PROJECT FINAL REPORT

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4.1 Final publishable summary report

4.1.1 Executive summary

CO_2SOLSTOCK was funded from Theme Energy under the Future emerging technologies (FET) scheme, with the aim of developing a biomimetic approach to CO_2 sequestration using bacteria to induce calcium carbonate precipitation. Specific objectives of the project were (i) To map-out alternative sustainable solutions based on different microbiological pathways of sequestration of CO_2 through carbonate precipitation; (ii) To establish a testing toolkit enabling research teams to undertake a scientific evaluation of the feasibility of each solution and (iii) To validate this technological strategy with at least two novel approaches ready for a proof of concept test. Work focussed on four avenues designed to cover a range of potential carbon and calcium sources.

Subsurface pathways under CCS conditions using halophilic bacteria in deep saline aquifers were shown to be potentially complex and energy intensive for low results in terms of carbon sequestration. They might still prove to be of interest for maintaining the integrity of cap rocks sealing saline aquifers used to sequester supercritical CO_2 in some CCS schemes.

Using halophilic bacteria, another approach sought to combine two sources of industrial by-products: desalination brines as calcium source & domestic wastewater as carbon source. The potential for precipitation of calcium carbonate in terms of bacterial strains was demonstrated in the lab, but the correct recipe has yet to be worked out and needs further experimentation. Issues remain around the stability of the chemical composition of the organic matter in wastewater and on the affordability of this carbon source.

Dual wastewater anaerobic treatment & silicate rocks weathering was the third pathway: in the first stage, a bacterial acid attack on silicate minerals frees the necessary calcium, while in the second stage, other bacteria produce the alkalinity needed to precipitate calcium carbonate and generate high-quality biogas. The energetic cost of providing and grinding the minerals will determine the potential implementation sites with a positive carbon balance for this method, which is protected by a patent.

Finally, an ecosystem management approach was developed based on the discovery of the interaction between some trees, fungi & bacteria, leading to the precipitation of calcium carbonate in acidic soils around and below the tree roots. This would allow reforestation projects using these types of trees not only to sequester additional amounts of carbon, but also the modification of soil pH (alkalinization) makes them more suitable for agriculture. Already known in dry areas of West Africa, the phenomenon was shown during the project to exist in other distant tropical countries (Bolivia & India). *It has already been taken up by agro-forestry projects, such as the one recently launched in Haiti, combining their specific benefits (carbon sequestration, soil fertility) to the usual agro-forestry advantages (sustainable agriculture, biodiversity, soil maintenance, water balance*).

4.1.2 Summary description of project context and objectives

Climate change linked to intensive fossil fuels use is increasingly considered as the most important challenge facing mankind for this century. The response will be two-fold: mitigation, to reduce as much as possible the man-made greenhouse gas emissions, and adaptation, since important societal consequences are already unavoidable. In any case, climate change is a long inertia phenomenon (Figure A) and whichever action is undertaken to tackle it will decrease its speed.

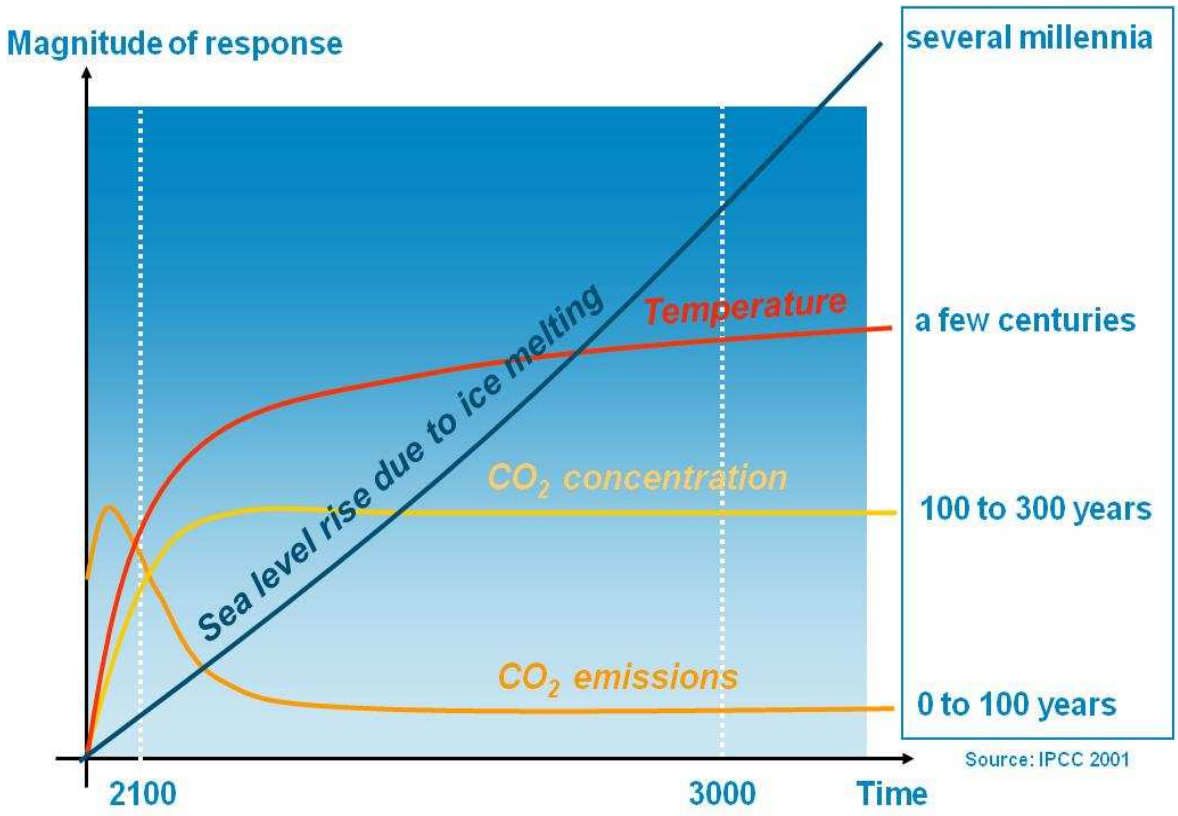

Figure A: *Time range of various consequences of CO_2 emissions in the course of the millennium, corresponding to a still hypothetical scenario of drastic reducing of CO_2 emissions after 2050.*

Currently, Carbon Capture & Storage (CCS) is the most promising technology to limit CO_2 emissions to the atmosphere. CCS aims to store CO_2 as a buoyant supercritical fluid at pressures > 80 atm under geological traps previously exploited for oil and gas, or in saline aquifers. However, CCS faces problems with regard to societal acceptability, owing to poorly understood/researched risk from leakage of the supercritical CO_2 (e.g. Haszeldine, 2009).

CO_2SOLSTOCK was funded *via* the Energy Theme under the Future Emerging Technologies (FET) scheme. The aim was to develop a technology based on new alternative sustainable solutions to reduce CO_2 emissions from anthropogenic activities, by investigating a biomimetic

approach relying on the capacity of organisms (Figure B) to induce calcium carbonate precipitation, as a complement to CCS.

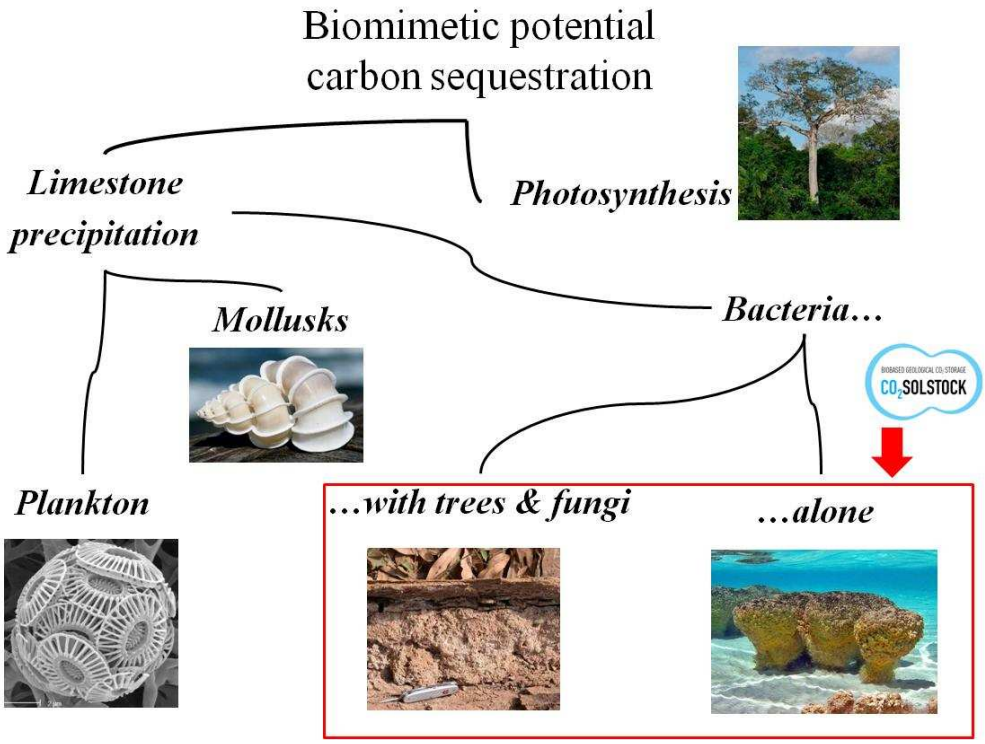

Figure B: *The main pathways used by living organisms leading to fixed carbon: photosynthesis (plants), limestone formation as shells (various planktonic organisms & molluscs) or as deposits that can become rocks.*

Bacteria were chosen, in part because of the possibility of using diverse metabolic pathways, fast turnover and potential ease of handling. Specific objectives of the project were:

1. To map-out alternative sustainable solutions based on different microbiological pathways of sequestration of CO_2 through carbonate precipitation.
2. To establish a testing toolkit enabling research teams to undertake a scientific evaluation of the feasibility of each solution.
3. To validate this technological strategy with at least two novel approaches ready for a proof of concept test.

Such a biomimetic approach is appealing on several grounds:

- A more stable storage form: sequestration of CO_2 as a solid, even if it could be dissolved in the long-run.
- No capture needed: this biomimetic approach would fix either atmospheric CO_2 or organic carbon, hence saving energy.
- In line with some of the most recent IPCC scenarios: it can sequester past emissions, thus opening the avenue for decreasing atmospheric CO_2 concentration itself.

Foreseen disadvantages were:

- Calcium & other cations should not originate from carbonate dissolution in the first place.
- Demands cheap & large volumes of calcium & other cations, as the volumes of CO_2 to sequester are considerable.

- Mass balance and flow: the difficulty will be to keep the biological systems functioning at all times, i.e. without slowing down to a dormant state.
- Up-scaling difficulties, due to the dynamism and the complexity of microbial communities.

Some of these anticipated problems were built into our experimental program in order to assess potential impacts of each investigated avenue.

4.1.3 Description of the main S & T avenues and results

4.1.3.1 METABOLIC PATHWAYS FOR MICROBIAL CO_2 SEQUESTRATION UNDER CCS CONDITIONS

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Carbon Capture & Storage (CCS) is the most promising technology to limit CO_2 emissions to the atmosphere (Figure 1). CCS aims to store CO_2 as a buoyant supercritical fluid at pressures > 80 atm (Bruant et al., 2002, Dupraz et al., 2009). A requirement for CCS realisation is therefore the presence of geological traps previously exploited for oil and gas, or in saline aquifers (Bruant et al., 2000).

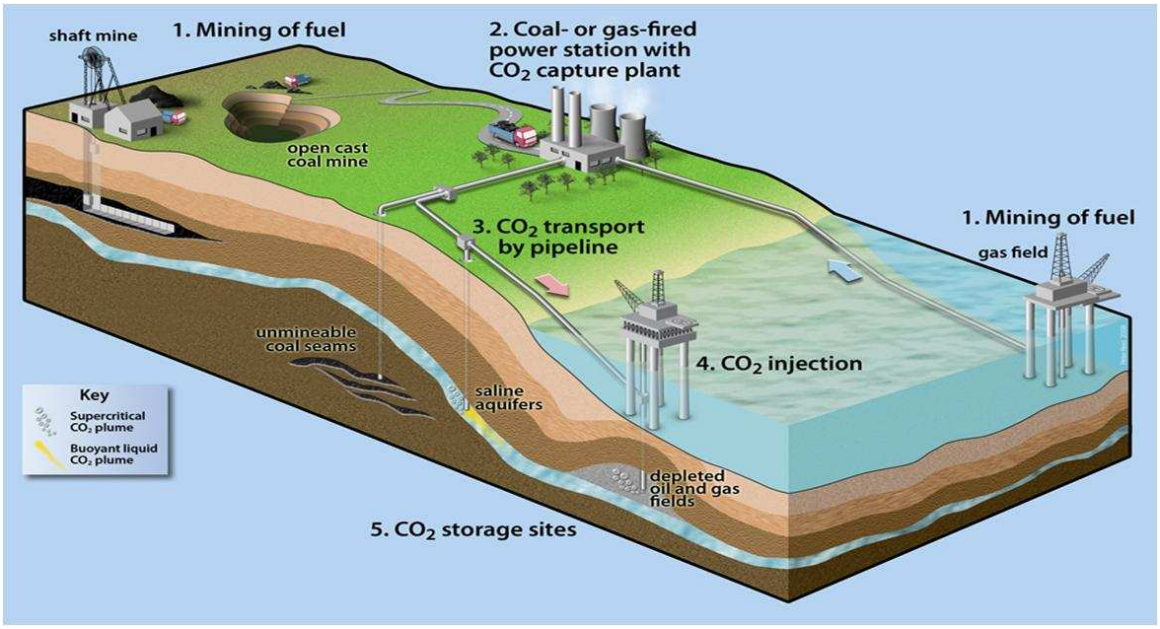
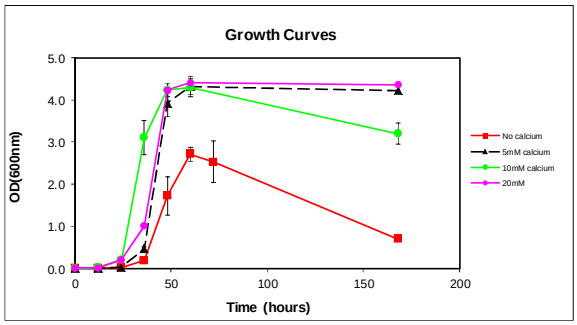


Figure 1*: Schematic view of the different options available for subsurface storage of CO_2. Note focus on saline aquifers, depleted oil & gas reservoirs and coal seams (credit: SCCS: http://www.geos.ed.ac.uk/ccs/what-is-ccs/).*

CCS faces significant hurdles with regard to societal acceptability, particularly for onshore sites, owing to poorly understood/researched risk from leakage (e.g. Haszeldine, 2009). The perceived risk is because the injected CO_2 exists mostly as a separate supercritical fluid and may escape through a variety of pathways, including through the pore system in cap rocks, through openings in the cap rock or fractures and faults, and through anthropomorphic

pathways, such as poorly completed and/or abandoned pre-existing wells (IPCC 2005). Turning some of the CO_2 into minerals is the safest form of storage since CO_2 cannot escape to the surface. However, mineral trapping takes thousands to billions of years (IPCC 2005, Xu et al. 2003). Microbiological pathways appear to offer faster routes to carbonate precipitation (e.g. Wu et al., 2011) but there are significant challenges in using microbes in such systems. (i) Supercritical CO_2 has been shown to reduce viable cell numbers in a range of microorganisms in planktonic culture (Zhang et al., 2006). (ii) The presence of CO_2 will make the formation fluids acidic (Dupraz et al., 2009) and compromise the rate and yield of carbonate precipitation. (iii) CO_2 has low 4% v/v) solubility under CCS conditions, thus fluid-gas mass transfer is likely to be the rate determining step in carbonate precipitation. (iv) The subsurface environment is anaerobic, relying on lower energy-yielding electron acceptors such as nitrate and sulphate to support energy generation, with potentially toxic by-products, as well as being under pressure and hence bacteria must be piezophilic or at least piezo tolerant. These challenges limit the type of microbial metabolisms that can be deployed for CO_2 sequestration via carbonate precipitation. With these limitations in mind, subsurface avenues focussed on three metabolic pathways with the ability to increase pH during microbial growth, a critical requirement for carbonate precipitation (Figure 2).

(a)



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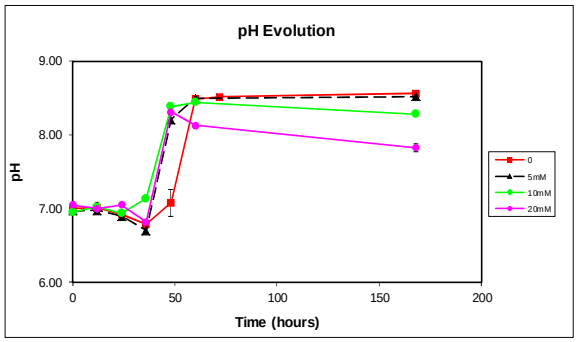

(c)

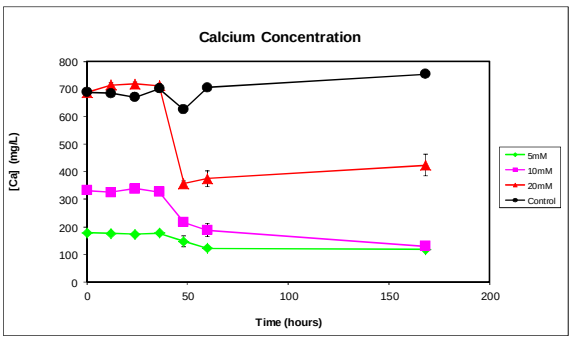

Figure 2*: Growth curves (a) changes in pH (b) and calcium removal (c) following inoculation of growth media containing different calcium concentrations with the halophilic Halomonas denitrificans strain. The pH rises by about 1.5 units during the 2-4 hour exponential growth phase, which coincides with calcium loss from solution.*

Methanogenesis: A preliminary study explored hydrogenotrophic methanogenesis, in which CO_2 is reduced by hydrogen to produce methane, a dominant reaction in the subsurface when all other more energetically favourable electron acceptors have been used.

$$4H_2 + CO_2 \Leftrightarrow CH_4 + 2H_2O$$

A secondary motivation is that the CO_2 is recycled to a useful by-product i.e. methane

Ureolysis: A range of bacteria can hydrolyse urea as a source of nitrogen.

$$CO(NH_2)_2 + H^+ + 2H_2O \Leftrightarrow 2HN_4^+ + HCO_3^-$$
$$Ca^{2+} + 2HCO_3^- \Leftrightarrow CaCO_3 + CO_2 + H_2O$$

Hydrolysis increases pH and produces HCO_3^-, promoting carbonate precipitation. 1 mole of CO_3^{2-} is produced in the urea hydrolysis reaction, but the shift in pH due to the production of NH_3 allows more CO_2 to dissociate in the fluid. Urea hydrolysis is one of the best routes to alkalinization in heterotrophic systems and has been tested successfully to clean up Ca^{2+} containing wastewater from paper recycling facilities (Hammes et al., 2003) and in groundwater aquifers (Fujita et al., 2008). Studies have shown that calcium carbonate precipitation by ureolytic bacteria can be achieved at CO_2 pressure of 1 bar, but there was only marginal alkalinisation at 5 bars, with no calcium carbonate precipitation (Dupraz et al, 2009). Significantly, some questions remain as to whether the widely promoted *Sporosarcina pasteurii* strain is active under anaerobic conditions.

Denitrification: Denitrification is the process whereby oxidized nitrogen compounds serve as electron acceptors during anaerobic respiration. Nitrate is reduced to nitrogen gas during the process

$$NO_3^- \Leftrightarrow NO_2^- \Leftrightarrow NO \Leftrightarrow N_2O \Leftrightarrow N_2$$

Denitrification uses H^+ during the process, producing an increase in pH and therefore increased precipitation of carbonates where favourable concentrations of cations exist. Denitrification has been used to cement sand columns via calcium carbonate precipitation (Van Paassen et al., 2009) and has the added advantage that the waste products are nitrogen gas and CO_2, rather than potentially toxic ammonium produced during ureolysis. The fact that it is an anaerobic process means the metabolism can be used in the deep subsurface where conditions are favourable, although the potential for denitrification and carbonate precipitation in halophilic organisms at pressure is unknown.

Sources of calcium

Bruant et al (2002) suggested that storage in deep saline aquifers holds the most promise in terms of storage capacity, proximity to emission sources and the fact that deep saline aquifers have little foreseeable economic or societal benefit because of their depth and high concentration of dissolved solids. The assumption is that there is sufficient Ca supply in the saline fluid to precipitate a significant proportion of the dissolved CO_2 under metabolic drivers. As shown by the Ketzin fluid data, the concentration of calcium can be locally variable and depends on the rock formation (Forster et al., 2006). Typical values range from those close to seawater (e.g. 426 mg/L at Sleipner to very high concentrations in the Frio Formation

(~2640mg/L). Calcium availability is thus not a major limitation in saline aquifers. The injection of CO_2 also accelerates the dissolution of calcium-containing minerals in the rock matrix. However, this source is considered relatively slow and likely to be of negligible contribution to the overall calcium budget.

Summary of experimental methods

Experiments were carried out in triplicate microcosms using serum vials (Figure 3a,) sealed with butyl rubber septa or in syringes, with Ca added as $CaCl_2$. For experiments at high pressure, the vials/syringes were filled to the brim with media and placed in confined gasket pressure vessels, pressurized (Figure 3b) and incubated. Sampling was carried out for analysis of optical density (OD600), pH, Ca concentration (ICP-OES) and mineralogy (XRD and coulometry). A scaled up (proof-of-concept) experiment was designed to demonstrate carbonate precipitation using *H. halodenitrificans* in a column filled with sand (22 L in volume) to replicate subsurface aquifers and various parameters such as pressure, pH, nitrate and $CaCO_3$ were measured (Figure 3c). The sand was inoculated with *H. halodenitrificans* and growth medium containing nutrients, nitrate and $CaCl_2$ pumped through the column.

(a) (b) (c)

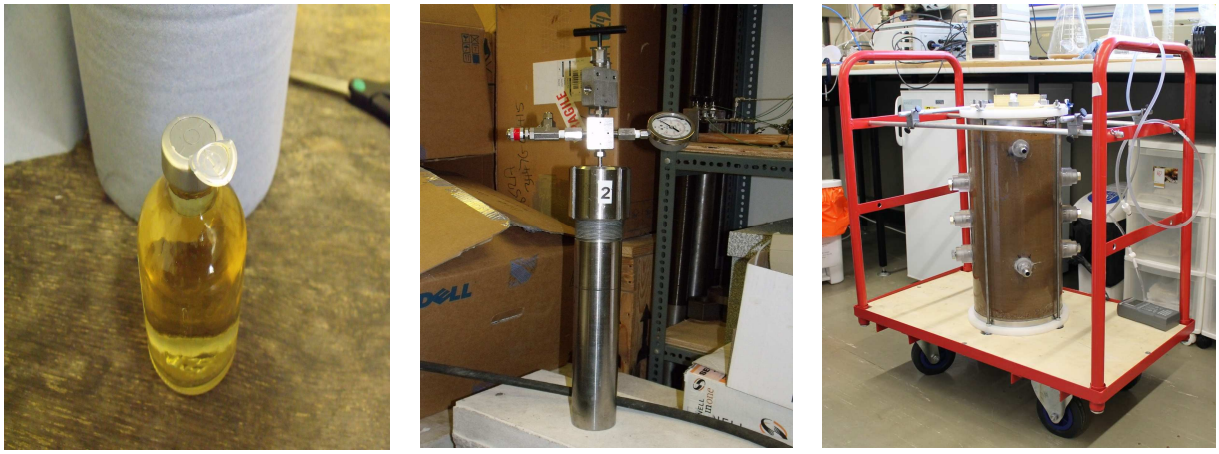

Figure 3*: (a) Media vials for ambient, (b) pressure vessels used in high pressure experiments and (c) large column filled with sand used for the proof-of-concept experiments. For the latter medium (1/4 strength phosphorus) containing 100mM calcium was pumped through the column from the bottom at a rate of ~10L day^{-1}.*

Major S&T results

Methanogenic and ureolytic pathways are unlikely candidates under CCS conditions

Experiments were performed using the methanogen *Methanobacterium espanolae* (DSM 5982), chosen for its ability to grow under acidic conditions. It has a growth optimum at a pH of 5.6-6.1, but growth has been observed at a pH of 4.7, and a temperature optimum of 35˚C, which is at the low end of the temperature range in formations used for possible CCS. Results showed significant methane production and loss of calcium from the fluid. However, examination of the solids using XRD and scanning electron microscopy revealed hydroxyapatite precipitation

instead of calcium carbonate and the pH did not rise significantly. This metabolic pathway was abandoned in subsequent investigations.

Sporosarcina pasteurii has been widely studied for its role in ureolysis driven $CaCO_3$ precipitation and its potential application in soil stabilization (Whiffin et al., 2007), radionuclide stabilization (Mitchell and Ferris, 2005) and carbon sequestration (Mitchell et al., 2010). It has been proposed that *S. pasteurii* could be used as a foreign inoculum for microbially induced carbonate precipitation (MICP) in anaerobic environments if indigenous bacteria lack the required ureolytic activity (Tobler et al., 2011). However, the growth of *S. pasteurii* under anaerobic conditions is equivocal. Our study has shown that no cell growth occurs under anaerobic conditions, although both pH and NH_4^+ increased during incubation, resulting in some $CaCO_3$ precipitation. We conclude that some ureolytic activity occurs under anoxic conditions (Tobler et al., 2011), linked to residual abiotic enzyme activity, which questions long term sustainability as the inoculum dies and the injected enzyme degrades.

Denitrification by *Halomonas denitrificans* was optimised to 65% efficiency in laboratory microcosms

Halomonas denitrificans is a halophile that can grow both aerobically and anaerobically using nitrate as an electron acceptor (Rivadeneyra et al., 2006). It offers potential for MICP under CCS conditions in saline aquifers. $CaCO_3$ yield depends on phosphate concentration in the fluid, which can be reduced to increase $CaCO_3$ yield (Figure 4a) without compromising microbial growth and metabolism. Moreover, rates of calcium removal and hence carbonate precipitation are statistically similar between ambient conditions and subsurface pressures of proposed/active CCS saline aquifers (Figure 4b).

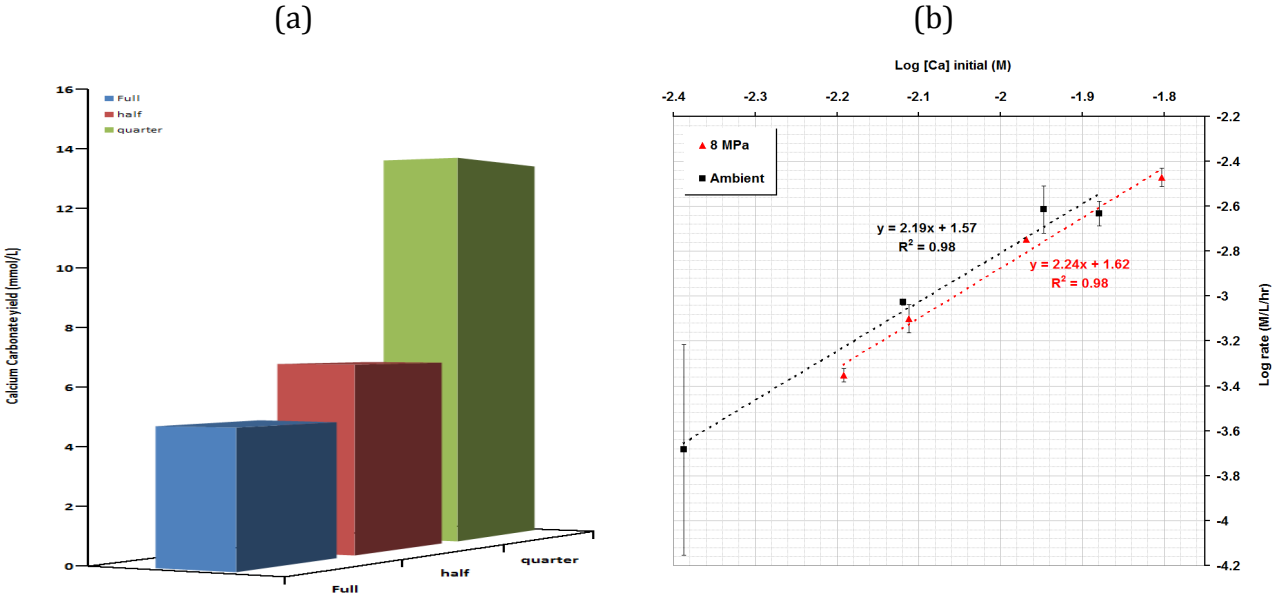

***Figure 4**: (a) Yield of $CaCO_3$, after incubation of H. halodenitrificans for 7 days. The initial concentration of calcium in the media was 20mM, hence 13.5mM yields ~65% $CaCO_3$ when phosphate concentration is reduced by diluting growth media of Rivadenyra et (2006) to 25%. (b) Calcium removal from solution during growth of H. halodenitrificans under anaerobic conditions at different pressures, showing that MICP would not be affected under most proposed saline aquifer CCS conditions.*

The fact that *H. halodenitrificans* can grow under pressure opens up its application to precipitate carbonates in saline CCS aquifers, both as a means to sequester CO_2, and in a bioengineering context, with the added advantage that there are no environmental concerns due to ammonium production. However, the relevant nutrients and nitrate must be supplied and since the metabolism is heterotrophic, adding to the CO_2 pool, this metabolism is likely to have relatively low impact on CO_2 sequestration above bioengineering aspects.

Carbonate precipitation in mesoscale flow through sand columns is spatially heterogeneous and consistent with reaction fingering.

Biocementation of the Proof-of-Concept column (Figure 5) developed as i) a uniformly cemented plug across the diameter of the column close to the nutrient injection point and ii) scalloped or fingered reaction fronts or zones in the downflow direction which were both laterally and medially discontinuous. This result is an example of reactive infiltration instability (Ortoleva et al., 1987) or reaction front fingering (Wei and Ortoleva 1990) in this case the reaction front fingering is a result of both physical flow processes and microbial denitrification promoting $CaCO_3$ precipitation. Specifically, flow fingering develops as a consequence of extensive $CaCO_3$ precipitation and permeability alteration in the cemented plug formed superjacent to the fluid inlet. Flow focusing results in preferential flow paths for nutrients within the porous media, promoting scalloped or fingered flow fronts in which pH is raised sufficiently to supersaturate and nucleate $CaCO_3$.
The development of biocementation and flow focusing proximal to injection points for nutrients may present considerable practical problems for approaches use of biocementation to restrict permeability pathways in the deep subsurface. However, microscopic examination of biocemented samples shows that the porosity is largely marinated so that the near-well bore is not necessarily compromised to further injection of nutrients. Modelling approaches to understand the progressive development of flow focussing and reaction front fingering are relatively well established (e.g. Ortoleva et al., 1987; Wei and Ortoleva 1990; Chen and Liu, 2004) and will be important tools for the development of subsurface applications.

Figure 5*: Cementation of the column (Top left) the whole of the bottom of the column was cemented in place (bottom left) side view of cemented pieces showing cemented gravel and cemented sand on top (Top right) broken pieces removed from column. (Bottom right) A continuous 30cm section of biocemented sand recovered from the body of the column.*

X-ray micro-computed tomography quantifies biocement volumes in porous media.

X-ray micro computed tomography (x-ray μCT) methods were developed to visualize carbonate precipitation in porous media (The model ureolytic organism *Sporosarcina pasteurii* was used since it has been extensively studied and is known to be able to precipitate large amounts of carbonate[1,2]. High-resolution μCT scans reveal the spatial distribution of the carbonate precipitates (Figure 6). $CaCO_3$ precipitation occurs distributed across grain surfaces, with a small proportion of precipitation contributing to cementation and strengthening of the sand pack. Significantly, $CaCO_3$ precipitation does not impact adversely upon the porosity of the sand pack.

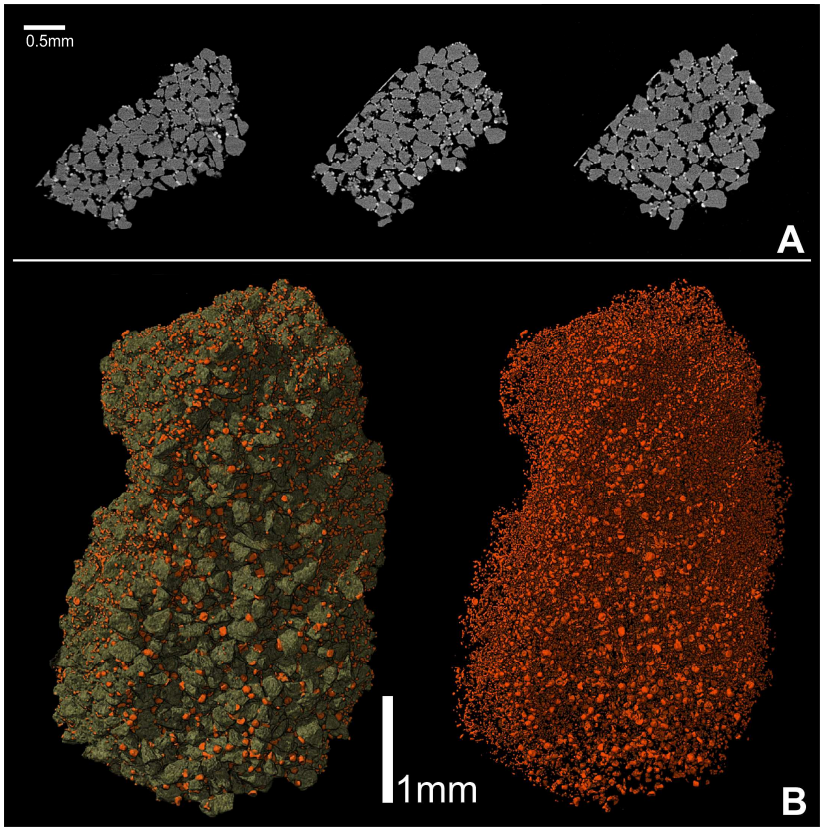

***Figure 6**: (A) Back-projected 2D μCT slices from a scan of a 2x5mm subsample of a carbonate cemented sand pack. Phases are discriminated by x-ray attenuation proportional to density. Free space is black, quartz sand is mid grey and calcium carbonate is white (B) 3D volume rendering of the sample, $CaCO_3$ grains are shown red and the quartz sand grains brown. In the right hand image the quartz grains have been rendered as invisible to demonstrate the mass of $CaCO_3$ precipitated throughout the sample. Note that this particular image was not from the mesoscale experiment and instrument access issues did not permit us to scan those samples between the end of the experiment and the report deadline.*

4.1.3.2 INTEGRATION OF MINERAL CO_2 SEQUESTRATION INTO TWO-STAGE BIOTECHNICAL PROCESSES.

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There are a number of biotechnological processes that involve a sequence of an acid and an alkalinity producing process. An example of such system are biological wastewater treatment that are characterized by a sequence of an acid producing process, and an alkalinity producing process which enables enhanced alkaline silicate dissolution in the first stage and subsequent $CaCO_3$ precipitation in the second stage. Figure 7 has described the concept of the proposed system.

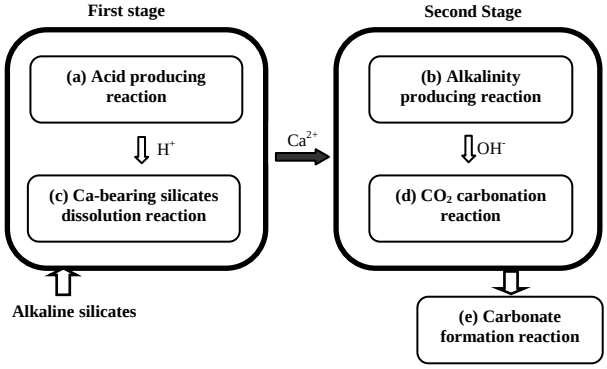

Figure 7*: Proposed scheme of mineral CO_2 sequestration integration into two-stage wastewater treatment systems.*

The processes and their metabolic pathways that are characterized by a sequence of an acid and an alkalinity producing step are:

- Anaerobic digestion; substrate fermentation to volatile fatty acids (reaction a) and subsequent methanogenesis (reaction b). Both reactions are biological.

$$(a)\ \frac{2}{3}C_6H_{12}O_6 \rightarrow 2CH_3COO^- + 2H^+$$

$$(b)\ 2CH_3COO^- + 2H_2O \rightarrow 2CH_4 + 2CO_2 + 2OH^-$$

- Biological nitrogen removal; aerobic nitrification (reaction a) and anoxic denitrification with organic carbon (reaction b).

$$(a)\ 1.2NH_4^+ + 2.3O_2 \rightarrow 1.2NO_3^- + 2.8H^+ + 2H_2O$$

$$(b)\ 1.2NO_3^- + 0.8C_2H_3O_2^- + 2H^+ \rightarrow 0.6N_2 + 1.5CO_2 + 2.1H_2O$$

- Flue gas desulphurisation; weak acid hydrogen sulphide absorption (reaction a) and subsequent aerobic oxidation to elemental sulphur (reaction b). While the reaction (a) is a chemical the reaction (b) is a biological reaction.

$$(a)\ 2H_2S + 2OH^- \rightarrow 2HS^- + 2H_2O$$

$$(b)\ 2HS^- + O_2 \rightarrow 2S^0(s) + 2OH^-$$

- Microbial fuel cells; physical separation of the acid producing anode reaction and the alkalinity producing cathode reaction.

$$(a)\ \frac{1}{2}C_2H_4O_2 + H_2O \rightarrow CO_2 + 4H^+$$

$$(b)\ 4H^+ + 2O_2 \rightarrow 2H_2O$$

Reaction (a) in all the above mentioned processes would provide the acidity required for silicate mineral dissolution (i.e. reaction c), while reaction (b) is an alkalinity-producing reaction which can neutralize carbonation of CO_2 (i.e. reaction d). Carbonate mineral precipitation can occur as result of these reactions (reaction e).

$$(c)\ CaSiO_3 + 2H^+ + H_2O \rightarrow Ca^{2+} + H_4SiO_4$$

$$(d)\ CO_2 + H_2O \leftrightarrow CO_3^{2-} + 2H^+$$

$$(e)\ Ca^{2+} + CO_3^{2-} \leftrightarrow CaCO_3\ (s)$$

Calcium or similar divalent cation supply

In order for carbonate ions to form carbonate minerals, a counter ion should be present. Evaluation on the feasibility of the method based on the divalent-cation availability from silicate minerals has shown that there are potentially adequate amounts of alkaline silicates available for capture and sequestration of the total known fossil fuel reserves (Lackner et al., 1995). Olivine, as one of the main silicate minerals of peridotite rocks containing high magnesium oxide/iron oxide, is estimated to have a global accessible reservoir of over 30Mt (McKetta (2005). The applicability of this method has been limited so far (Huijgen & Comans, 2005) due to the slow release rate of divalent cations from silicates at neutral/alkaline pH and ambient temperature (Brantley et al., 1991). Dissolution of silicates take place best in low pH conditions; however because of basic nature of most of these minerals the pH of the solution would increase over time (reaction c) which would result in decrease of the dissolution rate.

Certain microbial processes can help to increase the dissolution rate of Ca-silicate minerals. Microbes can potentially increase mineral dissolution rate by modification of the environment. As an example, the production of protons and (organic) ligands in an acid-producing biological process can increase the mineral dissolution rate by metal-proton exchange and organic ligand complexation mechanisms, respectively (Ullman et al., 1996, Wogelius & Walther, 1991, Pokrovsky et al., 2009). Nitrification and fermentation are two examples of widely used acid-producing microbial processes in wastewater treatment systems. During this project, fermentation of wastewater was tested as a route to accelerate dissolution of silicates in order to supply cations for the precipitation of carbonates in a downstream denitrification/methanogenesis reactor. The concept is the subject of a patent application.

Fermentation/Methane production system

*Chemical batch experiments***.** Batch (abiotic) experiments were conducted in order to investigate impacts of pH, silicate mineral concentration, and the organic products on the

mineral dissolution rate. Olivine and wollastonite were the two silicate minerals tested in various environmental conditions.

*Fed-batch (biotic/abiotic) Experiments***.** Fed-batch experiments were conducted to investigate the exact role of bacteria on silicate mineral dissolution. By performing biotic and abiotic fed-batch experiments the direct impact of microbes on silicate mineral dissolution could be identified. The biotic experiments were inoculated from a local anaerobic digestion reactor.

All experiments were conducted in a double jacket glass reactor with working volume of 2 litters (Applikon, The Netherlands). The reactor was temperature controlled at 30 ± 1 °C with a water jacket and a thermo bath (Figure 8). The pH was measured with a pH electrode and the solution was sparged with N_2 at 1 $l.min^{-1}$, all controlled by a biocontroller (BIOSTAT B plus, Sartorius). The pH, the N_2 flow rate, the stirring rate, and the temperature was controlled by a bio-controller (BIOSTAT B plus, Sartorius). Data acquisition of the online measurements (O_2, CO_2 and H_2 concentration, acid dosage, temperature, pH, and stirring rate) was made by MFCS/win (Sartorius Stedim Systems, U.S.A.). Elemental analysis (Ca^{+2}, Si, Mg^{+2}, Al^{+3}, and Fe^{+2}) in the solution used the inductively coupled plasma atomic emission spectroscopy (ICP-OES).

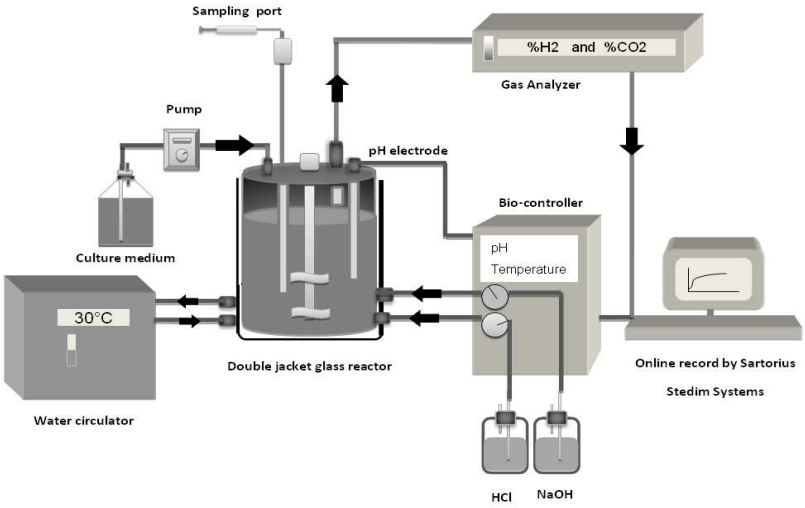

Figure 8*: Schematic setup of the reactor system.*

Nitrification/Denitrification system

Abiotic and biotic experiments were conducted to identify the exact roles of microbes on silicate dissolution.

*Chemical Batch experiments***.** To be able to quantify the biological effect on mineral dissolution, chemical experiments with wollastonite were performed. The chelates used in these chemical experiments are products of nitrification, nitrite and nitrate and the nitrogen source, ammonium. The effects of these compounds on wollastonite dissolution are observed to explain the possible biological enhancement.

Biotic Experiments. The biological experiments performed consisted of two parallel column experiments where chemolithoautotrophic nitrifying biofilm is grown on wollastonite with NH_4HCO_3 and NH_4Cl as a nitrogen source. The bed of wollastonite used is displayed in Figure 9. To prepare the bed, the column was filled with 170 gram of washed wollastonite. Both columns were inoculated with a SHARON sludge from a local reactor located in Rotterdam since this contained the organism of interest.

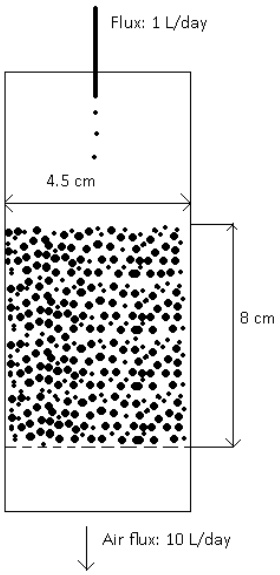

Figure 9*: Schematic representation with dimensions of the used column*

Major S & T Results

Among silicate mineral dissolution and CO_2 carbonation processes, silicate dissolution is the rate-limiting reaction. Different approaches were applied in order to increase silicate dissolution rates using acidifying microbial cultures such as fermentation and nitrification.

Fermentation process. The microbes enhanced dissolution rates by production of protons, organic ligands and complexing agents. Based on comparison between the biotic and abiotic experiments, microbes did no show detectable direct impacts on dissolution rate (Figure 10). Therefore, the Ca^{+2} release rate is lower in abiotic experiments which have comparable chemical conditions to the biotic experiments. Enhanced impacts of organic ligands produced during fermentation were determined by conducting a series of chemical experiments. It was shown that organic acids with more carboxyl groups, such as succinic acid, can enhance the dissolution rate more. Thus, the fermentative organisms enhance wollastonite dissolution mainly by production of organic acids and the resulting reduction of the pH which was an indirect impact. The results of this study show that the fermentation process would result into continuous Ca^{2+} and alkalinity release from the silicate mineral.

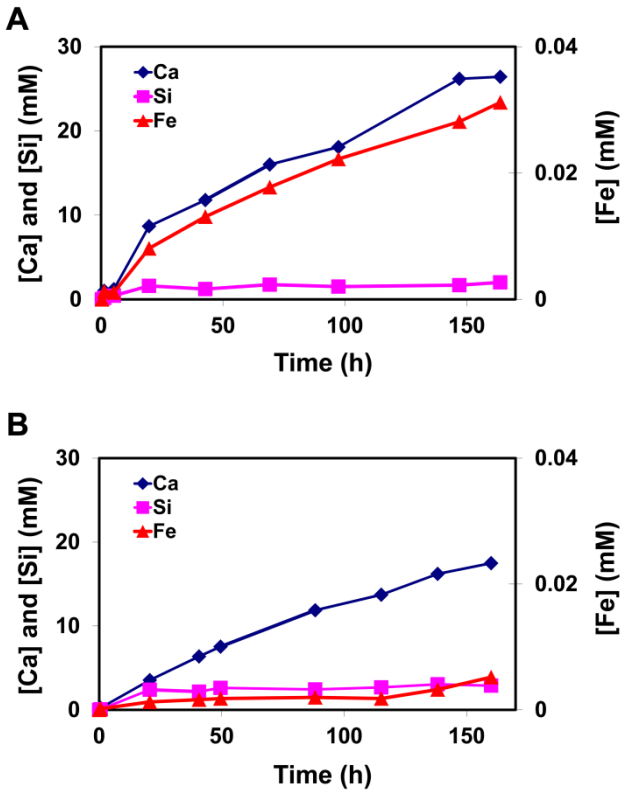

***Figure 10**: Accumulative Ca, Si and Fe ion concentrations from wollastonite dissolution. (A) During biotic fermentation. (B) During abiotic (control) fed-batch experiment.*

*Nitrification process***.** The main road block to enhance silicates dissolution rate during nitrification process is that nitrifiers' activity can be inhibited at pH values lower than 6. This can greatly decrease the dissolution rate of certain Ca/Mg-bearing silicates. To overcome this problem experiments were designed in a way to form biofilms on basic silicate surfaces. Using this approach the culture could gradually adapt to the lower pH values while benefiting from the relatively high pH values resulting from silicate mineral dissolution. Parallel column experiments where nitrifying biofilm was grown on wollastonite (a common silicate mineral) with NH_4HCO_3 and NH_4Cl as a nitrogen source were carried out. The culture showed better adaptation to lower pH with time as shown in Figure 11.

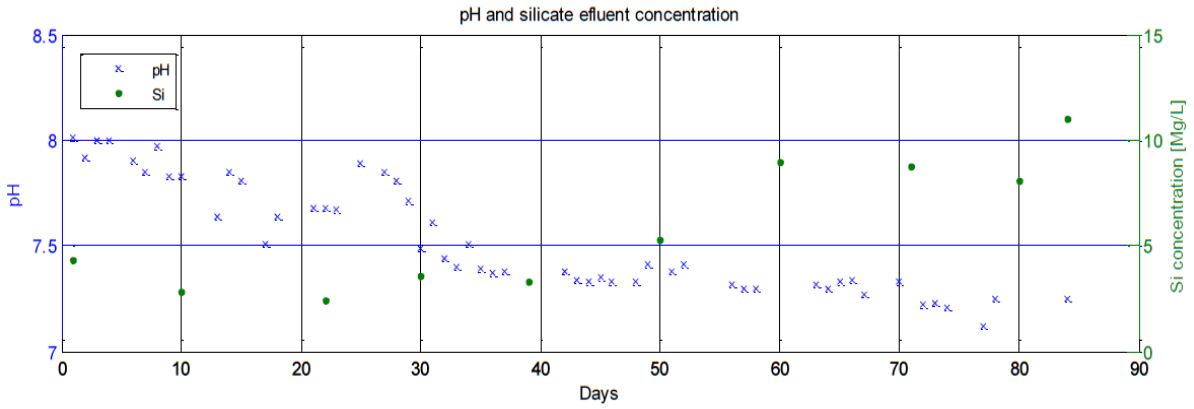

***Figure 11**: The development of the pH and silicate concentration in the effluent.*

Synthesis of major breakthroughs

The use of acid-producing microbial processes in wastewater treatment systems which consume waste materials as feed-stock at an ambient temperature can greatly reduce economic and energy costs for such a process. Continuous release of Ca^{+2} or other similar divalent cations as one of the essential materials for CO_2 sequestration can be obtained by introducing such alkaline silicate minerals into biological acid producing processes such as nitrification and anaerobic fermentation.

Remaining obstacles

The obtained rate of Ca/Mg ions as result of silicate minerals dissolution is the main road block for industrial feasibility of the method. Although microbes could continually release Ca/Mg by silicate mineral dissolution in a cost-efficient approach, the CO_2 production rate in industries is higher, and faster cation release is required in order for CO_2 to be totally sequestered. The present study showed that on average wollastonite has a 12 times higher dissolution rate than olivine, in the pH range of 3-8. The drawback is that wollastonite availability on Earth is far lower than olivine reserves. There might be microbial cultures in other biotechnical processes such as desulphurization that can tolerate lower pH values. Since the silicates dissolution rate increases exponentially at low pH values it might be helpful to test such system.

4.1.3.3 STUDY OF CARBONATE PRECIPITATION BY BACTERIA: POSSIBLE USE TO REDUCE ATMOSPHERICAL CO_2 EMISSIONS FROM WASTEWATER

Rivadeneyra, M.A.[1]; Uad, I.[1]; Silva-Castro, G.A.[1]; Vílchez, I.[1]; Rivadeneyra, A[3].; Martin-Ramos, D.[2]; Martínez-Toledo, V.[1]; C. Calvo[1]; A .Martín-Algarra; González-López, J[1].

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Bacteria can precipitate carbonates in natural and laboratory cultures. Some mechanisms of precipitation have potential to be used to reduce further atmospheric emissions of CO_2. The main objective of the current study was to analyze the feasibility of using bacterial carbonate precipitation to limit the impact of atmospheric CO_2 emissions from wastewater**.** Specific objectives were (a) to investigate the feasibility of precipitating carbonate from CO_2 produced from organic matter degradation in wastewater treatment plants and (b) selection of the best bacterial strains and culture conditions compatible with good precipitation yield at low costs (Calcium and organic matter source).

Carbonate precipitation by heterotrophic bacteria from wastewater and biological bioreactor water.

Since 1914, when Arden & Locket took out their patent for active-mud systems, this technology has been used to treat urban wastewater. This system allows the disposal of organic matter by amplifying natural biodegradation processes. Organic matter is mineralized or assimilated by different microbial groups and removed by sedimentation.

Solid media: The carbonate precipitation capacity of heterotrophic bacteria has been studied using solid natural culture media of wastewater and biological bioreactor water, as well as artificial media M1 (yeast extract, 10 g/l, peptone protease, 5g/l, glucose, 1 g/l, $Ca(C_2H_3O_2)$, 4 g/l) and M4 (yeast extract, 4 g/l; and $Ca(C_2H_3O_2)$, 2.5 g/l). Isolation, recount, selection and identification of microorganisms with high carbonate precipitation capacity were also performed. Strain identification was by means of 16s rRNA gene sequencing. Precipitated carbonates were examined by x-ray diffraction (XRD) and scanning electron microscopy (SEM and FESEM). Results showed wastewater total bacteria recounts were $23x10^5$ UFC/ml, with a 66% of carbonate precipitating colonies. Nine strains were selected for taxonomic identification. These strains precipitated $CaCO_3$ as calcite and vaterite in different proportions, which depended on the specific strain.
In the biological bioreactor water, the number of bacteria was $5x10^5$ UFC/ml, with a 90% of carbonate precipitating colonies. 17 strains were selected with capability for crystalline calcite precipitation No precipitation was detected in natural media with wastewater or mixed liquor.
Figure12 illustrates carbonate crystal forming colonies.

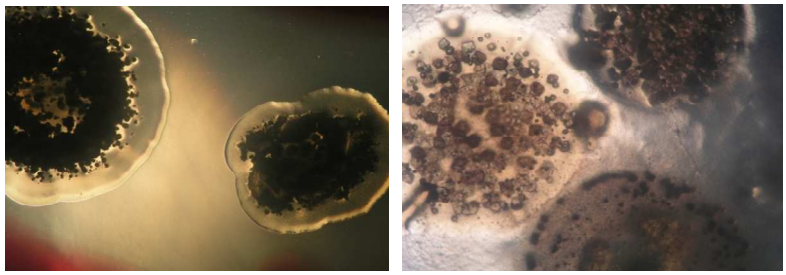

Figure 12*: Carbonate crystal forming colonies.*

Figure 13 illustrates carbonate bioliths. Bacterial footprints are clearly observed in these carbonate precipitates, confirming the role of bacteria in their formation.

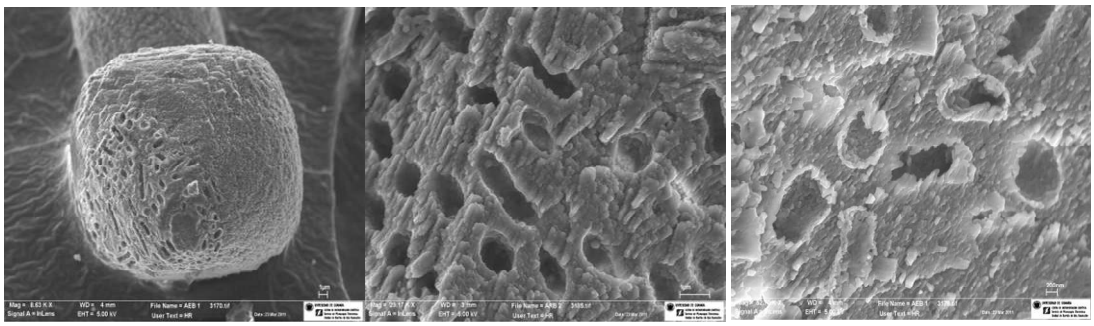

Figure 13*: Micrographs obtained by SEM and FESEM analysis.*

We concluded that

- Bacteria isolated from domestic wastewater and from aerobic submerged-filter bioreactors can induce carbonate precipitation in suitable media. No selective inoculation is needed to produce carbonate precipitation in domestic wastewater treatments plants.

- No precipitation was detected in natural media with wastewater or mixed liquor, possibly due to shortage of calcium or other cations suitable for precipitation. However, reduction in CO_2 emissions in biological wastewater treatment plant could be obtained by modification of wastewater treatment plant technology.

Liquid medium studies: The objective was to study the influence of calcium, organic matter sources and bacteria in carbonate precipitation. The sources investigated were as follows:

- Organic matter sources: wastewater and mixed liquor.
- Calcium sources: Brine (Aqualia, Ibiza); Ca $(C_2H_3O_2)_2$; $CaCl_2$.
- Microbial population from: Wastewater (WWMP); Mixed liquor (BWMP); Brine (BMP).

In all cultures and controls we monitored the evolution of pH, Ca and Mg concentration, TC, TOC and IC. To measure Ca and Mg, a Perkin-Elmer 5100ZL atomic absorption spectrophotometer and automatic sample analyzer equipment was used. To measure total carbon (TC), total organic carbon (TOC) and inorganic carbon (IC), a Rosemount analytical TOC analyser was used. Each culture was grown in duplicate and results presented are the average values of two cultures. The purified precipitates produced in all cultures were examined by XRD and SEM.

Results show that the use of brine as a Ca source was not successful. No significant changes were detected in: pH, Ca and Mg evolution, and we observed a decrease in organic carbon and total carbon only in the culture made with the microbiota present in the brine. This indicates that the use of brine as Ca source inhibits growth in natural wastewater and bioreactor populations. Precipitation was obtained only in the cultures with liquor mixed with brine and the microbiota present in the brine. Organic and inorganic carbon and calcium decreased (Figure 14), in media with added calcium acetate. Calcium carbonate precipitation was obtained in all wastewater and mixed liquor cultures containing calcium acetate and calcium chloride.

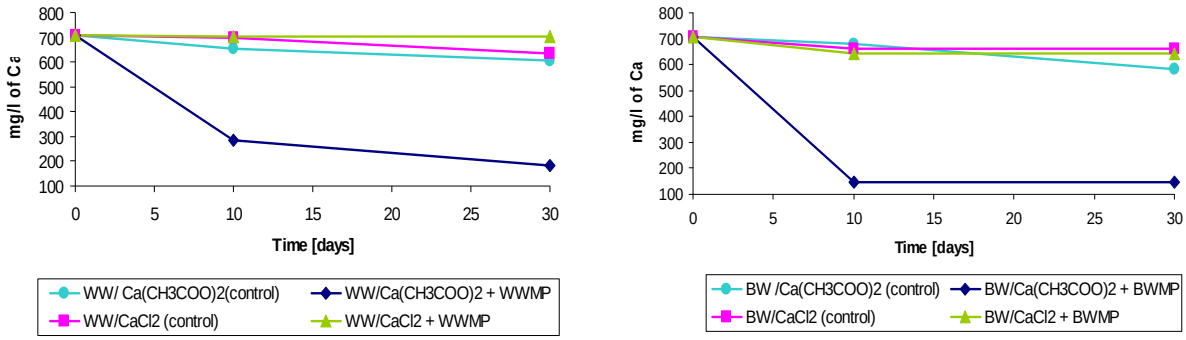

***Figure 14**: Evolution of calcium concentration in cultures with calcium acetate and calcium chloride as calcium source and wastewater (WW) and bioreactor water (BW) as organic matter source.*

The precipitates obtained in all cultures containing Ca $(C_2H_3O_2)_2$ and $CaCl_2$ were calcite (Table 1). In brine cultures the minerals formed were halite, anhydrite and a little calcite.

Samples	Calcite $CaCO_3$	Anhydrite $CaSO_4$	Halite ClNa	Amorphous	mg Ca theoretical	mg $CaCO_3$ theoretical	mg $CaCO_3$ experimental	% efficiency
Sterile BW/brine	3.9%	25.0%	65.9%	5.2%	-	-	-	-
BW/$Ca(C_2H_3O_2)_2$	95.5%	0	0.9%	3.5%	138	345	182	52.7
BW/$CaCl_2$	96.6%	0	0.8%	2.6%	138	345	14	4.0
WW/$Ca(C_2H_3O_2)_2$	100%	0	0	0	138	345	200	57.9
WW/$CaCl_2$	100%	0	0	0	138	345	31	9.6

***Table 1**: Summary of quantitative analysis of crystalline components and efficiency of carbonate precipitation in the culture media (200ml) added with calcium acetate or calcium chloride (Theoretical = Amount of precipitates assuming 100% Ca precipitation; Experimental = Amount of precipitates in the culture) .*

We conclude that:

- In presence of calcium, carbonate precipitation may occur in wastewater treatment plants.
- Brine addition inhibits growth of natural wastewater populations and results in extremely low precipitated carbonate yields (<0.003%).

However experiments in closed cultures do not resemble results obtained in a treatment plant. To get closer to understanding carbonate precipitation using domestic wastewater substrates, we initiated lab scale experiments in a submerged-filter biological reactor at the Water Research Institute of the University of Granada.

Studies in a laboratory scale-up.

The pilot plant consisted of an acrylic column (65 cm height, 15 cm diameter) packed with an inert plastic (BIO-NET ®) which was used as support media for biofilm growth, filling the column to the height of 50 cm. The wastewater was pumped at 15.8 l/day giving a hydraulic retention time (HRT) of 15 h. The wastewater entered at the top exited at the base. Air was supplied by a sliding vane blower to a single membrane air diffuser at the base of the cylinder (Figure 15).

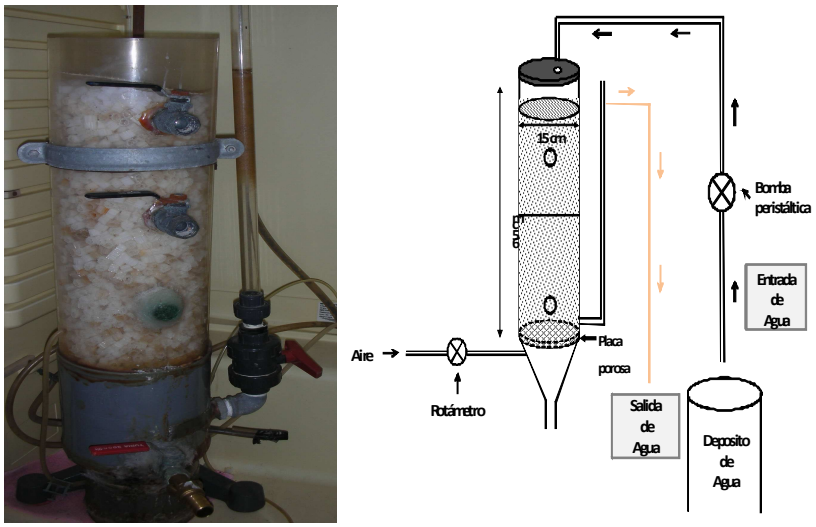

***Figure 15**: Laboratory-scale plant: aerated submerged biofilter inlet and outlet tanks, and air compressor.*

To initiate biofilm growth domestic wastewater was recirculated over 5 days until a visible biofilm was formed. After this, different influents (real wastewater and synthetic wastewaters amended or unamended with $CaCl_2$ (2.8 g/l) were treated in the aerated submerged biofilter. The addition of Ca as $CaCl_2$ was performed to avoid interference with the concentration and type of organic matter present. The capacity for organic matter removal was evaluated as reduction of COD and BOD_5. Calcium concentration at the inlet and outlet was measured daily by ion selective electrode (ISE) as was pH

We found that in synthetic wastewater assays, after 5 working days the organic matter elimination was less than detected when domestic wastewater was used. BOD_5 values decreased from 240 mg/l (bioreactor input) to 140 mg/l (bioreactor output), a sole reduction of 42%. This is related to a progressive decrease in the biofilm generated in the support possibly as a consequence of input nutritional deficiencies which determine modifications in biofilm. Addition of 2.8 g of calcium chloride didn't significantly change the depuration capacity of the plant. These results drove new investigations, with modifications in synthetic wastewater composition to improve the efficiency of organic matter elimination.
To determine the input-output ratio calcium ion concentration was measured. Wastewater calcium concentration was measured as a function of time, before and after biological treatment. Results demonstrate that calcium elimination was approximately 35%. Based on this study it is technically unviable to recover these carbonates from the pilot plant (Figure 16).

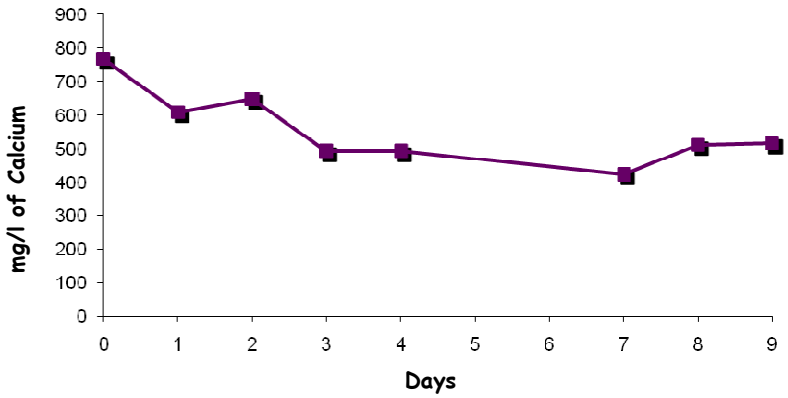

***Figure 16**: Calcium concentration evolution along the treatment cycle.*

Our results suggest that it's necessary to improve/optimize calcium carbonate precipitation in the depuration systems tested. Thus, it is necessary to establish optimal relations between organic matter and calcium concentration, and operational parameters in order to favor both precipitation and depuration processes.

Study of carbonate precipitation by halophilic bacteria using brine from desalination plants as a source of calcium.

We started with 12 strains isolated from deep seawater environments and 20 strains isolated from hypersaline habitats. We carried out preliminary experiments to verify the ability of these strains to precipitate carbonates. We used a rich culture M1 media very suitable for carbonates

precipitation. Later we studied carbonate precipitation with different sources and concentrations of calcium and organic matter. We selected M4 media (yeast extract, 4 g/l; $Ca(C_2H_3O_2)$, 2.5 g/l agar: 18 g/l) for future research with brine.. These media were added with artificial salt solution.

For the large scale use of carbonate precipitation, one of the main problems is to find cheap and abundant calcium, or other ion, source in nature. As a consequence of increased use of seawater desalination plants large volumes of brines are produced. These brines could be used as calcium, magnesium and other cation sources for carbonate precipitation, as well as providing the halophilic bacteria with an essential salt source.

In order to test the use of desalination brine as calcium source, we have utilised M4 medium, replacing the artificial source of calcium by natural brine supplied by Aqualia S.A. Carbonate precipitation was negative for all the strains assayed. These findings led us to the following questions:

1. Are the previously selected bacteria unsuitable for precipitation in these culture conditions?
2. Is the calcium concentration in brine too low?
3. Is the organic matter source in the culture medium too low?
4. Is there ionic interference?

To eliminate 1 above, isolation, selection and study of new strains from samples of brine and seawater of close area of desalination plant were carried out. Total bacteria counts and percentage of carbonate forming colonies were as follows. Brine sample: $4.16x10^3$(c.f.u./ml)with a 85% of carbonate forming. 3 strains were selected. Sea water sample: $5.83x10^3$ (c.f.u./ml) with 94% of carbonate forming. 3 strains were selected. The results of identification of 6 selected strains are show in Table 3.

To test if calcium concentration was too low, we tested different culture media with brine, with or without additional calcium acetate and calcium chloride. The results show that abundant carbonate precipitation occurred only when calcium acetate was added. With only calcium chloride and in the presence of glucose, carbonates were precipitated by two of the bacterial strains. We conclude that

- The bacteria, as well as the organic matter and calcium concentrations and sources play an important role in carbonate precipitation. These factors influence not only the precipitation efficiency but the bacteria´s ability to carry out the process under new environmental conditions.
- Calcium acetate is better than calcium chloride as calcium source because acetate degradation produces CO_2. In some cases, the addition of small amounts of glucose can substitute and allow precipitation.

If we want to investigate further the use of brine as a Ca source, we have to look for culture media with sources and concentrations of organic matter that allow carbonate precipitation at least in some bacteria. As a result of these research, we selected SMR2 medium (yeast extract: 7.5; peptone protease: 3.75; glucose: 0.75, brine 1000ml) as a basis for further research because this media so far has produced the most carbonate precipitation with our strains. The objective was to seek a deeper understanding of the process of carbonate precipitation and determine the carbonate precipitation yield**.** These studies were realized using SMR2 liquid media and four selected strains.

In all cultures we observed a significant diminution of the total organic carbon and an increase in the amount of inorganic carbon; Calcium concentration decreased by between 40 and 72% depending on the strain (Figure 17), while magnesium concentration decreased by less than 12%. The efficiency of carbonate precipitation results with four strains are less than 2% in all the cases.

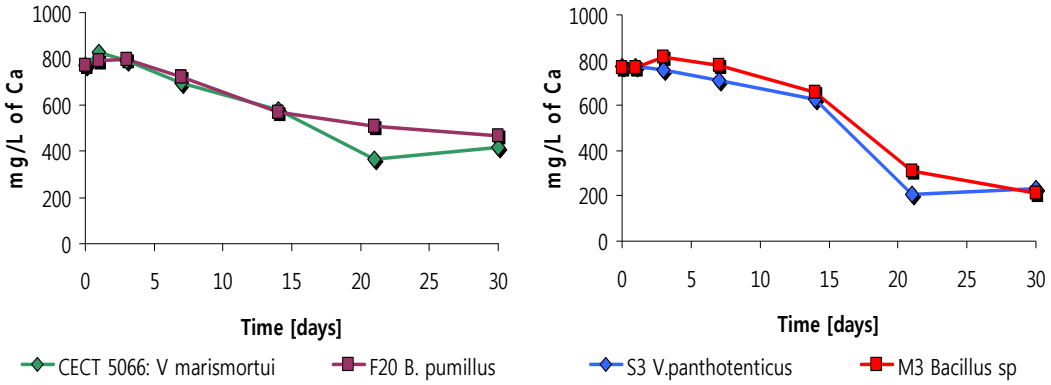

***Figure 17**: Calcium concentration evolution in the cultures of CECT 5066 V. marismotui , F20 B. Pumillus, S3 V. panthotenticus and M3 Bacillus sp during 30 days.*

Strains	m.h. calcite	Vaterite $CaCO_3$	Weddellite CaC_2O_4 $2H_2O$	Sylvine KCl	Halite NaCl	Struvite NH_4MgPO_4 $6H_2O$	Dittmarite $NH_4(MgCa)PO_4$
F20 *B.pumilus*	41,4	0,5	0,4	35,1	20,9	1,1	0,4
5066 *V. marismortui*	4,4	7,1	7,3	7,3	64,4	5,5	4
M3 *Bacillus* sp	24,9	2,2	3	60,4	3,5	5,5	0,5
S3 *V.panthotenticus*	41,4	1,3	0,7	32,2	21,4	1,8	1,2

***Table 2**: Quantitative analysis of precipitates and precipitation process efficiency.*

Studies using solid media with different organic matter sources

The objective of this study was to find sources and concentrations of compatible organic matter which promote good precipitation at low cost. This study was performed on solid media, using brine as the sole calcium source, since the study on solid media is a simple and rapid method for determining the ability of precipitation in different strains. The previously selected 31 strains were assayed and SMR2 medium was used as a reference. Two experiments were carried out. In the first experiment, different concentrations and sources of organic matter were tested, with variable concentrations and combinations of yeast extract, peptone (from different procedure –meat, soy, vegetal, milk and potatoes–) and glucose. In the second experiment, pig manure and fertilizers obtained as a byproduct of manure treatment, provided by a waste management company farm Vilches (Jaén, Spain) was used.

X-ray diffraction results have shown that precipitated crystals are mostly calcium sulfate (gypsum, anhydrite and bassanite). From all tested media carbonate precipitation (kutnohorite, aragonite) was observed only in media made with yeast extracts. Electron

microscopy verified that the calcium or calcium-magnesium carbonate spherulites produced show cell fingerprints; however sulphates did not show calcified bacteria or bacterial fingerprints.

The main conclusions from all these studies are that

- Desalination Brine isn't a good source of Ca for precipitation of carbonates, due to the presence of high concentrations of other ions that hinder the precipitation of carbonates.
- The results so far obtained in the experiments to search for abundant and economic organic matter sources, indicate that none of the sources investigated is appropriate. However these studies were carried out with brine and potentially this study might be more fruitful with other more appropriate sources of calcium.
- Carbonate precipitation process is complex and needs to be better understood before we can think about possible large scale applications and/or optimize conditions for a particular purpose. It is important to know the efficiency of the process in different strains, and to explore the possibility of increasing it. Therefore, we are conducting further studies to deepen in the knowledge of the process.

Other complementary studies

*Geochemical study***:** A geochemical study has also been carried out to obtain a better understanding of the degree of bacteria involvement in mineral precipitation. The geochemical modelling of the solutions assayed was carried out using the geochemical computer program PHREEQC Ver.2 (Parkhust and Appelo, 1999). The activity of dissolved species was determined and the degree of saturation evaluated. The comparison of results of geochemical modelling and identification of minerals precipitated clearly show that bacteria exert some control in the process of precipitation. Thus, in media with high enough concentrations of Ca^{2+} ions, the bacteria investigated promote the precipitation of carbonates instead of hydroxyapatite. In addition, in the same medium, different bacteria precipitate different minerals, or different proportions of minerals which confirms that bacteria play an active role in the type of minerals precipitated.

*Enzymatic carbonic anhydrase activity***:** We have studied the enzymatic carbonic anhydrase activity in all the strains assayed in order to understand the relationship between this activity and the capacity of carbonate precipitation. Carbonic anhydrase (CA) is an enzyme which catalyzes the reversible hydration of CO_2 to bicarbonate in many organisms. Carbonic anhydrase could be responsible of the kidnapping of a significant fraction of CO_2 by heterotrophic bacteria in underground natural environments (Sánchez-Moral et al., 2003). In addition, this enzyme could be used for capture and storage of carbon dioxide (Ramanan et al 2009, Sharma et al., 2008). However, despite being detected in many bacteria, its role in carbonate precipitation by bacteria is unclear.

Carbonic anhydrase (CA) assay was performed using the methodology described by Ramanan et *al.* (2009). The reaction is based on the hydration of p-nitrophenyl-acetate (p-NPA) to p-nitrophenol and acetate that in presence of CA enzyme produces a yellow coloration.

Main conclusions

Most of the strains tested produced carbonic anhydrase (Table 3), which confirms that CA production is widespread in the bacterial world. We have found carbonate precipitation in CA

positive and negative bacteria, indicating that it is not an essential enzyme for carbonate precipitation. Nor have we found that the presence or absence of this enzyme allows an improvement in bacteria tested ability to precipitate carbonates, since some CA negative strains produce a large amount of carbonates and initiate the formation even more rapidly than other CA positive strains. Some strains produce a very weak staining, possibly by the production of small amount of enzyme, and others have color only on the cell mass (possibly produce only intracellular CA), and even in these cases we have detected differences in precipitation between clearly positive strains.

	Strains isolated in this study	AC activity			Other strains	AC activity	
		1 day	5 days			1 day	5 days
Bioreactor	*Arthrobacter* sp LM2	+	+	Deep seawate	*B. thuringiensis W1*	+	+
	Bacillus sp LM3	+	+		*Thalassospira sp W5*	+	+
	Pseudomonas sp LM6	+	+		*Thalassospira sp W7*	+	+
	Bacillus sp LM7	+	+		*Halomonas sp W12*	+	+
	B. megaterium LM8	+	+		*B. pumilus W15*	-	+
	Enterococcus sp LM9	+	+		*B. pumilus W16*	+	+
	Bacillus sp LM10	+	+		*Brevibacterium casei F2*	+	+
	Bacilluc sp LM11	+	+		*B. pumilus F20*	D1	+
	B. flexus LM12	+	+		*Pseudomonas sp S21*	-	-
	Bacillus sp LM13	-	+		*Pseudomonas sp S23*	-	+
	Enterococcus sp LM15	D1	D1		*B. pumilus S30*	+	+
	Agromyces sp LM16	+	+		*B. pumilus S31*	D1	+
	Bacilluc sp LM17	+	+	CECT	*H. pacifica*	-	-
	Agromyces sp LM18	+	+		*H. halodenitrificans*	-	-
	Rhodococcus sp LM19	+	+		*S. ruber*	+	+
	Rhodococcus sp LM20	D1	D1		*I. Loihiensis*	+	+
	Bacillus sp LM21	+	+		*H. hitroreducens*	+	+
Brine (S) and seawater (M)	*Bacilluc* sp S1	+	+		*V. salinus*	D1	+
	Bacilluc sp S2	+	+		*V. pantothenticus*	+	+
	V.panthotenticus S3	+	+		*S. costicola*	-	D1
	B. pumilus M1	+	+		*V. marismortui*	D2	D2
	B. pumilus M2	+	+		*H. meridiana*	-	-
	Bacillus sp M3	+	+		*H. anticariensis*	D1	D1
					H. maura	+	+
					Salipiger mucosus	+(I)	+

+: intense yellow coloration (few seconds); **(I):** rapid reaction with stain only in cell mass (possibly CA intracellular production); **D1:** doubtful: very weak staining and stain only the cell mass (possibility of Intracellular producction of CA, in small amounts); **D2:** very weak staining and color changing´s very slow.

***Table 3**: Identification of selected strains and carbonic anhydrase activity in all studied bacteria.*

4.1.3.4 THE OXALATE-CARBONATE PATHWAY

Daniel Bravo, Martin Clerc, Sabrina de los Rios, Daniel Job, Pilar Junier
University of Neuchatel
Guillaume Cailleau, Fabienne Dietrich, Anne Golay, Gaëtan Martin, Eric Verrecchia
University of Lausanne

The production of oxalic acid ($H_2C_2O_4$) and oxalate minerals is observed among a wide variety of organisms including plants, animals, fungi, and bacteria present in soil (Tamer*, et al.*, 2002). Oxalic acid is often accumulated as a metabolic end-product in plant cells and can be released by root systems as free acid mineral salts of calcium, sodium, potassium, ammonium or magnesium. Deposition of calcium oxalate occurs in a wide range of plant taxa -215 plant families reported so far - (Nakata, 2003), and it can comprise up to 85% of the dry weight of some plants (Koyama, 1988). After the death and decay of plants, oxalate is released into the soil litter (above and below ground) where it can fulfill important roles (e.g. in plant nutrition by increasing the availability of phosphorous and other micronutrients). Interestingly, and despite oxalate's relative insolubility and chemical stability, the accumulation of metal oxalates has not been observed in geological records (Schilling & Jellison, 2004). This supposes a microbiologically mediated process as the main oxalate sink in natural environments (Robbel & Kutzner, 1973). Most bacteria may not use oxalate, the simplest of the dicarboxilic acids (Dijkhuizen*, et al.*, 1977), as the first choice for an energy source (Clausen*, et al.*, 2008). However, because oxalate is a highly oxidized substrate, its oxidation to CO_2 provides two low potential electrons that enter in the respiratory chain through formate-dehydrogenase, allowing direct reduction of NAD^+ (Blackmore & Quayle, 1968). Oxalotrophic bacteria, which use oxalate as source of carbon and energy (e.g. Anbazhagan*, et al.*, 2007), do not constitute a phylogenetic group but rather a functional guild (Sidhu*, et al.*, 1997).

Oxalotrophic bacteria were studied in three sampling sites located in tropical soils from Bolivia, India, and Cameroon. The three tree systems studied were *Terminalia oblonga* (Bolivia), *Terminalia bellirica* (India), and *Millicia excelsa* (Cameroun). At each sampling site, two pedological profiles were prepared. Profile A corresponded to the soil influenced by the presence of the oxalogenic tree and was dug aside the tree trunk. Profile C was dug in soil away of any oxalogenic influence to be used as a control.

Oxalotrophic bacteria in soil samples were enriched in a solid Schelgel AB medium supplemented with calcium oxalate (CaOx) as carbon and energy source (Braissant*, et al.*, 2002). The use of this mineral medium is considered the first step in order to obtain oxalotrophic viable bacteria (Aragno & Schlegel, 1991). Based on the number of colony formation units (CFU), the number of culturable oxalotrophic bacteria for the three sites studied ranged from $3x10^6$ (profile under the tree, at 30 cm deep, Bolivia) and $1x10^8$ (profile under the three at 20 cm deep, Africa). In all the cases, oxalotrophic bacteria were more abundant in the upper part of the profile, generally over the first 20 cm. A decrease in the number of culturable oxalotrophic bacteria was detected for the deeper part of the profiles, reaching values close to 0 below 40-50 cm. Overall, the results of the culturable fraction of the oxalotrophic community show that this bacterial guild is ubiquitous and can be enriched and isolated from soil independently of the presence of an oxalogenic tree.

A total of 160 bacterial morphotypes were isolated by using the same medium. This corresponds to 32 isolates from Bolivia, 88 from India and 40 from Cameroon. For the

identification of the isolates, a DNA extraction (InnuPrep Bacteria DNA extraction kit) followed by amplification and sequencing of the 16S rRNA gene was used. Amplification of almost the entire 16S rRNA gene was carried out using the primers Eub9_27 forward, and Eub1542 reverse (Muyzer, *et al.*, 1993) and according to the optimal temperature for the *Taq* DNA polymerase (NEBiolabs). From the 160 isolates, 67 strains (23 in Bolivia, 33 in Cameroon and 11 in India) have been already identified (Table 4). The most commonly identified genera included *Stenotrophomonas*, *Agrobacterium*, *Alcaligenes,* and *Variovorax*. Among the gram-positive bacterial genera observed, it is possible to mention *Bacillus* and *Streptomyces*, the last group being particularly important in India. Despite the fact that some of the isolates were related to the same phylogenetic group, great phenotypic diversity was observed. Another important aspect to be considered is the fact that the *frc* gene, which is a functional marker associated to oxalotrophy (see below), could not be amplified in all the different strains, regardless of their phylogenetic affiliation.

First Identified BLAST hit	*frc*	Bolivia	India	Cameroon
Achromobacter sp. QUEBA08	-			1
Acidovorax sp.	-	1		
Afipia sp. D1	+		1	
Agrobacterium sp. W14	-			3
Alcaligenes sp. ETH1	+/-			3
Arthrobacter sp. 41-3	+			1
Bacillus sp. PA27, isolate PA27	-	1		
Cupriavidus metallidurans CH34	-			1
Cupriavidus respiraculi strain LFS88	-			1
Ensifer adhaerens strain S-30.7.5	-			1
Lysobacter sp. ITP09 strain JA110	+/-		2	1
Lysobacter sp. MHS036	-			1
Methylobacter sp. CRR17	-		1	
Oligotropha sp. enrichment culture clone z108	+	1		
Paenibacillus sp. JDR-2	+			1
Rhizobium sp. CCBAU 65048	-			1
Stenotrophomonas maltophilia	+/-	12		6
Stenotrophomonas sp. Dy034	-			2
Stenotrophomonas sp. ROI7	+/-	1		1
Streptomyces achromogenes strain S4BS5	+		1	
Streptomyces flavogriseus ATCC 33331	-	1		
Streptomyces purpurascens LMG 20526	+		3	
Streptomyces sp. AB5B	+		1	
Streptomyces sp. 2SM-1	-			1
Streptomyces sp. HaNXJ11	+	1		
Streptomyces sp. HCD24	+		1	
Streptomyces sp. SUZ-B	+		1	
Streptomyces sp. Xzfj-27 16S	-	1		
Streptomyces variabilis strain BAB1536	+	1		
Variovorax soli strain: NBRC 106424	+/-			3
Variovorax sp. Aek21	-			1
Variovorax sp. CRF3-Va-1	-	1		
Variovorax sp. SaNR1	+/-			4
Xanthomonas campestris	-	1		
Xanthomonas sp. 33DCP	+	1		

Table 4*: Identification of strains from Bolivia (B), Cameroon (C) and India (I) after a BLAST analysis of the 16S rRNA gene sequence. The first hit and the first identified hit in BLAST are presented. The results of the amplification of the frc gene are also shown in the table.*

Culture-independent quantification of oxalotrophic bacteria

The fact that oxalotrophs are a functional group and not a taxonomic clade implies that their study *in situ* cannot rely on traditional phylogenetic molecular markers such as the 16S rRNA gene. Therefore, the use of genes coding for enzymes involved in the catabolism of oxalate as molecular markers is a better-suited strategy. In the past, primers designed to amplify the gene *frc* have been used to assess diversity and abundance of oxalotrophic bacteria *in situ* (Khammar et al., 2009). The numbers of total and oxalotrophic bacteria obtained by qPCR of the *frc* gene are over two to three orders of magnitude higher than those obtained by the culture-dependent method. This result shows clearly the effect of the enrichment biases largely known in microbial ecology. In general, the comparison of the results obtained for the profiles under an oxalogenic tree and those of the control soil, in all the three sites, shows that

abundances of both oxalotrophic and total bacteria are stimulated by the presence of the tree (Figure 18). In all the cases, a difference of several orders of magnitude was observed when samples between profiles at equivalent depths are compared.

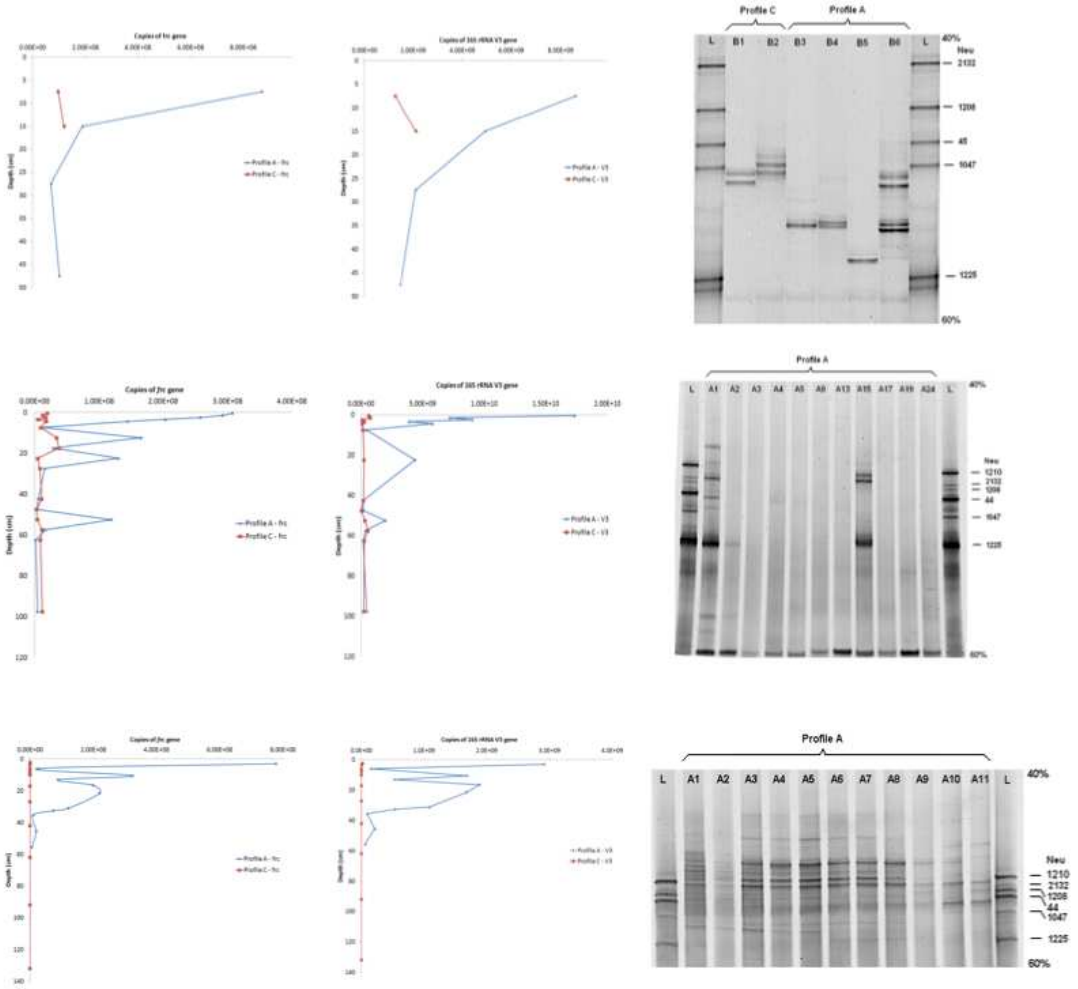

Figure 18*: Quantification of oxalotrophic bacteria (frc gene – left panel) and total bacteria (16S rRNA gene V3 region –middle panel) in soil from Bolivia (top), India (middle) and Cameroon (bottom). Profile A represents the profile under the oxalogenic tree. Profile C represents the profile for the control soil. In front of each qPCR profile, the results of denaturing gradient gel electrophoresis (DGGE) for the gene frc are shown.*

Similarly to the tendency observed for the culturable fraction of the community, the abundance of oxalotrophic bacteria decreased with depth. An exception to this was the sample at 45 cm in the profile under the tree in India, in which a "peak" was observed in abundance of both oxalotrophic and total bacteria. A closer analysis of the sample demonstrated the presence of deeper roots of the oxalogenic tree that can produce a local increase in oxalate at this depth. In the profiles from India and Cameroon, which contained a larger number of samples, the existence of three different regions with high abundances of oxalotrophic bacteria can be inferred. The first of these regions corresponds to the litter in the upper most part of the profile, in which a major exchange ad turnover of organic matter can be expected. The other two regions (15 cm and 25 cm) might correspond to less transient pools of oxalate, either

linked to a direct secretion by the tree or leaching from the upper part of the profile. This has to be confirmed. The effect of these different pools over the composition of the oxalotrophic communities is starting to be addressed as well (Figure 18, left panel).

Overall, the results from the culture-independent study show, for the first time, a clear correlation between the presence of an oxalogenic tree and the oxalate-consuming bacterial communities in the soil, *in situ*. This correlation is also emphasized across samples from the different sites, in which the oxalate-carbonate pathway has shown to be active.

The calcium cycle in an oxalogenic ecosystem: an update

The amount of calcium needed to maintain the carbon accumulation rate calculated for the iroko tree (Bertoua site, southeastern Cameroon) is a critical factor. Considering the net balance between the inputs and the outputs of the system (ca. 60g/yr), the calculated input value is insufficient to support the flux of calcium constituting the pedogenic carbonate compartment (on average 5700g/yr over the tree lifetime) (Figure 19).

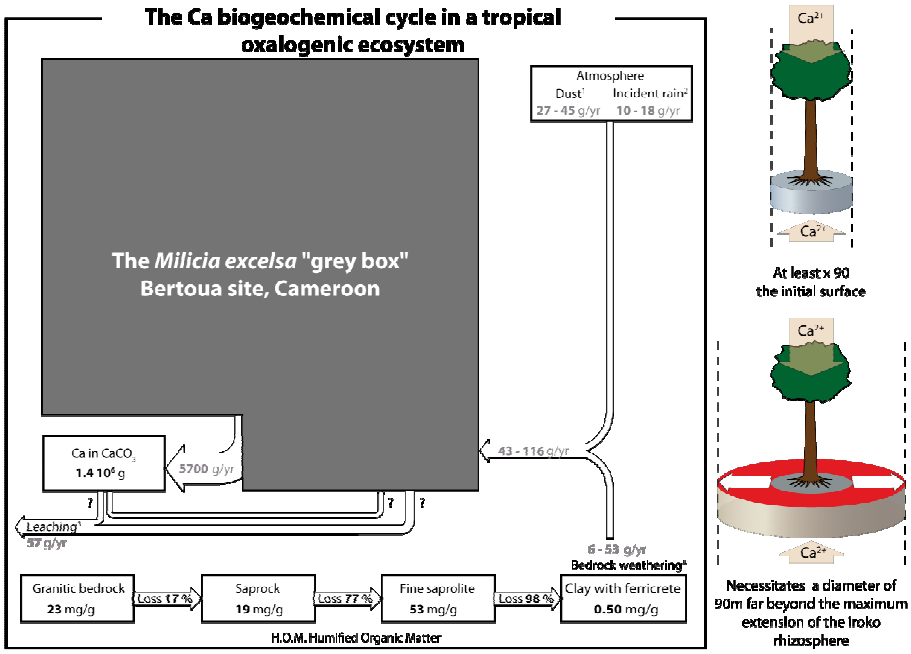

Figure 19*: Calcium biogeochemical cycle around an iroko ecosystem in Bertoua (Cameroon). The flux of calcium needed to support the carbon accumulation rate cannot be supplied by the external inputs calculated for the reference surface. In order to match what is needed and what can be provided by external inputs, it is necessary to increase the surface taken into account far beyond the possible extension of the iroko root system.*

The calculation was made for an arbitrarily chosen surface equal to the projection of the iroko foliar crown onto the soil corresponding to a diameter of 9m. Obviously the root system spreads over this circle. But if a supply through the soil is the only source, in order to get enough calcium from external sources to feed the carbon accumulation rate, it is necessary to take into account a disk of 90 m in diameter, an extension that is far too large for the iroko root system. So the calcium needs to be at least partly fed by pre-existing calcium pools present in the iroko ecosystem, in addition to the external inputs. These pre-existing pools are the soil

itself and the local biomass, both possibly impoverished in favor of the carbonate pool, which is built-up during the tree's lifetime.

A specific study of both these compartments has been performed. We investigated an oxalogenic system at the Bertoua site (southeastern Cameroon). Four pools of calcium were identified and quantified around an oxalogenic tree *Milicia excelsa*. These results provide the first detailed snapshot of the local Ca cycle driven by the oxalate carbonate pathway. Considering the calcium present in the soil, calcium mapping and a sequential extraction used to determine the calcium content in some sub-compartments, has been performed (Figure 20). There is more calcium close to the trunk than in the soil unaffected by an efficient oxalate-carbonate pathway. This was expected as the calcium carbonate accumulates around the iroko tree. This expected pattern is now proven, as well as the distribution of low Ca concentrations in a "typical acidic soil". Regarding the distribution of calcium in the different compartments, it varies according to the soil profile position. For instance, in the deep soil horizons at a distance from the tree, the calcium is mainly present in the exchangeable calcium pool, whereas below the trunk, the calcium is the most abundant in the carbonate fraction pool. As a consequence, a pumping effect, generated by the iroko tree and the oxalate-carbonate pathway, can be inferred. Unfortunately, although the enrichment zone is clear, the impoverishment area remains to be documented. Also, the extent in which the low amount of calcium in the distant soil is the expression of a normal poor soil or is it a consequence of an impoverishment, is not yet clear and at this stage further work is needed.

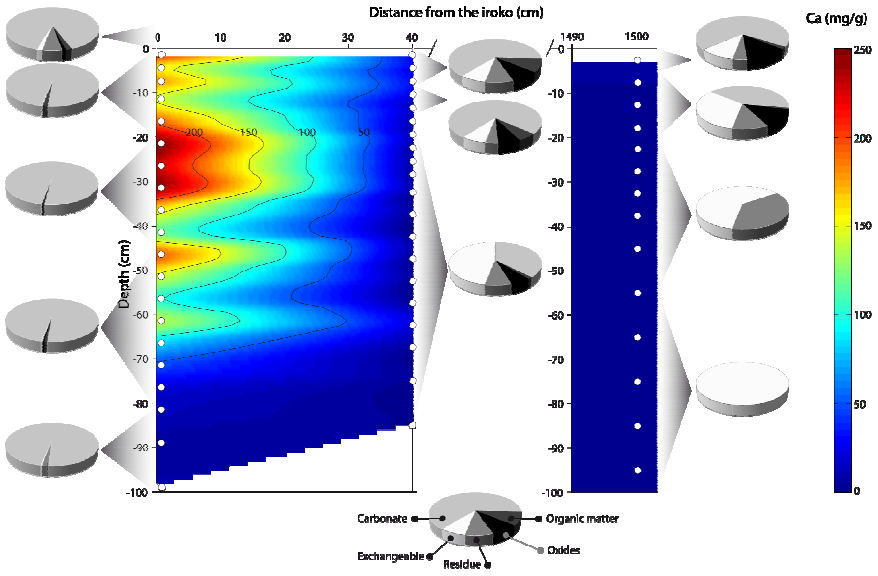

Figure 20*: Soil calcium map and calcium distribution in different soil sub-compartments around an iroko ecosystem in Bertoua (Cameroon). White dots: analysed samples.*

Regarding the calcium present in the biomass, vegetation surveys have been conducted and the results are given in Table 5. In this table, there are comparisons of calcium contents between the same species. If the oxalogenic system works as a "Ca sponge", the surrounding plants were suspected to be impoverished regarding Ca. The data shows that this is apparently not the case. The plants close to the iroko tree present greater amounts of Ca than the same species further away from the tree. This was surprising, as an impoverishment in the soil seemed intuitively more likely. Perhaps the Ca depletion in the vegetation can be observed a little bit further from

the trunk, but this has yet to be tested. Another assumption is that the Ca depletion magnitude could be low but is spread over a large area. Further work is needed to explain the pattern of calcium distribution on biomass at different distances from the iroko ecosystem.

Genus species	Ca (mg/g)	distance to the iroko	DBH
Capoifeira mildbraedii	2.2	away	170
Capoifeira mildbraedii	19.6	8.1	170
Capoifeira mildbraedii	29.2	2	190
Theobroma cacao	0.8	away	35
Theobroma cacao	1.2	5.1	85
Theobroma cacao	2.5	3.2	30
Mussa ssp	3.3	away	21
Mussa ssp	8.9	4.1	30
Aframonum ssp	1.4	away	5
Aframonum ssp	7.9	1	5

***Table 5**: Comparison of calcium contents measured in the same species close to the tree as well as those growing on a soil unaffected by an active OCP at the Bertoua site (Cameroon).*

The new results allow a more complete calcium cycle to be proposed (Figure 21). First of all, it is clear that all the compartments of calcium present in the "grey box" (Figure 19) as well as the pedogenic calcium carbonate compartment have been constituted over a large period of time by the external inputs. Indeed, the soil components (organic and mineral) are inherited from the basement weathering and the atmospheric inputs (dry and wet).

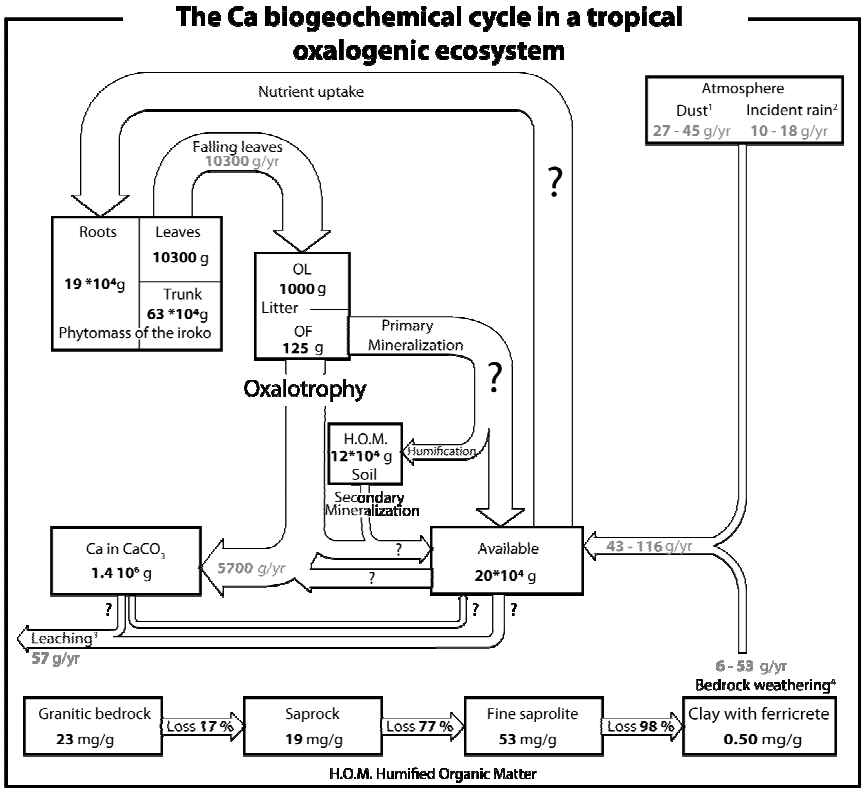

***Figure 21**: Synthetic sketch of the whole calcium biogeochemical cycle close to the iroko tree (Bertoua, Cameroon).*

It is well known that in tropical acidic soils, the various nutrients are present in low amounts (low CEC, low organic matter content, etc.), and these nutrients are mostly retained by the biomass reservoir constituted over centuries. Secondly, the amount of calcium required to build-up the carbonate compartment means that the annual rate of calcium entering the system is insufficient by itself, and the calcium intra-cycle, mainly driven by the "organic matter turnover", must supply a part of it. Considering the soil compartment, it is difficult to discuss its contribution, as a surrounding depletion has to be demonstrated, especially because the importance of the various soil compartments depends on profile positions. Considering the above-ground biomass compartment, unexpectedly, it has greater amounts of calcium close to the tree than at distance. In consequence, the role of plant biomass seems to be less obvious than previously thought.

It is difficult to draw a final and definitive conclusion. Regarding the supply of calcium to the Iroko ecosystem, the important flux of calcium recycled in the forest ground litter (Figure 21) through the fast turnover of organic matter, could intrinsically provide an important amount of calcium or at least a part of what the system needs.

Major S&T Results

Quantification of microbial process and modeling of microbial contribution

Bacteria alone are sufficient to shift the pH from acidic to alkaline in cultures with calcium oxalate as sole carbon source (Braissant et al 2002, Braissant et al 2004, Jayasuriya 1955). Under these experimental conditions, the pH shift allows the precipitation of calcium carbonate crystals (Braissant et al 2002). However, to date, there is no direct evidence showing that bacteria can oxidize calcium oxalate under natural environmental conditions and induce the pH shift required for calcium carbonate precipitation. Furthermore, although fungi are recognized as major players in the oxalate cycle in soils (Dutton and Evans 1996, Tuason and Arocena 2009), their role in the oxalate-carbonate pathway, and more generally in the functioning of soils, needs to be clarified.

Consequently, two major questions were addressed in this study: i) can oxalotrophic bacteria alone cause the shift in pH required for the precipitation of calcium carbonate in soil? ii) to what extent are fungi-bacteria interactions instrumental for the pH shift to occur? Microcosm experiments were conducted to fill the gap between experiments with pure cultures and field observations in order to answer these two questions. In a first experiment, a sterile soil was inoculated with a mix of pure bacterial and fungal cultures. In a second one, fresh soil collected near an oxalogenic tree and containing its own complex native microbial community was treated to selectively inhibit the activity of bacteria or/and fungi and to test their individual contribution to soil pH shift.

We evaluated the influence of amendments with bacteria, fungi, and oxalate on changes in soil pH in seven different microcosms (Figure 22). For most of the systems assayed, pH values remained unchanged for more than 90 days. However, in the treatment amended with bacteria, fungi, and oxalate (FBox), an increase in soil pH was observed after 20 days of incubation. After 90 days, soil pH had reached a final value of 7.5, being 2.5 pH units higher than the initial value. This coincided simultaneously with a decrease in the oxalate concentration from 34.2 ± 24.5 mg g^{-1} to 7.9 ± 5.2 mg g^{-1}. This pH shift was sufficient to induce calcite precipitation, which was

not observed in treatments where the pH remained constant. X-ray diffraction analysis revealed small yet characteristic peaks for calcite in FBox soil but not in SSox soil.

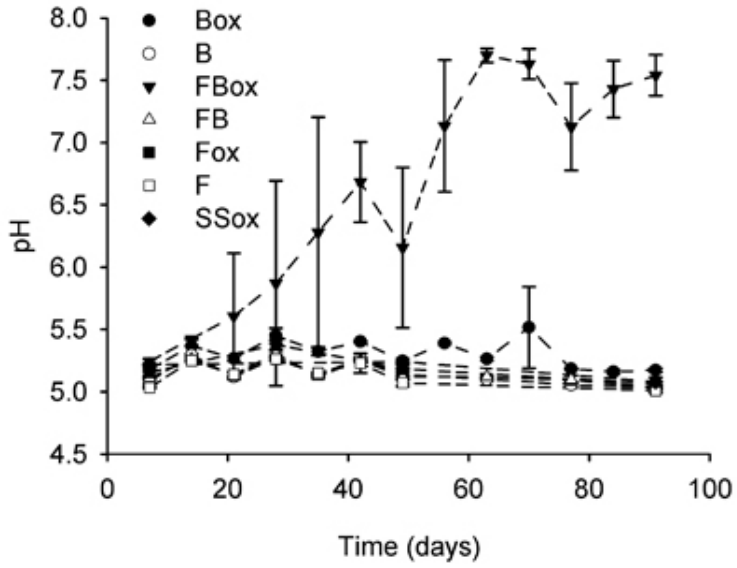

Figure 22*: Evolution of pH in microcosms created with a sterile soil and inoculated with allochthonous microbial community. The different treatments were: inoculation with bacteria and Ca-oxalate amendment (Box), inoculation with bacteria, without Ca-oxalate amendment (B), inoculation with bacteria and fungi, with Ca-oxalate amendment (FBox), inoculation with bacteria and fungi, without Ca-oxalate amendment (FB), inoculation with fungi and Ca-oxalate amendment (Fox), inoculation with fungi, without Ca-oxalate amendment (F), and finally sterile microcosms with Ca-oxalate amendment (SSox). Data points represent mean values of samples (± standard deviations) from three independent microcosms.*

Bacterial metabolism of oxalate has been shown to induce a pH increase in experiments on Petri dishes (Braissant et al 2004, Jayasuriya 1955). Surprisingly, amendment of oxalotrophic bacteria was not sufficient to induce such a pH change in oxalate containing microcosms (Box). Addition of fungi alone (with or without oxalate) did not lead to a change in the soil pH either, raising the question as to whether the added microorganisms survived and developed in these microcosms. In order to test this we followed copy numbers of *frc* and 16S rRNA genes of oxalotrophic and total bacteria, respectively, by qPCR. As for soil pH, bacterial abundance increased only in the FBox treatment, where copy numbers of both marker genes increased by three to four orders of magnitude (*frc*: 3.7×10^4 to 2.8×10^8; 16S rRNA gene: 2.3×10^6 to 3.8×10^9). In all other treatments containing bacteria (alone or in the presence of fungi), bacterial abundance remained close to the values recorded 7 days after inoculation. Active fungal biomass was assessed by the ergosterol content in soil. In contrast to bacteria, fungi developed in all microcosms independently of the presence of bacteria or oxalate.

The results obtained for the microcosms with an artificially recreated microbial community prompted us to verify the findings in a less artificial system. Therefore, a second set of microcosms with tropical acidic soil containing a native microbial community was carried out. In these microcosms, the various treatments of the previous experiment were mimicked by the

addition of domain-specific biocides. In microcosms with the native microbial community, the pH rose by about one unit within two weeks after calcium oxalate addition but remained unchanged in the treatments with the biocide mix (SSox analogue). Furthermore, maximum pH (pH 8) was reached in less than ten days as compared to eight weeks in the first experiment (Figure 23). A shift in soil pH was observed under two experimental conditions: no biocide treatment (FBox analogue) and cycloheximide-treated soil (Box analogue).

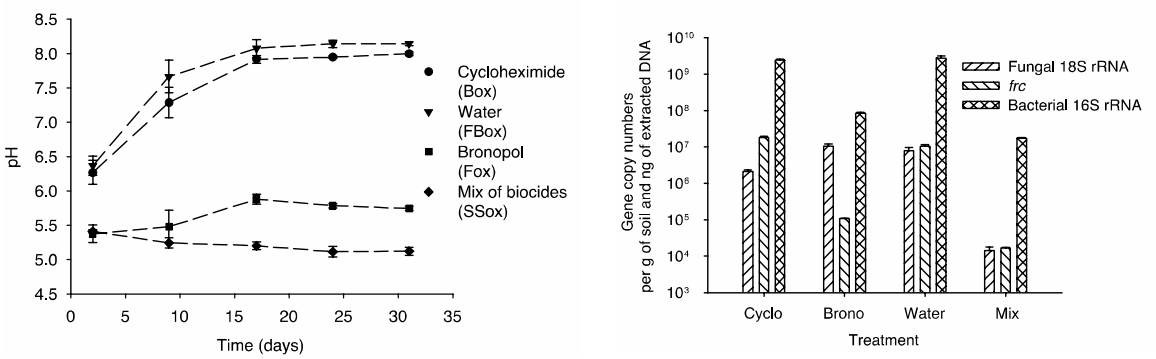

***Figure 23**: Evolution of pH in soil microcosms with native microflora and treated with specific biocides (left panel). Values are means (± standard deviations) of measurements performed on three separate microcosms. For abbreviations in brackets see caption of Figure 22. The right panel presents the quantification of frc, 16S rRNA gene and 18S rRNA gene copies at the end of the experiment performed in soils with native microflora and treated with biocides. Values are means (± standard deviations) of quantifications performed with DNA from three replicate microcosms.*

The results from the microcosms with native microbial community (FBox analogue) confirmed those of the first microcosm series. However, the pH shift in the Box analogue suggests that the bacterial activity alone acted as the driver of soil pH shift for a native microbial community. In order to confirm the effect of biocides, the abundance of bacteria and fungi was determined at the end of the experiment. Although ergosterol was also measured, several additional peaks affected the interpretation of results and therefore fungal abundance was estimated by qPCR amplification of the 18S rRNA gene.

The results for the FBox analogue system confirmed the presence of bacteria, oxalotrophic bacteria, and fungi in this soil. The bacterial and fungal abundances in this treatment were at least three orders of magnitude higher than in the control (Sox analogue). In the microcosms treated with bronopol alone (Fox analogue), bacterial abundance (total and oxalotrophic) dropped by one and a half orders of magnitude compared to the untreated soil (FBox). This system did not show a change in soil pH over time confirming the role of bacteria in the process. Finally, in the cycloheximide treated microcosm (Box analogue), fungal abundance decreased by less than an order of magnitude. A statistical analysis on the qPCR results shows that fungal abundance was significantly different between the water versus bronopol and cycloheximide treatments (p-value = 0.05), but not between water versus cycloheximide (p-value = 0.11), or bronopol (p-value = 0.54) alone. Therefore the cycloheximide treated microcosm (Box analogue) should be regarded as a less performing FBox analogue instead, still supporting the idea that bacteria and fungi are required simultaneously to cause the shift in soil pH observed in the oxalate-carbonate pathway.

The experiments carried out with either an artificial or a native microbial community showed consistently that the simultaneous presence of bacteria, fungi, and oxalate is essential to induce the alkalinisation of the soil pH by up to 2.5 units and the precipitation of calcite, which are two key effects observed for the oxalate-carbonate pathway in nature (Cailleau et al 2005). These results confirm for the first time that the oxalate-carbonate pathway could be reproduced artificially in a microcosm, and that the parameters measured in the field (soil pH and presence of carbonates) effectively described an active pathway.

In order to identify active bacteria involved in the oxalate-carbonate pathway, the same approach, i.e. a microcosm experiment, was set up with the purpose to establish how calcium oxalate concentration influence the diversity and the identity of active oxalotrophic bacteria in the soil. The microcosms had calcium oxalate concentrations ranging from 0% to 4 % (w/w) and they were set in order to allow sampling of three replicates for each concentration every 5 days during 15 days. In addition, for each calcium oxalate concentration, microcosms were treated with Bromodeoxyuridine (5-bromo-2'-deoxyuridine, BrdU) in order to label DNA of organisms replicating their DNA, i.e. active bacteria. When incubation was over, collected microcosms were kept at – 80°C and samples were taken for further DNA extractions or have been dried for pH measurement. DNA samples containing labelled DNA were all treated at the Lawrence Berkeley national lab. The treatment consisted of an immune-separation of BrdU labelled DNA in order to obtain a DNA solution enriched in DNA from actively growing bacteria.

The comparison of the community profiles for oxalotrophic bacteria obtained by denaturing gradient gel electrophoresis (DGGE) of the gene *frc* amplified from BrdU-labelled versus un-labelled DNA shows that the active community is distinct from the total oxalotrophic community (Figure 24). Currently the stimulated bacteria are being identified by band sequencing and comparing it with the isolate collection at UNINE.

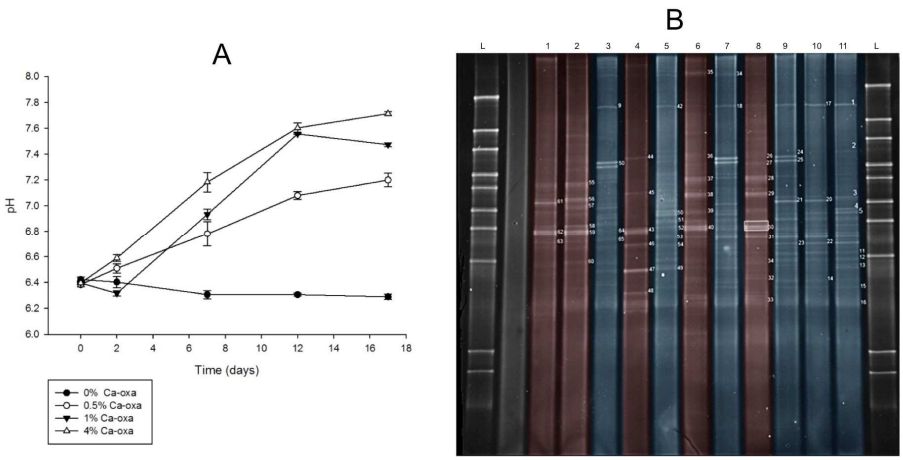

Figure 24*: Evaluation of the active oxalotrophic community during stimulation of the oxalate-carbonate pathway in soil microcosms amended with 0.5%, 1% and 4% of calcium oxalate. A. Evolution of soil pH in the amended and control (0%) microcosms. B. DGGE profiles of the oxalotrophic bacterial community revealed by amplification of the frc gene in BrdU-labelled (red shade) and unlabelled (blue shade) DNA. L= ladders. 1= 1% day 2; 2-3= 1% day 12; 4-5= 0% day 12; 6-7= 4% day 12; 8-9= 0.5% day 12; 10= 1% day 0; 11= 0.5% day 0.*

Quantification and ecosystem

Within the last ten years, many field trips have been conducted in Africa and iroko trees were studied in order to gather data related to the quantification issues of the OCP. In the Ivory Coast, a 170-yr old iroko tree has been studied, and the amount of calcium carbonate present in the soil was quantified. Approximately 8160 kg of $CaCO_3$ were present in the soil, which represent a carbonate accumulation rate equal to 48.02 kg of $CaCO_3$ /yr, averaged over the tree's lifetime (i.e. 21.13 kg CO_2 /yr). Obviously, this figure cannot be applied to the tree during its youth. Indeed, before calcium carbonate can accumulate, the OCP has to buffer the original acidic soil pH, in order to reach the stability pH for calcite (8.4 in normal temperature and pressure, i.e. 25°C and 1 atm).

In southeastern Cameroon (CO2SS project field campaign), the amount of calcium carbonate accumulated close to an at least 200 yr-old iroko tree has been estimated to 1420 kg of $CaCO_3$. This figure represents a carbonate accumulation rate averaged over the tree lifetime equal to 17.71 kg of $CaCO_3$ /yr, if the tree is 200 yr-old (7.81kg CO_2 /yr) and 14.17 kg of $CaCO_3$ /yr if the tree is 250 yr-old (6.23 kg CO_2 /yr). These results provide only an incomplete picture of the efficiency of the OCP related to iroko trees. However, considering Carozzi's work (1967) describing "irregularly shaped blocks, 1 to 1.5 m in size" in African soils, further work is needed to get a complete and accurate model of the iroko tree ecosystem.

	Lower estimation	Upper estimation	
	170 yr old iroko (Biga site)	**170 yr old iroko (Biga site)**	**170 yr old iroko (Biga site)**
Carbonate accumulation rate (kg C/yr)	5.76	5.76	5.76
Estimation of mineralized irokos (%)	33.3333	100	100
Iroko density (foot/ha)	0.1416 (SEFN)	1(FAO)	3 (FAO)
Sequestration deficit over the whole iroko tree African distribution (PgC/yr)	$1.22x10^{-4}$	$2.59x10^{-3}$	$7.77x10^{-3}$
Sequestration deficit over the whole iroko tree African distribution (tons CO_2/yr)	44f209	9497684	28492992
	250 yr old iroko (Bertoua site)	**200 yr old iroko (Bertoua site)**	**200 yr old iroko (Bertoua site)**
Carbonate accumulation rate (kg C/yr)	1.7	2.13	2.13
Estimation of mineralized irokos (%)	33.3333	100	100
Iroko density (foot/ha)	0.1416 (SEFN)	1(FAO)	3 (FAO)
Sequestration deficit over the whole iroko tree African distribution (PgC/yr)	$1.22x10^{-4}$	$2.59x10^{-3}$	$7.77x10^{-3}$
Sequestration deficit over the whole iroko tree African distribution (tons CO_2/yr)	132307	3503912	10511736

Table 6*: Calculations of the carbon sequestration deficit using various scenarios based on available information related to the iroko density in Tropical Africa and the potential fraction of mineralised trees in the iroko population.*

In addition, a new approach has been developed during the CO2SS project to have a better understanding of the dynamics of the iroko ecosystem and its carbon accumulation rate. The aim was to adjust the rates to the tree lifetime in order to get a more realistic time-dependant rate (taking into account the environmental change that occurred during the tree growth). This approach has been possible by using dendrochemical methods: as sedimentary rocks record the past, trees do the same in their growth rings. Considering the OCP, one of its important environmental effects is its drastic increase in soil pH, which should be recorded in the wood tissues by a specific element sensitive to pH variations in the soil solution, manganese (Mn). Indeed, it has been demonstrated in the literature that there is a correlation between high Mn content and acidic soil conditions. As a consequence, Mn is considered as a good proxy of the soil pH. The environmental variation is discussed based on the CaO/MnO ratio (Figure 25), as

the tree record is not linear due to the decrease of the cation binding capacity (this cation binding capacity is well described by the calcium record in the tissues). It appears in these preliminary results that between 65 to 90 years before the tree was cut down, the CaO/MnO ratio recorded a drastic change in the soil pH. Does this mean that the soil reached favorable conditions for soil calcium carbonate accumulation? At this stage, only hypotheses can be made. This drastic shift in the record likely represents an increase in the soil carbonate accumulation rate, which must occur as the OCP increasingly impacts the environment with the age of the tree.

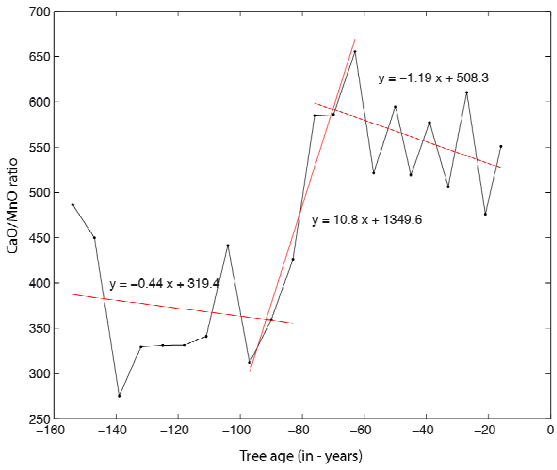

***Figure 25**: Scatterplot of the recorded CaO/MnO ratio (y axis) on a radial wood slab from the trunk of a 170 year-old iroko tree (Cameroon; time on x axis – negative as past). This ratio is used as a proxy of the soil pH evolution and change.*

4.1.4 Potential impact (including the socio-economic impact and the wider societal implications of the project)

4.1.4.1 Potential Impacts

The impact of CO2SOLSTOCK in terms of CO_2 sequestration is dependent on the various CO_2 emitter situations and position within the global carbon geo-cycle. Emission magnitude, capture and process, raw material supply, carbon and especially calcium, and end-product valorisation call for very different impact considerations:

- Emissions themselves have an extremely broad spectrum of magnitude and contexts
- Very high emitters call for reaction and end-product usage processes of considerable scales
- Calcium sourcing has a considerable impact, positive or negative, depending on its source
- Agro-forestry impacts should be viewed in a tropical livelihood and conservation context.

Apart from their CO_2 sequestration impact, the CO_2SolStock technologies might provide side benefits of social and economic added-value. When relevant, these aspects are detailed in this section within each CO_2SolStock opportunity, as well as under "Exploitation of results".

CO_2 magnitudes

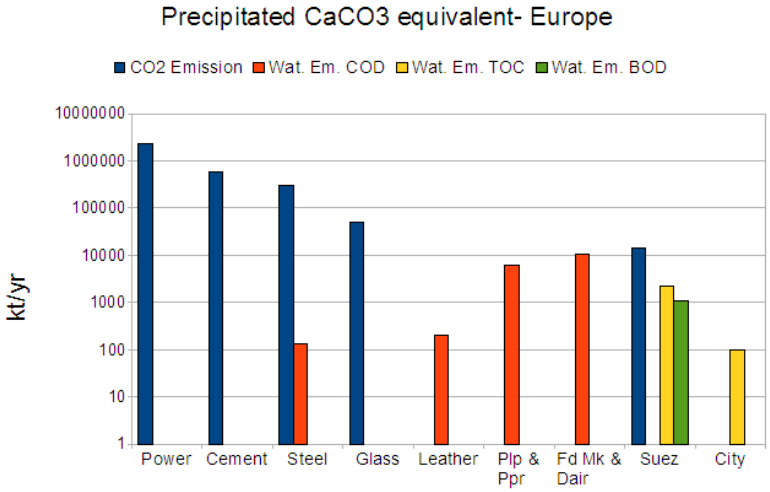

***Figure 26**: European industry CO_2 sequestration need, on the basis that zero emission is required, so as to re-absorb passed excessive emissions. In CO_2 sequestration, carbonate is the production. Industrial yardstick are measured more readily as "production" than as "raw material usage".*

Figure 26 has been obtained by compiling Best Available Techniques Reference Documents published by the European Commission:

- On large combustion plants (2006),
- Cement, lime and Magnesium Oxide Manufacturing Industries (2010),
- Production of iron and steel (2008),
- Glass Manufacturing Industry (2008),
- Tanning of Hides and Skins (2008),
- Pulp and Paper Industry, Food, drink and milk industries (2006).

In the case of water treatment, chemical oxygen demands estimated in typical cases have been transformed into equivalent CO_2, then into $CaCO_3$, assuming a 100% yield, while using typical plant throughput, multiplied by European production, as defined in said reports. From time to time, authors made extrapolations, because of scarcity of comparable data, but were satisfied with the orders of magnitudes. For more general water treatment, two specific examples were used:

- The Suez Environment Sustainable Development Report 2007/2008,
- A typical city of 1 million inhabitants that is assumed to consume 300 kt water/day, resulting in 100mg/l Total Organic Carbon (TOC).

High emitters

While CO_2 emissions are considerable in all circumstances, they are at least three orders of magnitude higher in the energy area (power generation, cement, steel and glass industries) than in the water treatment cases. In cooperation with one energy producer, BIOMIM has

calculated that *via* treatment of emissions by a CO_2SolStock technology, the daily emissions of a single 1 GW power station would produce 30 000 tons of calcium carbonate each day!

Disposal is the main impact bottleneck

Underground: Conventional CCS has the potential to achieve such large quantity disposal, for example in saline aquifers, where microbiological pathways (halophilic and ureolytic) are considered to provide added sealing capabilities (UEDIN), and where calcium ions are readily available. Once carbon is returned underground, in any form, it might be considered sequestered. Further treatment by microbiological or other form of precipitation provides stabilization. The added impact can be measured against the leakage risk, which is currently evaluated in the form of a fine amounting to 300 €/ton CO_2 leaked. In a market which has difficulty in establishing itself, it is not possible, at this stage, to further quantify the impact of such an option, which depends on the leakage risk which might ultimately be taken.

Polders, decantation: Another disposal of large amounts of carbonates, having been considered in case of successful high productivity, would be decantation fields which could lead to deposition of large quantities of carbonate slurries, with a positive impact of reclaiming land areas. Clean, compressed, carbonate would have land value. It would also have raw material value to the chemical and pulp and paper industry, for example, with low quarry cost (no crushing). Given reclaimable land, the impact is positive.

High throughput for impact

Anhydrase has been known to accelerate considerable CO_2 transfer and absorption. It has also been discovered that bacteria have anhydrase transfer capability. UGR has further discovered that this capability is extended to many species. Whether such impact has a chance to materialize can, initially, be scope-designed and simulated in a scrubber process. TUDelft has demonstrated its ability to do so, in the case of a sulphur scrubber. However, this ambitious project is out of CO_2SolStock, as being far too remote from any proof of concept.

Forestry opportunity *via* the oxalate-carbonate pathway

Global statistics on a given species, albeit commercial, is difficult to come-by, although some numbers can be found on the Status of Tropical Forest Management reports of the International Tropical Timber Organisation. For example, between three tropical countries, Cameroon, Central African Republic and Ivory Coast, about 200 000 m^3 of *Milicia excelsa* (the iroko tree) seem to be harvested every year. Iroko typically accounts for 5 to 10 % of tropical round wood production, and its value commands professional management. It grows in several different tropical forest ecologies, but thrives in traditional agroforestry systems, where its density stands around 1 to 4 stems per hectare. Extending the 200 000 m^3 to world tropical forests, and to similar species with the same calcification properties, might provide a ceiling of 5 M m^3. This is based on the assumption that irokos are harvested at maturity, allowing the carbonate slab to grow, and that they are replaced. Carbonate accumulation is of the same order of magnitude as the tree weight itself (Braissant et al., 2004).

Limiting the assumption to Africa and strictly iroko trees puts the ceiling around 1 M t/yr. This positive sink capability is powerfully coupled with soil amendment capability because of the alkalinity it induces, has good trading value and lends itself to agroforestry practices, which

provide low energy consuming sustainability and independence to populations. There are further carbon conservation credits, difficult to quantify, but very real in providing such support to populations.

4.1.4.2 Impact by sector

Figure 27 shows an overview of the projected carbon sequestration potential and investment risk of the investigated pathways, the basis of which is detailed below.

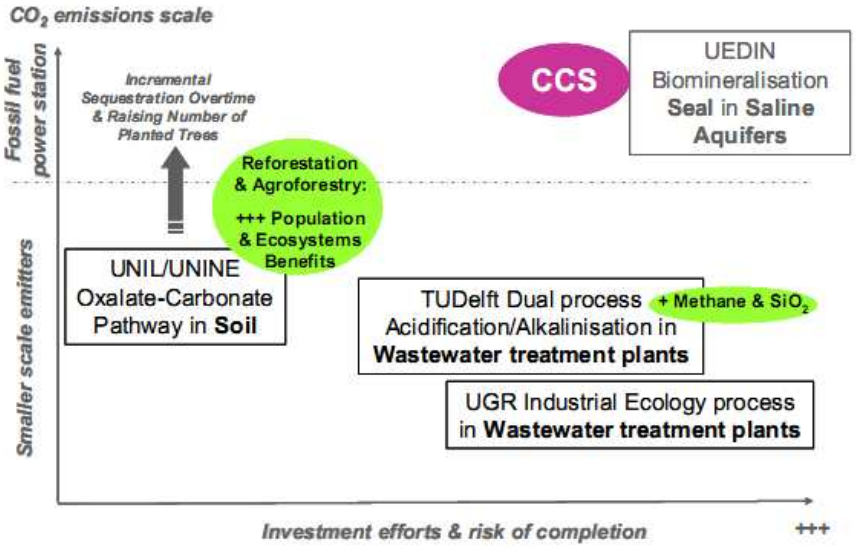

***Figure 27**: Impact overview of the carbon sequestration potential and investment risk of the investigated pathways.*

Forestation with oxalotrophic trees for log production

The reforestation case, with iroko and similar trees does not need any further proof of concept. The oxalate-carbonate pathway is fully demonstrated, and its extension to the total tropical habitat of the concerned trees has also been demonstrated. It falls within existing socio-forest ecosystems, which need exemplary extension as the next step. The raw material source is from the atmosphere for carbon, and from the soil gradients for calcium, where bedrock weathering might be the origin. This is a low investment, with a long term and sustainable payout.

The carbon sequestration potential of this case depends greatly on the tree species, the tree's age and the availability of calcium in the tree surroundings.

- In average up to 21 kg of CO_2 can be stored as $CaCO_3$ per tree and per year
- The deficit of CO_2 capture due to deforestation between the 60's and present day in African soils under iroko trees are estimated to be in the orders of magnitude of 10E5 to 10E7 t/year of CO_2. These figures do not take into account either the CO_2 trapped in the biomass of trees or the soil organic matter (SOM) incorporated inside the soil matrix.
- The OCP enhances soil fertility, which also contributes substantially to CO_2 capture by affecting the quality of the SOM and its residence time.

- An agroforestry project where 200 “OCP trees” per hectare are planted could bring up to 4.2 tons of CO_2 stored as $CaCO_3$ per hectare and per year, in addition to the carbon stored as biomass (plants, soil organisms, decaying organic matter).

More generally and when compared to “classical” reforestation schemes, the OCP trees have the big advantage of fixing part of their carbon as calcium carbonate: if dead trees eventually release CO_2 during their decay (also known as leakage in the REDD discussions), limestone is stable in dry soils for at least thousands of years.
While these CO_2 sequestration impacts are further encouragements to use such species for reforestation, the socio-ecosystem benefits extend value of this CO_2SolStock pathway well beyond. In a sustainable forestry management scheme, the trees command the highest value of the traded tropical log woods. In the case of the Irokos, it is exported worldwide, and used in Europe and elsewhere as a high quality “tek” substitute. The database of tropical belt OCP trees that was generated during CO_2SolStock will be of great value for future agroforestry projects and will positively impact on new afforestation campaigns in tropical areas by helping actors in their choice of tree species. Extending the project by involving reputable European tropical knowledge institutions will be of clear benefit for the North-South cooperation, i.e. CIRAD in Montpellier and CFF in Southampton.

Water treatment related opportunities, and their spin-offs

While carbon laden effluents are by no means as a considerable CO_2 emitter as energy and cement producers, they nevertheless constitute a worthwhile sizable sector for carbonate precipitation. For example, the pulp and paper European industry accounts for close to 10 M ton of carbonate equivalent emission treated in their water. Furthermore, carbonate is of high value to this industry, as an ingredient in the paper produced. Bacterial based water treatment is an established technology, and any investment required is within the realm of established expertise for this market; the investment and the pay-out are well within the original project criteria. Furthermore, CO_2SolStock partners have within their team world-class industrial application expertise in said area.

TU Delft dual process Industrial wastewater treatment

The dual acidification and methanation pathways can be applied in an industrial water treatment unit.

- 100% of the original calcium is demonstrated to convert into calcium carbonate.
- A typical modern pulp and paper plant with a production of 1000 t/day treats 20,000 m^3 water each day, with a COD of 30 kg/m^3. On the basis of the proof of concept so far reached, it would require 4.8% w/w silicate mineral to waste water, which could sequester 100t CO_2/day, e.g. 250t carbonate/day. Yields, achieved so far using wollastonite would result in a larger quantity with the use of olivine.
- Including the cost of silicate treatment, the sequestration cost is currently estimated at 30-50 €/ton CO_2.
- High quality biogas recovery will add the benefit of avoiding further fossil carbon extraction.

Impact might be significantly improved in situations where the industrial system in place provides both the source of calcium bearing mineral, and the carbon source from wastewater.

This is the case of slag producing industries, such as steel mills. The process would associate microbial slag treatment (acidification), with steel mills water treatment (alkalinization). COD of wastewater from coke oven plants is 2250-4450 mg/l, which amounts to 430-1700 g/t of coke. Depending on the steel manufacturing process, slag production ranges in the 10% over steel production. The world steel production is close to a billion tons. Slag contains plenty of calcium and is currently finding applications, including road and cement.

A spin-off in the field of landfill and dredged material stabilization: Landfills are known to mineralize with time in a two steps natural mechanism: an acidification phase and a methanogenic phase.

- The methanogenic phase leads to carbonatation; it can be accelerated, according to patent application EP2354237, proposed by BG as CO_2SolStock foreground.
- Applying TUDelft's multistage biological process (patent application NL2006819, CO_2SolStock foreground) can enhance and accelerate both acidification & methanogenesis mechanisms.
- Either of the two pathways analyzed by TUDelft (acidification/methanation and nitrification/denitrification) is applicable. Sequestration capacity is measured in terms of cementation fill.
- A 10 Mt/yr discharge case site would yield 0.5 to 1 Mt carbonate sequestration years earlier than in the natural process, with its associated benefit of earlier alternate use of land.

The same process might also be applicable in the maturation of dredged material, while sequestering CO_2. For example, the port of Rotterdam dredges 20 million cubic meters/year, yielding over 1 Mt/yr carbonate fixation, if applicable. In both cases, the proof of concept has still to be demonstrated, including a credible carbon source, and indicate an impact potential. CO_2SolStock partners are now seeking external collaborations to explore those avenues, which were put on hold during the CO_2 SolStock project duration.

A spin-off in the field of road construction and civil engineering: This technique, now being considered by TUDelft and BG, in conjunction with a major European road constructor, should be of reasonably easy proof-of-concept demonstration of the two-step process. A first step in a quarry, and a second in laying the highway base course. Currently, most major roads, and motorways use a sub-base, which is “mildly” treated with binders, usually Portland cement (3-5%), fly ash or other binding industrial residues in combination or not with cement. 1Kt/km constructed could use bacterial based cementation, instead of Portland cement. The European sequestration might be of the order of 1 Mt carbonate, at the current rate of road construction, plus the avoidance of the usage of cement. The carbon source considered might be industrial, agricultural and potentially ammonia containing waste such as manure, which will require a safe handling approach, as part of the needed proof of concept. This opportunity might also provide a European based export technology.

Industrial ecology at the University of Granada

An impact opportunity in associating desalination plants in cooperation with a water treatment industrialist has indicated a potential of 150 000t carbonate a year, for a very large desalination plant (as in the Aquasur project). Difficulty for achieving microbial-based precipitation with pure desalination brines having nevertheless being uncovered, further opportunities can be explored with brines mixed with regular wastewaters. In the latter case, precipitation occurs, as the potential for precipitation of calcium carbonate in terms of

bacterial strains was demonstrated in the lab, but the correct recipe has yet to be worked out and needs further experimentation. Issues remain around the stability of the chemical composition of the organic matter in wastewater and on the affordability of this carbon source. A proof of concept needs to demonstrate that impact potential.

Subsurface biomineralisation in CCS systems

Deep aquifer biomineralization opportunities are proven as potentially feasible & effective, as sealant of prospective CCS reservoirs. The impact of such a process is measured more by the effectiveness of diminishing leakage risk, than their actual sequestration capability:

- The CO_2 sequestration impact was assessed as potentially high as a form of stabilizing injected CO_2 (but no net CO_2 sequestration). In effect, once carbon is returned to the lithosphere, it is sequestered. Biomineralization fixes carbon in a stable form, and can thus be used to further seal CCS reservoirs, which may contain ample calcium cation as deep saline aquifers.
- This impact is dependent on carbon and urea availability.

Both halophilic and ureolytic pathways are of good potential, and the carbon source can be waste water, industrial or agricultural effluents. Under pressurized conditions occurring in CCS candidate saline aquifers, conversion into carbonate has been demonstrated at lab scale *via* the halophilic pathway. A proof of concept has been demonstrated to constrain effectiveness in a granular medium mimicking deep aquifer seems.

4.1.4.3 Dissemination activities

This chapter gives an overview on the major dissemination actions that have been performed, while the details of each action are given in WP8 deliverables.

CO2SolStock website

The CO_2SolStock website can be consulted at the following link: www.co2solstock.eu. Regular updates allowed us to disclose general information about the project, scientific publications within the consortium as well as dissemination actions on TV, radio and newspapers. Moreover, a twitter account (@CO2Solstock) was created to post interesting news related to climate change and carbon emissions mitigation. Moreover, this site was used as a collaborative platform by consortium members where presentations, minute documents can be uploaded and shared.

Logo

The CO_2SolStock logo was designed within the first months of the project and is now visible on the project's website as well as on all documents that have been produced by the consortium.

Publication of scientific results in scientific journals

Five publications were submitted, among which 4 to scientific journals and one (accepted) to an international congress. Moreover, two posters were presented during international scientific congresses, as well as one oral communication. The details are given in deliverable 8.5.

Press file

In addition to the two press releases, dissemination activities in various media were performed. Indeed, the project was advertised in 51 conference events for the general public and/or businesses, in 2 TV and 6 radio interventions, as well as in 17 web articles.

CO_2SolStock outreach conference

This event, which program is detailed in deliverable 8.6, was held on the 9th of March 2012, on the campus of Ecole Centrale Paris (ECP), a prestigious graduate and doctoral engineering Institution and Research Centre. Other venues had been previously considered. Indeed, the initial strategy sought to promote the project was to link the CO_2SolStock outreach conference to existing major sustainability events, in order to secure the presence and attendance of targeted stakeholders. Unfortunately, venues and initiatives such as the European Commission and the Climate KIC activity program, our first choices, could not be secured.

Participation to international congresses addressing climate change and carbon emissions

As displayed in paragraph 8.3, oral communications and posters were submitted and/or presented during major events focussing on sustainability challenges such as the 8th IWA Leading-Edge Conference on Water and Wastewater Technologies (2011, Amsterdam), the Goldschmidt 2011 conference (Prague), the Planet Under Pressure 2012 conference (London) and the Sustainability through Biomimicry conference to be held in Dammam on the 26th and 28th of November 2012.

Scientific training

This pedagogical event, held on the 10/02/2012 at the Brussels Natural Science Museum, was aimed at post-doctoral researchers and scientists willing to work and teach on the topic of CO_2 sequestration by microbial carbonatation. The material produced to that end is detailed in deliverable 8.7.

Expertise network

The CO_2SolStock consortium is the first European expertise network on CO_2 sequestration by microbial carbonatation. As the first dissemination activity, this team of experts focussed on the generation of a science education material that was used during the CO_2SolStock training day in Brussels. In parallel to the CO_2SolStock program, the network built up a Marie Curie ITN proposal consisting of the core research teams of the CO_2SolStock consortium with additional associated partners to address microbial carbonatation as a means to sequester pollutants, such as CO_2 and heavy metals.

Outreach synthesis report

Targeting international bodies and industries, this report summarises the achievements of the consortium and give hints at the development opportunities of the proposed CO_2SolStock technologies.

4.1.4.4 Exploitation of results

Compared to CCS, the bio-carbonation pathways represent a real paradigm shift, as:

- They could use all the industrial opportunities rejecting organic carbon as a source of "fixable" CO_2.
- They mimic the natural-geological CO_2 storage mechanism, thus increasing the natural CO_2 carbon fixation route as $CaCO_3$.
- They fix CO_2 as a stable solid, which can be either stored or could potentially be used as a building material under various forms.
- Hence storage sites do not need necessarily to be big, nor subterranean with cap rock sealing them.
- They can fix past emissions, as the organic carbon is from photosynthetic origin, hence originally pumped by plants from the atmosphere (instead of fixing future emissions like CCS).
- They make a moderate use of high temperature/high pressure chemistry, if at all
- In the case of ecosystem-management pathways (the oxalate-carbonate pathway), they generate multiple beneficial side-effects.
- In the case of integration in biogas systems the biogas will consist of a substantial increase in methane content and thereby need less or no upgrading to reach natural gas quality.
- They need an additional ingredient, the counter ion necessary to form carbonates:
    - calcium either as an industrial by-product, or weathered from silicate rocks.
    - other cations that could be turned into stable carbonates (especially Mg & Fe) but also undesired elements such as heavy metals, thus leading the way to new bio-assisted remediation techniques.

Because it can deal with CO_2 that could be out of reach of classical CCS (even the CO_2 in the atmosphere might be used), these differences allow the CO_2SolStock approach to be complementary to CCS.

Furthermore, once fossil fuels exploitation will stop (and CCS with it) the CO_2SolStock approach will remain both needed (to sequester the excess CO_2 from the atmosphere) & applicable.

Further exploitation to be developed:

- The TU-Delft Project approach (the wollastonite experience, patent Delft/CO_2SolStock) could be extended to (i) Quarries, civil engineering industries which may have suitable calcium silicate source, as their regular output, typically road construction industry (which could then make good use of the carbonates obtained such as biocementation, avoiding Portland cement), (ii) Steel, and more generally, alkaline slag by-product producing industry.
- The Granada approach (desalinization brines + wastewater) could benefit from (i) The acceleration of atmospheric CO_2 intake in the system via the carbonic anhydrase biocatalyst directly produced by living microbial populations, (ii) A collaboration with teams working on micro-algae that pump atmospheric CO_2 to generate more readily bio-available carbon source for calcifying microbial guilds
- The oxalate-carbonate pathway with trees, after its initial R&D effort on understanding the interaction between trees, fungi & bacteria and the calcium cycling needs to join forces with (i) World-class agro-forestry researcher teams (like the World Agroforestry

Centre in Nairobi or the CIRAD in Montpellier), to optimize the plants associations in terms of side-benefits (soil & biodiversity conservation, fertility, water regimes, etc), (ii) Local communities & field NGOs (like the ones of the IAFN network), to extend the search for other suitable sites, to select the companion crops, and to optimize the economical returns (as early as possible), (iii) International NGOs specialized in carbon markets and reforestation (see UN-REDD programme), to work on an official acceptance of carbonates as a carbon storage & to sell it to foundations searching for philanthropic innovating projects and big companies in need for carbon compensation. This network has already been built and is growing (Figure 28 and Figure 29).

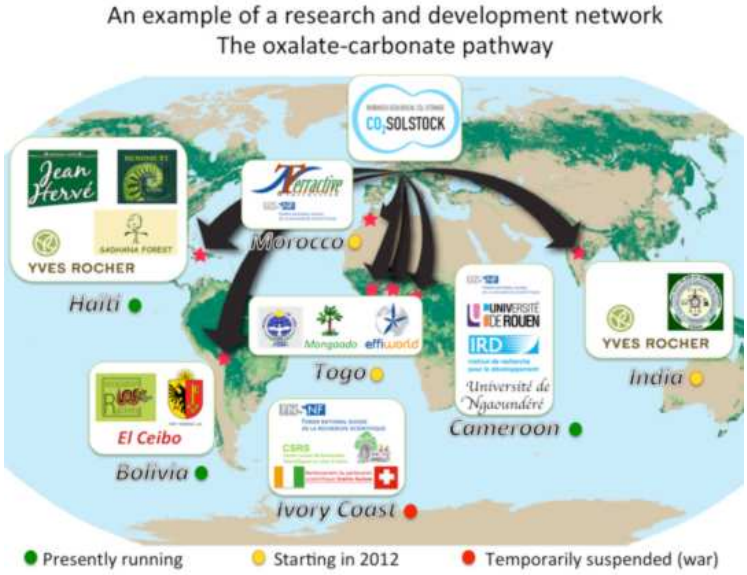

***Figure 28**: A network of NGOs, Foundations, and Universities has been built from CO_2SolStock in order to apply and develop the OCP in the tropical belt. Presently operating: in Haïti, Foundation Yves Rocher (France), Jean Hervé SA (France), Biomimicry Europa (Belgium), Sadhana Forest (USA); Bolivia, Racines (NGO, Switzerland), Canton de Genève (Switzerland), El Ceibo (cooperative, Bolivia); in Cameroon, SNF (Switzerland), Université de Rouen and IRD (France), Université de Ngaoundéré (Cameroon). Starting in 2012: Morocco, N'Terractive and SNF (Switzerland); India, Fondation Yves Rocher (France) and Pantnagar University (India); Togo, Effiworld (Switzerland), Mongaado (NGO, Togo), Université de Kara (Togo). To be restarted: Ivory Coast, SNF (Switzerland), CSRS (Ivory Coast), agreement for scientific cooperation (Ivory Coast – Switzerland).*

Figure 29*: Left: the kitchen and the dormitories for scientists, Sapecho, Bolivia (Racines and Canton de Genève). Right: oxalogenic tree nurseries in Haïti (Fondation Yves Rocher).*

The landfill process (patent BG/CO_2SolStock): this opportunity proposes to exploit industrial ecology principles to valorize industrial by-products and drive their maturation and stabilization at waste disposal sites towards carbonatation.

References

Anbazhagan, K. *et al*., (2007). Oxalotrophic *Paracoccus alcaliphilus* isolated from *Amorphophallus* sp rhizoplane. *World J. Microbiol. Biotechnol.,* **23**, 1529-1535.

Aragno, M. & Schlegel, H.G. (1991). The mesophilic hydrogenoxidizing (Knallgas) bacteria. In: *The prokaryotes a Handbook on Habitats, Isolation and Identification of Bacteria*. (Balows A, Trüper HG, Dworkin M, Harder W & Schleifer KH, eds.), 344 – 384. Springer-Verlag. Berlin Heidelberg New York.

Blackmore MA and J. R. Quayle, (1968). Choice between autotrophy and heterotrophy in *Pseudomonas oxalaticus*. Growth in mixed substrates. *Biochem J*., **107**, 705–713.

Braissant, O *et al.*, (2002). Is the contribution of bacteria to terrestrial carbon budget greatly underestimated? *Naturwissenschaften*, **89**, 366–370

Braissant O, *et al.,* (2004). Biologically induced mineralization in the tree *Milicia excelsa* (Moraceae): its causes and consequences to the environment. *Geobiol.,* **2**, 59–66.

Brantley, S.L*., et al.* (2003). Reaction kinetics of primary rock-forming minerals under ambient conditions. In *Treatise on Geochemistry*, **5**, 73-117, Pergamon.

Bruant, R.G., et al. (2002). Safe storage of CO_2 in deep saline aquifers. *Environ. Sci. Technol* **36**, 240A-245A.

Cailleau, Guillaume, (2005). Cycle du carbone et biominéralisation carbonatée en milieu continental: la diagenèse des phases oxalate-carbonate. Thèse de l'université de Neuchâtel.

Cailleau G., *et al* (2005). Biologically induced accumulations of $CaCO_3$ in orthox soils of Biga, Ivory Coast. *Catena*, **59**, 1–17.

Carozzi, A. V., (1967). Recent calcite-cemented sandstone generated by the equatorial tree iroko (Chlorophora excelsa), Daloa, Ivory Coast. *J. Sed. Petrol.*, **37**, 2, 597-600.

Chen J.S. & Liu C.W., (2004). Interaction of reactive fronts during transport in a homogeneous porous medium with initial small non-uniformity. *J. Contam. Hydrol.*, **72**, 47– 66

Clausen CA, et al., (2008). Oxalate analysis methodology for decayed wood. *Intern. Biodet. Biodeg.*, **62**, 372-375.

Dijkhuizen, L., et al., (1977). Energy production and growth of *Pseudomonas* oxalaticus OX1 on oxalate and formate. *Arch. Microbiol*. **115**, 229-236.

Dupraz, S. et al. (2009). Experimental approach of CO2 biomineralization in deep saline aquifers. *Chem. Geol.,* **265**, 54-62.

Dutton, M. & Evans, C. (1996). Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment. Canad. J. Microbiol., **42**:881-895.

FAO http://www.fao.org/docrep/n6845f/n6845f03.htm.

Ferris, F.G. et al. (2003). Kinetics of calcite precipitation induced by ureolytic bacteria at 10 to 20 ˚C in artificial groundwater. *Geochim. Cosmochim. Acta* **68**, 1701-1710.

Forster, A. et al. (2006). Baseline characterization of the CO2SINK geological storage site at Ketzin, Germany. *Environ. Geosci*. **13**, 145-161.

Fujita, Y. et al., (2008). Stimulation of microbial urea hydrolysis in groundwater to enhance calcite precipitation. *Environ. Sci. Technol.,* **42**, 3025-3032.

Hammes, F., et al., (2003). A novel approach to calcium removal from calcium-rich industrial wastewater. *Wat. Res.,* **37**, 699-704.

Haszeldine, R.S. (2009). Carbon Capture and Storage: How Green Can Black Be? *Science* **325**, 1647-1652.

Huijgen, W.J.J. & Comans, R.N.J. (2005). Carbon dioxide sequestration by mineral carbonation: Literature review update 2003-2004.

Intergovernmental Panel on Climate Change (IPCC, 2005). *IPCC Special Report on Carbon Dioxide Capture and Storage. Prepared by Working Group III of the Intergovernmental Panel on Climate Change*, Cambridge University Press.

Jayasuriya, G. (1955). The Isolation and Characteristics of an Oxalate-decomposing Organism. *J. Gen. Microbiol*., **12**, 419-428.

Khammar N. et al., (2009). Use of the *frc* gene as a molecular marker to characterize oxalate-oxidizing bacterial abundance and diversity structure in soil. *J. Microbiol. Methods* **76**, 120-127.

Koyama H, 1988. Purification and Characterization of Oxalate Oxidase from *Pseudomonas* sp. OX-53 (Biological Chemistry). *Agric. Biol. Chem*., **52**, 743-748.

Lackner, K.S*., et al.* (1995) Carbon dioxide disposal in carbonate minerals. *Energy* **20**, 1153-1170.

McKetta, J.J. (1999). *Encyclopedia of chemical processing and design*. CRC Press.

Mitchell, A.C. & Ferris, F.G. (2005). The coprecipitation of Sr into calcite precipitates induced by bacterial ureolysis in artificial groundwater: Temperature and kinetic dependence. *Geochim. Cosmochim. Acta* **69**, 4199-4210.

Mitchell, A. & Ferris, F. (2006). The Influence of Bacillus pasteurii on the nucleation and growth of calcium carbonate. *Geomicrobiol. J.,* **23**, 213-226.

Mitchell, A. et al. (2009). Biofilm enhanced geologic sequestration of supercritical CO2. *Internat. J. Greenhouse Gas Control* **3**, 90-99.

Mitchell, A.C., et al. (2010). Microbially Enhanced Carbon Capture and Storage by Mineral-Trapping and Solubility-Trapping. *Environ. Sci. Technol.,* **44**, 5270-5276.

Muyzer G. et al., (1993). Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.,* **59**, 695-700.

Nakata, P.A. (2003). Advances in our understanding of calcium oxalate crystal formation and function in plants. *Plant Sci*., **164**, 901-909.

Ortoleva P. *et al*., (1987). Geochemical self-organisation II: The reactive infiltration instability. *Am. J. Sci.* **287**, 1008-1040.

Parkhust, D.L. & Appelo, C.A.J. (1999). User's guide to PHREEQC (Version 2) – a computer program for speciation, batchreaction, one-dimensional transport, and inverse geochemical calculations. Water-Resources Investigations Report 99–4259, US Geological Survey, Denver, CO.

Pokrovsky, O.S*., et al.* (2009). Effect of organic ligands and heterotrophic bacteria on wollastonite dissolution kinetics. *Am. J. Sci.* **309**, 731-772.

Ramanan, R., et al., (2009). Bio-sequestration of carbon dioxide using carbonic anhydrase enzyme purified from *Citrobacter freundii*. *World Microbiol. Biotechnol.*, **25**: 981-987.

Rivadeneyra, M.A., et al. (2006). Precipitation of minerals by 22 species of moderately Halophilic bacteria in artificial marine salts media: Influence of salt concentration. *Folia Microbiologia* **51**, 445-453.

Robbel, L. & Kutzner, J.J. (1973). *Streptomyces* taxonomy: utilization of organic acids as an aid in the identification of species. *Naturwissenschaften*, **60**, 351–352.

Sánchez-Moral,L., et al., (2003). Bioinduced precipitation of calcium carbonate metastable phases in hypogean environments: a short review *Geomicrobiol. J*., **20**: 491-500.

Schilling, J. & Jellison, J. (2004). High performance liquid chromatographic analysis of soluble and total oxalate in Ca and Mg-amended liquid cultures of three wood decay fungi. *Holzforschung*, **58**, 682-687.

Sharma, A., et al., (2008). Characterization of carbonic anhydrase from diversified genus for biomimetic carbon-dioxide sequestration. *Indian J. Microbiol*. **48**, 365–371.

Sidhu, H. et al (1997). Identification and classification of *Oxalobacter formigenes* strains by using oligonucleotide probes and primers. *J. Clinic. Microbiol*., **35**, 350-353.

Tamer, A.U. et al (2002). Isolation and characterization of a new type of aerobic, oxalic acid utilizing bacteria, and proposal of *Oxalicibacterium flavum* gen. nov., sp. nov. Systematic and Appl. Microbiol., **4**, 513-519.

Tobler, D.J. et al. (2011). Comparison of rates of ureolysis between Sporosarcina pasteurii and an indigenous groundwater community under conditions required to precipitate large volumes of calcite: *Geochim. Cosmochim. Acta* **75**, 3290-3301.

Trinchant, J.C. & Rigaud, J. (1996). Bacteroid oxalate oxidase and soluble oxalate in nodules of faba beans (Vicia faba L.) submitted to water restricted conditions: possible involvement in nitrogen fixation. J. Exper. Bot., **47**, 1865-1870.

Tuason, M. & Arocena, J. (2009). Calcium Oxalate Biomineralization by *Piloderma fallax* in Response to Various Levels of Calcium and Phosphorus. *Appl. Environ. Microbiol*. **75**:7079-7085.

Ullman, W.J*., et al.* (1996). Laboratory evidence for microbially mediated silicate mineral dissolution in nature. *Chem. Geol.* **132**, 11-17.

Van Paassen, L.A. et al. (2009). Potential soil reinforcement by biological denitrification. *Ecologic. Eng*., **36**, 168-175.

Wei C. & Ortoleva P. (1990). Reaction front fingering in carbonate-cemented sandstones. *Earth*

Sci. Rev., **29**, 183-198.

Whiffin, V.S. et al., (2007). Microbial Carbonate Precipitation as a Soil Improvement Technique: *Geomicrobiol. J.,* **24**, 147- 423.

Wogelius, R.A. & Walther, J.V. (1991) Olivine dissolution at 25°C: Effects of pH, CO2, and organic acids. *Geochim. Cosmochim. Acta* **55**, 943-954.

Xu, T.F., et al. (2003). Reactive geochemical transport simulation to study mineral trapping for CO2 disposal in deep arenaceous formations", *J. Geophys. Res.*, **108**, B2, 2071.

Zhang, J. et al. (2006). Sterilization using high-pressure carbon dioxide. *J. Supercrit. Fluids* **38**, 354-372.

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