

$$yCO_3^{2-} + yCO_2 \rightarrow 2yHCO_3^-$$ (acidification in a carbonate system) eq. 7

For the experiment at pH 9.5, bicarbonate and carbonate concentrations have been calculated during the photosynthesis period, according to the equation:

$$y = [C1(r-1) + C2r + x]/[2-r] \text{ with,} \qquad \text{eq. 8}$$

- x = µmol nitrate consumed during time interval considered (t_2-t_1), known values
- $(HCO_3^-)_{t1} = C_1$, data t_1
- $(CO_3^{2-})_{t1} = C_2$, data t_1
- r = 0.71 = ratio $bicarbonate/(bicarbonate + carbonate)$ at pH 9.5, determined experimentally.
- y = unknown value, representing CO_2 injected (µM) to counterbalance the nitrate consumption.
- $(HCO_3^-)_{t2} = C_1 + 2y - x$ and $(CO_3^{2-})_{t2} = C_2 + x - y$.

The calculated bicarbonate and carbonate values, determined as mentioned above, were obtained for seven successive light periods taking into account the variation of nitrate concentration (x). During this period, the calculated values are in good agreement with the analyzed values (**Figure 4**). A good correlation between nitrate consumed and nitrogen incorporated in the biomass was obtained, with a cyanobacterial nitrogen content of 8.87% (w/w). This value has been determined on a cyanobacterial cells grown on bicarbonate without nitrate limitation. The nitrogen estimated in the biomass produced represented 113 % of the nitrate disappeared (**Figure 5**).

The pH decreased during the night period and the CO_2 injection was not stimulated (no regulation acting below pH target value). During this night period, nitrate was not consumed and biomass was not generated. The two solenoid valves were systematically closed during this night period and the gas phase of the photoreactor was not in contact with the atmosphere. CO_2 has never been detected in the gas phase at the end of the night period. CO_2 has also never been detected, immediately after air/CO_2 mixture injection during the light period. Consequently the CO_2 excreted into the culture medium due to dark respiration was trapped by the carbonate system resulting in bicarbonate/carbonate ratio changes (acidification) according to the **equation 3**. The CO_2 produced during the dark respiration was estimated through the carbonate and bicarbonate concentration changes over the night period. The CO_2 values calculated on three successive night periods are respectively 250, 324 and 457 µM. During theses three successive night periods the biomass concentrations measured

are respectively 68, 97 and 138 mg.l^{-1}. A net respiration was estimated to 3.4 µmol CO_2 .12 h^{-1} per mg of biomass.

The carbon balance was also determined over a period of four days. Only 342 µmol (about 5.7 %) on a total of 6,062 µmol CO_2 injected has not been trapped by the carbonate system. The biomass carbon synthesized during this period was estimated to 6,209 µmol of C using absorbance data at 600 nm and 5,624 µmol of C using gravimetric method. These data well accredit the conversion of one mole CO_2 into one mole $C_{Biomass}$ during growth on gaseous CO_2 as carbon source.

Conclusions-discussions

The carbon source availability is of central importance for aquatic photosynthesis. The mineral carbon source for photosynthesis can be either CO_2 or bicarbonate depending on environmental conditions (marine environments, hard-lake or freshwater streams...). In the case of bicarbonate, the global reaction can be formalized by the following equation: $2HCO_3^- + \gamma \rightarrow [CO_2 biomass] + H_2O + 1CO_3^{2-}$ (equation 1).

In the present study, the first experimental approach was implemented with a limited mineral carbon source supply (atmospheric CO_2 diffusion into the liquid phase). During the cyanobacterial growth, variations presented in carbonate, bicarbonate and also in hydroxyl ions concentrations resulted from pervasive unbalances between CO_2 transport (across the air-culture medium surface) and the biological demand. The evolution of both carbonate species and hydroxyl ions contents due to CO_2 supply can be explained by the two proposed bicarbonate consumption situations, in excess of bicarbonate (equation 1) or with bicarbonate close to zero (equation 4). The increase of pH values during the first hours of the light cycle (after a dark period) reflects the rapid consumption of the renewed bicarbonate pool. In the ten following photosynthesis hours, with negligible bicarbonate concentration in the growth medium, assimilation of CO_2 operated. Such evolutions could be present in natural environments, as in mats in which permanent unbalances between supply and biological demand of CO_2 (with changes in water covered/non-covered periods) may result in drastic evolution of the interstitial water compositions (Krumbein et al., 1977; Merz, 1992; Merz-Preiß, 2000). For instance, in Fayetteville Green Lake (FGL) (Brunskill et al., 1969; Thompson et al., 1997), a DIC decrease in the surface water was directly related to a spring *Synechococcus* bloom, suggesting a limited Ci supply in the lake from weak atmospheric transfer. Despite of Ci consumption (bicarbonate) by the cyanobacteria, pH did not vary

significantly around 7.9, partly because of great lake volume and developed calcification trapping carbonate ions.

In the second experimental approach presented in this study, the CO_2 supply in a carbonate system was governed by the pH regulation at a target value. In this condition, the mineral carbon addition, via CO_2 injection, was steadily adjusted to the biological biomass demand (growth without C limitation). Using this carbonate system, the cyanobacterial growth was done exclusively on bicarbonate.

During the growth, the release of hydroxyl, as a result of the bicarbonate consumption, promoted CO_3^{2-} production according to the equation 1. The CO_2 injection in the culture medium counterbalanced the disequilibrium occurring between the two carbonate species. The nitrate consumption contributed also to additional OH^- production (one mole of nitrate consumed produced one mole of hydroxyl). Consequently, the bicarbonate/carbonate ratio is also affected. This nitrate consumption was responsible over time for a drift (increase) of the respective concentrations of the two species of the carbonate system. The nitrate disappearance was in consistency with the estimated amount of nitrogen in the synthesized biomass, taken into account the C/N ratio of the cyanobacterial biomass grown on bicarbonate without nitrate limitation. No nitrite was produced with the marine strain *Synechococcus* PCC 8806 when using nitrate as nitrogen source.

The cyanobacteria culture in a carbonate system which is able to convert incoming CO_2 from flue gas should be developed as a possible solution for the final objective of a biological CO_2 sequestration process in the form of $CaCO_3$. Elevated pH values are optimal conditions for both CO_2 capture by the carbonate system and high cyanobacteria growth rates. Moreover the drift in bicarbonate and carbonate concentrations potentially favours the $CaCO_3$ precipitation condition which is mainly dependent on the respective Ca^{2+} and CO_3^{2-} concentrations. Controlling the detailed precipitation mechanism (nucleation and growth of crystal) will be an important future goal to ultimately optimize the biomineralisation.

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Table Captions

Figure Captions

Figure 1: Cyanobacterial growth upon strong limited CO_2 supply.

Figure 2: pH evolution during succession of light/dark cycles.

Figure 3: CO_2 supply and bicarbonate assimilation in carbonated system controlled by pH.

Figure 4: Evolution of bicarbonate and carbonate concentrations during time course of the cyanobacterial growth (estimated versus analysed).

Figure 5: Correlation between nitrate consumed and N incorporated in biomass. The duration of the two cultures (assays 1 and 2) corresponded to the overall nitrate consumption (respectively after 8 and 16 days).

Table 1: Variations in OH^-, CO_3^{2-} and HCO_3^- during the 12h night period.

	Days	[OH] µM	[CO3] µM	[HCO3] µM	CO2 supply µM/12H
T0 Night cycle	61.2	37	194	0	
T12 Night cycle	61.8	0	189	38	
Δ[12H night cycle]		-37	-6	38	
		-37 **	-5 **	47 **	42 *
T0 Night cycle	68.2	128	174	0	
T12 Night cycle	68.8	0	208	65	
Δ[12H night cycle]		-128	34	65	
		-128 **	38 **	52 **	90 *

* CO_2 supply estimated from acidification equations 2 and 3

** Δ[OH], [CO3], [HCO3] calculated

Table 2: Variations in OH^-, CO_3^{2-} and HCO_3^- during the first 2h of the light period.

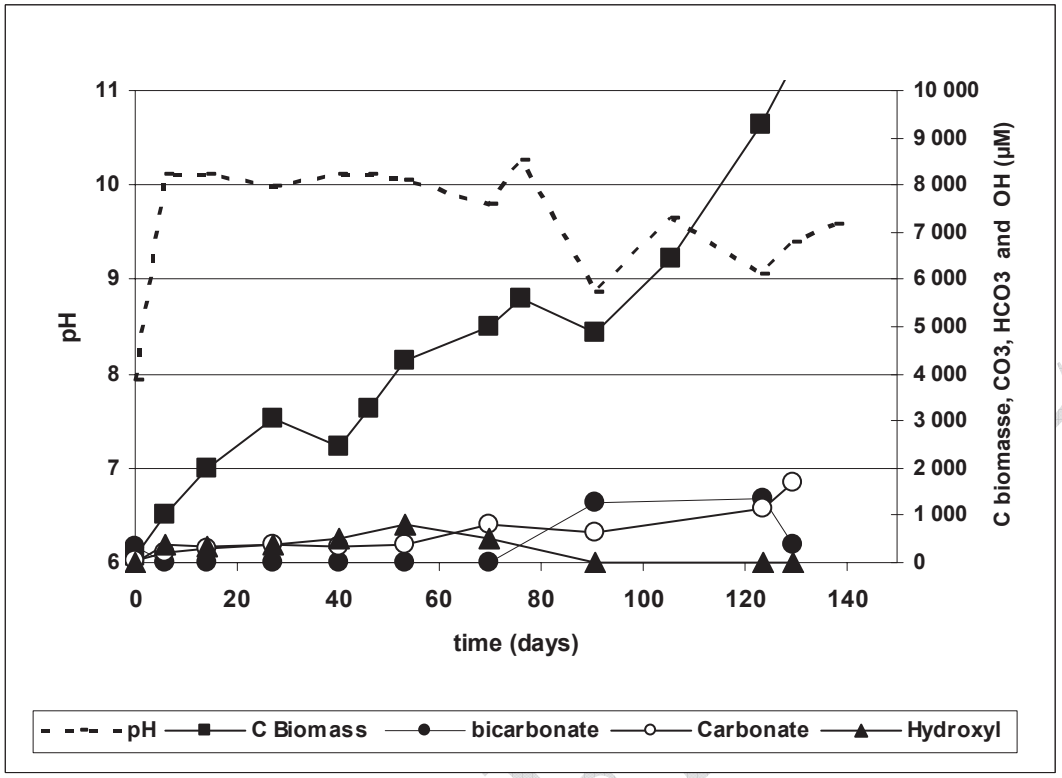
	Days	[OH] µM	[CO3] µM	[HCO3] µM	Δ[HCO3] µM/2H	CO2 supply µM/2H
T0 Photosynthesis	60.8	0	170	71		
T+2H Photosynthesis	60.9	59	175	0		
Δ[T0 - T2H photosynthesis]		59	5	-71		
		59 **	6 **	-71 **	26+59 (1)+(2)	7*
T0 Photosynthesis	61.8	0	189	38		
T+2H Photosynthesis	61.9	67	183	0		
Δ[T0 - T2H photosynthesis]		67	-5	-38		
		58 **	-10 **	-38 **	0+67(1)+(2)	10 *
T0 Photosynthesis	66.8	0	216	20		
T+2H Photosynthesis	66.9	113	186	0		
Δ[T0 - T2H photosynthesis]		113	-30	-20		
		50 **	-15 **	-20 **	0+50 (1)+(2)	15 *

* CO2 supply estimated from respiration period.

** Δ[OH], [CO3], [HCO3] calculated with CO2 supply reacted only with carbonate (equation 3)

(1) use of bicarbonate with equation 1

(2) use of bicarbonatewith equation 4



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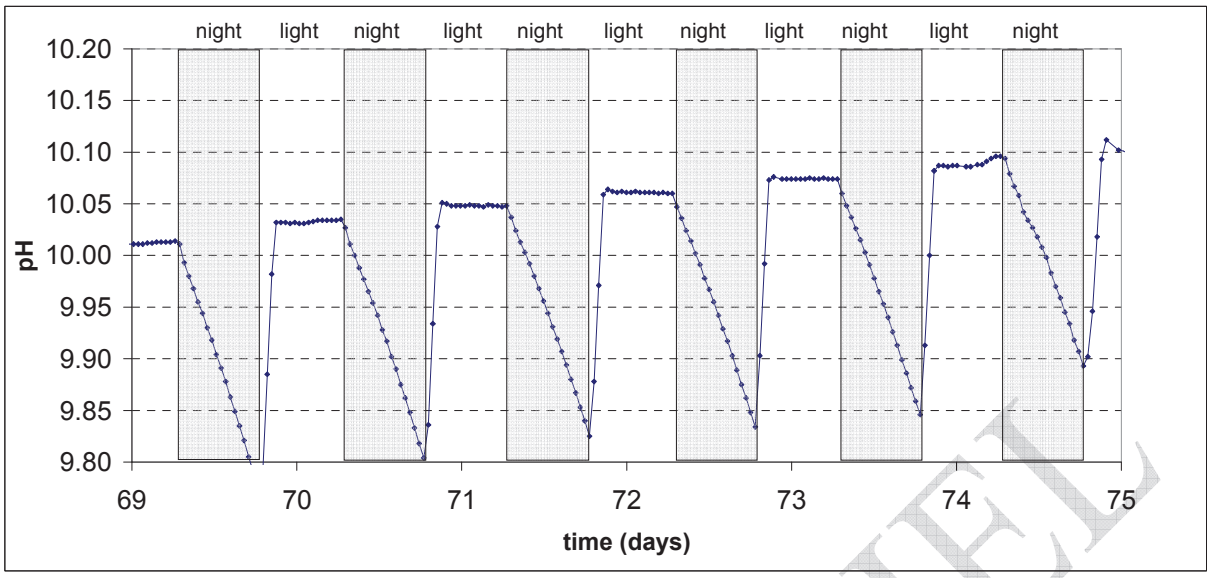


Figure 2: pH evolution during succession of light/dark cycles.

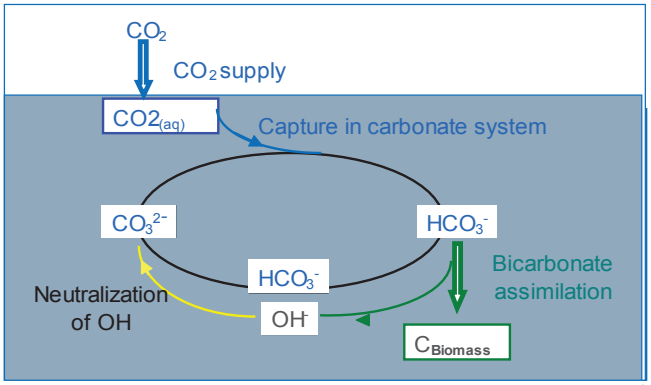
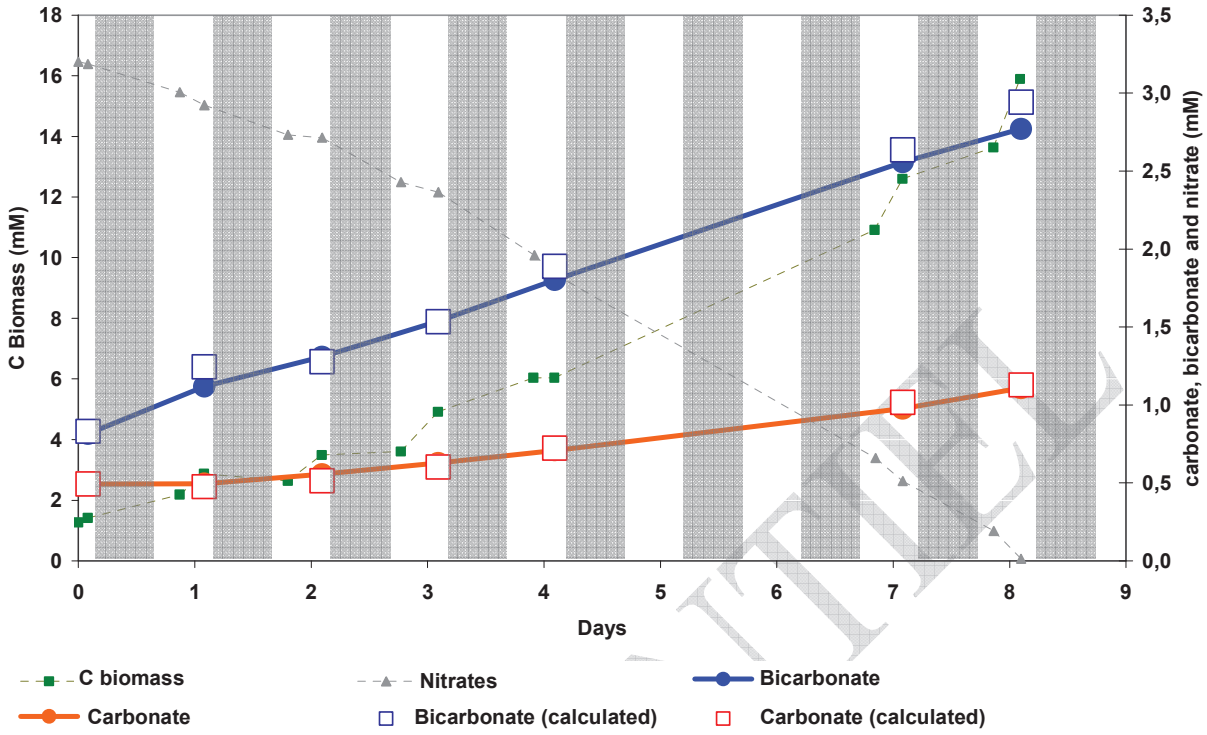


Figure 3: CO_2 supply and bicarbonate assimilation in carbonated system controlled by pH.



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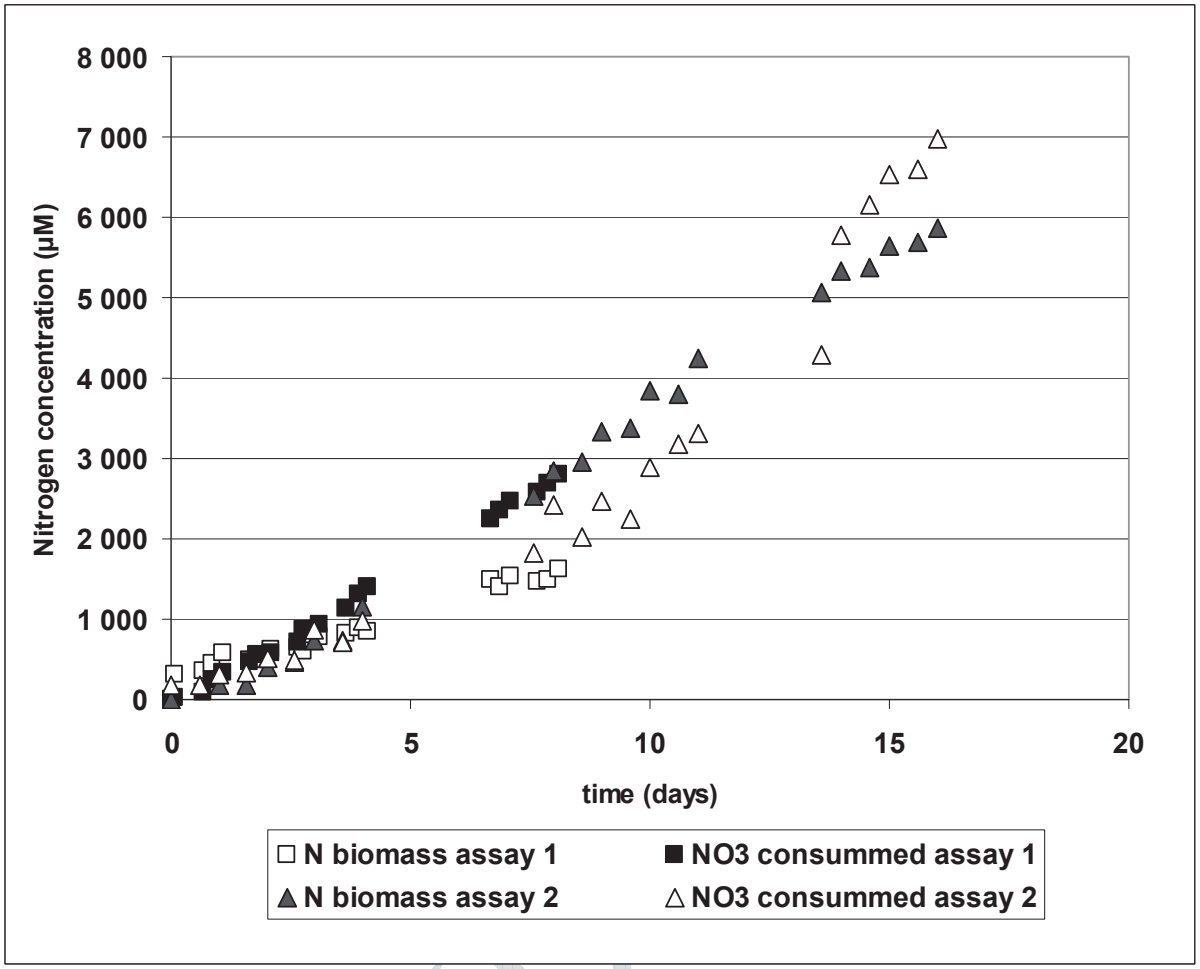

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