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# **Calcium isotope fractionation by intracellular amorphous calcium carbonate (ACC) forming cyanobacteria**

Neha Mehta\*<sup>1,2</sup>, Harold Bradbury<sup>3,4</sup>, Karim Benzerara<sup>1</sup>

<sup>1</sup>Sorbonne Université, Muséum National d'Histoire Naturelle, UMR CNRS 7590. Institut de Minéralogie, de Physique des Matériaux et de Cosmochimie (IMPMC), Paris, France

<sup>2</sup>Department of Geosciences, Environment and Society, Université libre de Bruxelles, Brussels, Belgium

<sup>3</sup>Department of Earth, Ocean and Atmospheric Sciences, University of British Columbia, 2020-2207 Main Mall, Vancouver, British Columbia, V6T 1Z4, Canada.

<sup>4</sup>Department of Earth Sciences, University of Cambridge, Cambridge, CB2 3EQ, UK

\*Corresponding author:

[neha.mehta@upmc.fr](mailto:neha.mehta@upmc.fr)

[hbradbury@eoas.ubc.ca](mailto:hbradbury@eoas.ubc.ca)

[karim.benzerara@sorbonne-universite.fr](mailto:karim.benzerara@sorbonne-universite.fr)

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## **Data availability statement**

The data reported in this manuscript are available in the supplementary information.

## **Abstract**

The formation of intracellular amorphous calcium carbonate (ACC) by various cyanobacteria is a widespread biomineralization process, yet its mechanism and importance in past and modern environments remain to be fully comprehended. This study explores whether calcium (Ca) isotope fractionation, linked to ACC-forming cyanobacteria, can serve as a reliable tracer for detecting these microorganisms in modern and ancient settings. Accordingly, we measured stable Ca isotope fractionation during Ca uptake by the intracellular ACC-forming cyanobacterium *Cyanothece* sp. PCC 7425. Our results show that *Cyanothece* sp. PCC 7425 cells are enriched in lighter Ca isotopes relative to the solution. This finding is consistent with the kinetic isotope effects observed in the Ca isotope fractionation during biogenic carbonate formation by marine calcifying organisms. The Ca isotope composition of *Cyanothece* sp. PCC 7425 was accurately modeled using a Rayleigh fractionation model, resulting in a Ca isotope fractionation factor ( $\Delta^{44}\text{Ca}$ ) equal to  $-0.72 \pm 0.05\%$ . Numerical modeling suggests that Ca uptake by these cyanobacteria is primarily unidirectional, with minimal back reaction observed over the duration of the experiment. Finally, we compared our  $\Delta^{44}\text{Ca}$  values with those of other biotic and abiotic carbonates, revealing similarities with organisms that form biogenic calcite. These similarities raise questions about the effectiveness of using the Ca isotope fractionation factor as a univocal tracer of ACC-forming cyanobacteria in the environment. We propose that the use of  $\Delta^{44}\text{Ca}$  in combination with other proposed tracers of ACC-forming cyanobacteria such as Ba and Sr isotope fractionation factors and/or elevated Ba/Ca and Sr/Ca ratios may provide a more reliable approach.

**Keyword:** cyanobacteria; biomineralization; amorphous carbonates; calcium isotope fractionation;

## **1. Introduction**

Cyanobacteria are phylogenetically and ecologically diverse photosynthetic bacteria, playing a pivotal role in the global cycling of elements such as carbon (C), calcium (Ca), nitrogen (N), and phosphorus (P) (Sánchez-Baracaldo *et al.*, 2022). Notably, their contribution to the global C cycle is of significant interest due to their capacity to sequester atmospheric carbon dioxide (CO<sub>2</sub>). In addition to the production of organic C, they also trigger a biomineralization process known as calcification resulting in the formation of calcium carbonate biominerals (CaCO<sub>3</sub>) (Riding, 2006). Cyanobacterial calcification is a significant process in the formation of carbonate sedimentary deposits, including stromatolites, with some of the oldest ones possibly formed by cyanobacteria dating back as far as 2.98 billion years (Golubic & Seong-Joo, 1999; Altermann *et al.*, 2006; Bosak *et al.*, 2013).

Traditionally, cyanobacterial calcification was considered as an extracellular process (Riding, 2000; Altermann *et al.*, 2006). However, this paradigm was challenged when several cyanobacterial strains were found capable of forming intracellular amorphous calcium carbonate (ACC) (Couradeau *et al.*, 2012; Benzerara *et al.*, 2014, 2022). The ACC-forming cyanobacteria are found in diverse environments, including soil, marine, freshwater, and brackish water (Ragon *et al.*, 2014; Benzerara *et al.*, 2022). Using comparative genomics, Benzerara *et al.* proposed that ACC formation in cyanobacteria is a genetically controlled process, involving a new gene family (Benzerara *et al.*, 2022). These strains exhibit the highest Ca demand among cyanobacteria, potentially driven by their intracellular ACC formation, suggesting a biological role for ACC, possibly as an intracellular pH buffer or a reservoir for inorganic carbon and calcium (De Wever *et al.*, 2019; Cosmidis & Benzerara, 2022).

The formation of intracellular ACC by cyanobacteria also holds biogeochemical implications. Among ACC-forming cyanobacteria, some can bloom and locally reach relatively high cell density, suggesting that they may produce significant amounts of ACC in these environments and perturb

C and Ca cycles, although this remains to be demonstrated (Gaëtan *et al.*, 2022). Moreover, the ACC inclusions formed by some cyanobacteria strains sequester high concentrations of alkaline earth elements such as Ba, Sr, and the radioactive  $^{90}\text{Sr}$  and  $^{226}\text{Ra}$  isotopes, which holds two implications: (1) these cyanobacteria may be overlooked actors in the biogeochemical cycles of these trace elements (Cam *et al.*, 2016; Blondeau *et al.*, 2018); (2) they may offer some potential for remediating pollution of these radioactive isotopes (Mehta *et al.*, 2019, 2022a).

The process of intracellular ACC formation has ancient origins, as ACC-forming cyanobacteria are deeply rooted in the cyanobacterial phylogenetic tree (Ponce-Toledo *et al.*, 2017; Benzerara *et al.*, 2022). Yet, their geological record remains unexplored, in part due to lack of reliable tracers of ACC-forming cyanobacteria. Stable isotope fractionation has emerged as one of the most valuable tools for this purpose (e.g. Johnston & Fischer, 2012). Specifically, the calcium isotope composition of geological archives has been widely utilized to identify and quantify different calcium pools in the environment. The Ca isotope compositions of marine calcifiers (e.g. corals, coccolithophores, foraminifera) have been extensively studied and debated owing to their potential relevance as proxy archives for constraining sea-surface temperature in which these organisms lived (e.g. (Böhm *et al.*, 2006; Inoue *et al.*, 2015; Mejía *et al.*, 2018; Roberts *et al.*, 2018)). By contrast, no studies have measured the Ca isotope composition of intracellular ACC forming cyanobacteria. Considering the significance of Ca in ACC-forming cyanobacteria, if the process of ACC formation is confirmed to be associated with a substantial Ca isotope fractionation, this could serve as a valuable diagnostic tool for assessing the presence of ACC-forming cyanobacteria in both modern and ancient environments. Additionally, the fractionation of Ca isotopes by ACC-forming cyanobacteria may provide a traceable record of how Ca moves from the surrounding extracellular solution to the interior of the cell. This, in turn, could offer valuable insights into the factors governing Ca regulation, uptake and storage. Accordingly, here, we experimentally

determine the stable Ca isotope fractionation occurring during Ca uptake by the intracellular ACC-forming cyanobacterium *Cyanothece* sp. PCC 7425.

## **2. Materials and methods**

**2.1 Growth of cyanobacteria cultures:** Axenic cultures of the intracellular ACC-forming cyanobacterium *Cyanothece* sp. PCC 7425 were incubated in the BG-11 growth medium in a batch reactor setup. We chose this strain for this study as it appears as a good model for ACC-forming cyanobacteria. Indeed, (1) it grows reproducibly, at a relatively high rate, (2) a significant set of information about this strain is available in the literature, including about its growth and Ca uptake under different conditions (e.g. Cam *et al.*, 2016, 2018), the identity of Ca channels and transporters found in its genome (De Wever *et al.*, 2019), and the characterization of the ACC granules formed by the strain and their relation with cell ultrastructure (e.g. Mehta *et al.*, 2022b, 2023b), and last (3) it is genetically tractable (e.g. Chenebault *et al.*, 2020). In the long-term compiling all these pieces of information on such a model strain may help developing an integrative view on ACC formation and its geochemical implications. The chemical composition of BG-11 is provided in Table S1. The initial concentration of dissolved Ca in the growth medium is 300  $\mu\text{M}$ . Temperature and luminosity were kept constant at 30°C and 30  $\mu\text{mol s}^{-1} \text{m}^{-2}$ , respectively. The reactors were agitated at 120 rpm in a rotating shaker. Evaporation was compensated daily by adding sterile de-ionized water prior to sampling. Triplicate incubations were performed for a total duration of 15 days. The growth of the cells was monitored by measuring the optical density at 730 nm ( $\text{OD}_{730\text{nm}}$ ) of the cell suspensions using a spectrophotometer. The pH of the cell suspension was measured over the growth of the cells. A control, non-inoculated experiment with the same initial conditions as in the cultures was also conducted. For measurements of dissolved Ca, 500  $\mu\text{l}$  of cell suspension were filtered on a 0.22  $\mu\text{m}$  polyvinylidene fluoride (PVDF) filter. The filtrate was subsequently

acidified with concentrated HNO<sub>3</sub> (70 wt%) and analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES).

**2.2 Calcium isotope measurements:** The calcium isotope compositions of Ca in both (i) the filtered solution, and (ii) the cell fraction, were measured. The cell fraction corresponds to intact cells containing ACC. At present, no protocol has been established to extract and preserve ACC outside of the cells. Therefore, here we used intact cells for the measurements. The solution fraction was collected by filtering the cell suspension. Aliquots of the samples containing 6 µg of Ca were initially acidified in conc. HNO<sub>3</sub> with 200 µl of ultrapure H<sub>2</sub>O<sub>2</sub> then refluxed at 80°C for ~6 hours to break down all organic material in the samples. There is no isotope fractionation occurring during these steps, as the protocol ensured that all the calcium was extracted from the cells and remained in the solution. This was formerly shown by mass balance calculations (Mehta *et al.*, 2023a). The samples were double spiked with equal amounts of <sup>42</sup>Ca and <sup>48</sup>Ca (a ratio of 1:1). Double spike containing 0.6 µg of calcium was added to each sample (a ratio of 1:10), before the spiked samples were dried to completion at 100 °C to ensure homogenization of the double spike and sample. The spiked samples were subsequently re-dissolved in 0.5% HNO<sub>3</sub>, and were run through a ThermoScientific Dionex 5000+ HPIC (High Performance Ion Chromatography) to separate the Ca following the methods of (Bradbury & Turchyn, 2018). The resulting Ca was converted to a nitrate form, dried to completion at 100°C, then re-dissolved in 1 µl of 2 M HNO<sub>3</sub> before being loaded on zone-refined rhenium filaments with phosphoric acid activator for analysis on a ThermoScientific Triton Plus TIMS. The samples were analyzed for their Ca isotope ratios following the method detailed in (Bradbury & Turchyn, 2018). The Ca isotope values are reported using the standard delta notation  $\delta^{44}\text{Ca} (\text{‰}) = ((^{44}\text{Ca}/^{40}\text{Ca})_{\text{sample}} / (^{44}\text{Ca}/^{40}\text{Ca})_{\text{standard}}) - 1) * 1000$ , where  $\delta^{44*}\text{Ca}_{\text{sol}}$  and  $\delta^{44*}\text{Ca}_{\text{bac}}$  are the relative changes in  $\delta^{44}\text{Ca}$  values from the initial isotope composition

of the media for the filtered solution and cell fraction respectively. The standard values over the course of the analysis for NIST 915A and 915B were  $-0.99\%$  ( $2\sigma = 0.09\%$ ,  $n = 8$ ) and  $-0.27\%$  ( $2\sigma = 0.05\%$ ,  $n = 7$ ), respectively, relative to bulk silicate Earth (BSE).

### 3. Results

#### 3.1. Uptake of Ca and growth of *Cyanothece* sp. PCC 7425

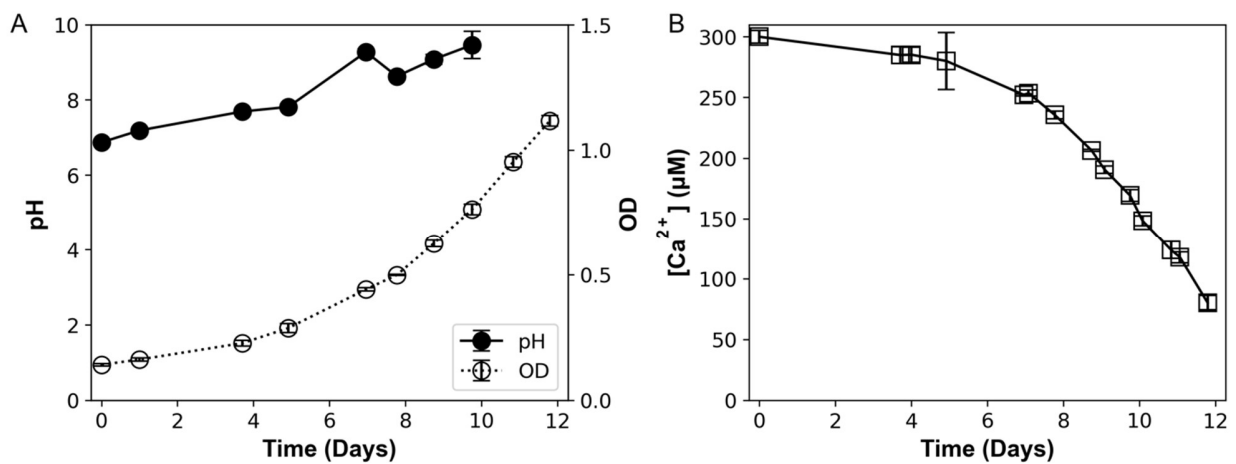


Figure 1: (A) The pH and OD of *Cyanothece* sp. PCC 7425 cultures over time. (B) Dissolved concentration of Ca in the cultures of *Cyanothece* sp. PCC 7425 cultures. The error bars indicate standard deviation of replicates. Error bars are smaller than the symbol when not visible. Numeric data corresponding to this dataset is available in the supplementary information (Table S2).

The  $OD_{730nm}$  of *Cyanothece* sp. PCC 7425 cell suspension increased continuously from 0.14 to 1.11 over 12 days (Figure 1A). The increase in  $OD_{730nm}$  was accompanied by an increase in pH from 6.8 to 9.5 and a decrease in the extracellular concentration of dissolved Ca from 300  $\mu M$  to 80  $\mu M$  (Figure 1B). By contrast, the concentration of Ca remained constant in the non-inoculated control (Figure S1). The extracellular solution remained undersaturated with respect to carbonate phases (i.e. calcite and aragonite) as shown in Table S3. The extracellular concentration of Mg in inoculated control remained relatively constant over time as shown in Figure S2. Intracellular concentration of Mg in *Cyanothece* sp. PCC 7425 is presently unknown. The time evolution of



OD, pH, and Ca concentration during the growth of *Cyanothece* sp. PCC 7425 cells were consistent with those observed by previous studies (e.g. Cam *et al.*, 2018).

### 3.2 Ca isotope composition and Ca fractionation factor

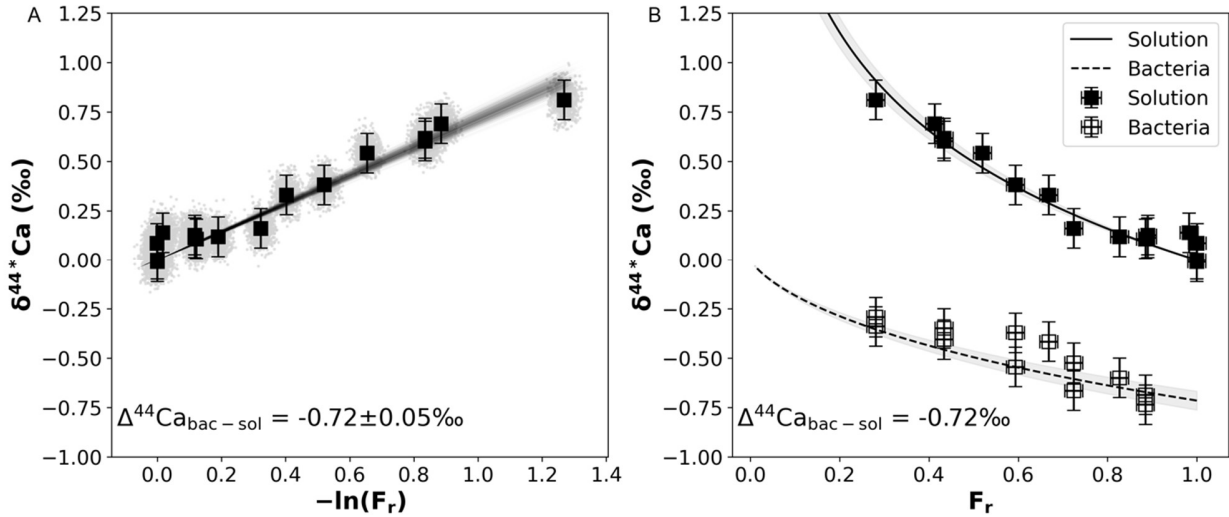


Figure 2: (Left) Ca isotope composition of the solution ( $\delta^{44*} \text{Ca}_{\text{sol}}$ ) plotted against  $-\ln(F_r)$ , where  $F_r$  is the fraction of Ca remaining in the solution. (Right) Ca isotope composition of the solution (solid square) and bacteria (empty square) plotted against the fraction of Ca remaining in the solution ( $F_r$ ). Error bars correspond to the analytical 2 SD. The solid and dashed lines represent Rayleigh fits to  $\delta^{44*} \text{Ca}$  values of the solution and bacteria.

With the removal of dissolved Ca, the Ca isotope composition of the solution ( $\delta^{44*} \text{Ca}_{\text{sol}}$ ) increased by 0.75‰ (Figure 2). Throughout the duration of the experiments, the cells were enriched in  $^{40}\text{Ca}$  relative to the remaining solution (Figure 2). To quantitatively assess the observed Ca isotope fractionation patterns during its uptake by *Cyanothece* sp. PCC 7425, the isotope data were modeled using a Rayleigh distillation equation (Equation 1).

$$R_t = R_0 F_r^{(\alpha-1)} \quad \text{eq (1)}$$

where  $R_t$  represents the ratio  $^{44}\text{Ca}/^{40}\text{Ca}$  at time  $t$ ,  $R_0$  represents the initial  $^{44}\text{Ca}/^{40}\text{Ca}$  ratio,  $F_r$  is the fraction of the initial calcium remaining in the system and  $\alpha$  is the fractionation factor.

Equation 1 can be recast into equation 2 by utilizing  $\delta^{44}\text{Ca}$  values (instead of  $^{44}\text{Ca}/^{40}\text{Ca}$  ratios) where the slope of the line between the calcium isotopic composition of the remaining medium

( $\delta^{44}\text{Ca}_{\text{sol}}$ ) and  $-\ln(F_r)$  is equal to the calcium isotopic fractionation factor between the cells and solution ( $\Delta^{44}\text{Ca}_{\text{bac-sol}}$ ) (Equation 2) (Mariotti *et al.*, 1981; Bradbury *et al.*, 2020).

$$\delta^{44}\text{Ca}_{\text{sol}} = \delta^{44}\text{Ca}_{\text{sol0}} + \Delta^{44}\text{Ca}_{(\text{bac-sol})} \times -\ln(F_r) \quad \text{eq (2)}$$

Using this approach, the Ca isotope fractionation factor between the cyanobacteria cells and the solution ( $\Delta^{44}\text{Ca}_{\text{bac-sol}}$ ) was estimated at  $-0.72 \pm 0.05\text{‰}$ . This visualization approach allows for easy assessment of the error associated with calculating the isotope fractionation using a Monte Carlo simulation (Bradbury *et al.*, 2020). A Monte Carlo simulation was conducted based on 1000 simulations using normal distributions based around the measured data points with a standard deviation of  $0.05\text{‰}$  for  $\delta^{44}\text{Ca}$  and 2% for Ca concentrations (Figure 2A). The calculated  $\Delta^{44}\text{Ca}_{(\text{bac-sol})}$  of  $-0.72 \pm 0.05\text{‰}$  is also displayed on a traditional Rayleigh distillation plot in Figure 2B, with the shaded regions representing the error on the isotope fractionation value calculated using the Monte Carlo simulation.

### 3. Discussion

#### 3.1. Calcium isotope composition of *Cyanothece* sp. PCC 7425

At the end of the experiment, 73% of the total calcium (Ca) was removed from the solution (Figure 1). As shown in previous studies, this removal is due to the Ca uptake by *Cyanothece* sp. PCC 7425 cells (Cam *et al.*, 2018; De Wever *et al.*, 2019). Moreover, electron microscopy and Ca K-edge XANES analyses revealed that ACC inclusions represent 84% of the total Ca in *Cyanothece* sp. PCC 7425 cells (De Wever *et al.*, 2019; Mehta *et al.*, 2023b).

The origin of Ca fractionation as observed from dissolved Ca measurements can be discussed. First, the role of evaporation in Ca isotope fractionation was null since no Ca goes to the vapor fraction and we compensated for evaporation by daily addition of (Ca-free) de-ionized water prior to

sampling. Second, while the formation of Ca-bearing extracellular phases could alter the dissolved Ca isotope composition, the experimental conditions did not thermodynamically allow such a precipitation, as shown by the undersaturation of the extracellular solution with respect to carbonate phases (Table S3). This is also consistent with several previous studies using electron microscopy and spectroscopy on the same strain cultured under similar conditions showing the absence of extracellular carbonate phases (e.g. De Wever *et al.*, 2019; Mehta *et al.*, 2022b, 2023b; Benzerara *et al.*, 2023). Overall, the enrichment of the solution in  $^{44}\text{Ca}$  relative to  $^{40}\text{Ca}$  is primarily attributed to the sequestration of Ca by *Cyanothece* sp. PCC 7425 with a preferential uptake of the lighter Ca isotope.

Enrichment of *Cyanothece* sp. PCC 7425 in  $^{40}\text{Ca}$  over  $^{44}\text{Ca}$  relative to the solution aligns with the kinetic isotope effects observed in Ca isotope fractionation during biogenic carbonate formation by marine calcifiers, where lighter isotopes are enriched in the precipitated biomineral (e.g. Böhm *et al.*, 2006; Gussone *et al.*, 2007; Kisakürek *et al.*, 2011; Inoue *et al.*, 2015). Among marine calcifiers, Ca isotope fractionation primarily occurs during cellular transport of Ca, possibly by dehydration of the Ca-aquocomplex at the surface of the channel or transporter, followed by a complete utilization of transported Ca for biomineral precipitation (Gussone *et al.*, 2006; Inoue *et al.*, 2015). The light isotope enrichment in biominerals arises from the differences in the dehydration reaction rates between light and heavy isotopes: lighter isotopes dehydrate more rapidly at the surface of the channel or transporter (DePaolo, 2011; Stevenson *et al.*, 2014). Previously, we measured Ba and Sr isotope fractionation during their uptake by another ACC-forming cyanobacterium *Gloeomargarita lithophora* (Mehta *et al.*, 2023a). Similar to Ca, the cells were enriched in lighter isotopes of Ba and Sr. However, the Ba and Sr isotope measurements of *G. lithophora* showed deviations from the Rayleigh distillation curve when the fraction of Ba and

Sr remaining in the solution became very small. These deviations were attributed to a back reaction of Ba and Sr between the cells and the solution, which eventually led to an isotopic equilibrium between the cells and extracellular solution, resulting in no apparent fractionation after some time. This back reaction was hypothesized to be induced by cellular stress resulting from the accumulation of high amounts of Ba and Sr, that led to the dissolution of Ba- and Sr-enriched ACC and the subsequent release of trapped light isotopes of Ba and Sr to the extracellular solution.

Unlike Ba and Sr, the kinetic isotope effect on Ca isotope fractionation by *Cyanothece* sp. PCC 7425 was not overridden by isotope equilibrium effects. To further test the presence/absence of a back reaction in the case of Ca uptake, we modeled the Ca isotope composition data using the Rayleigh model that incorporates the back reaction, as used in our previous study (Mehta *et al.*, 2023a).

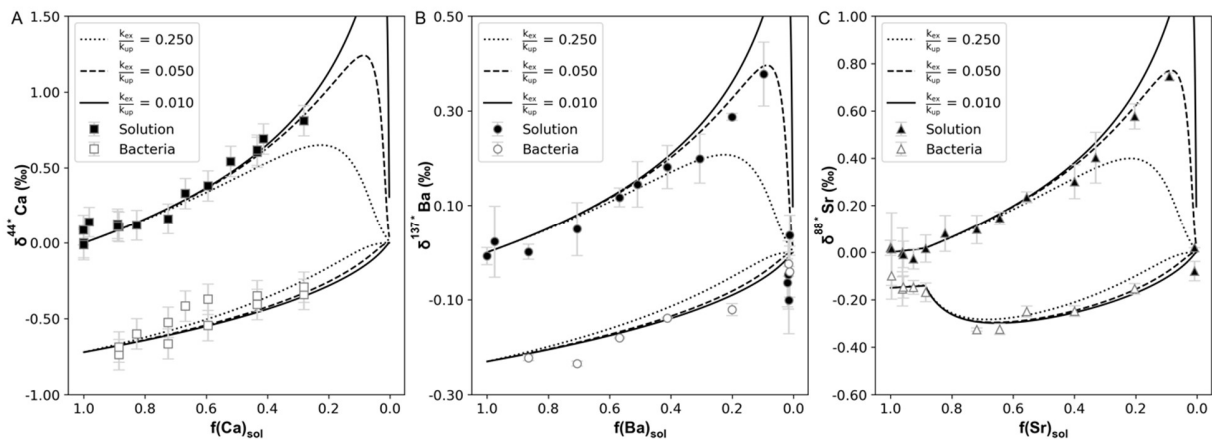


Figure 3: Visualization of the evolution of solution (filled markers) and cell fraction (empty markers) isotope values relative to the fraction of the element of interest remaining in the media for Ca (A: this study), Ba (B: Mehta *et al.*, 2023a), and Sr (C: Mehta *et al.*, 2023a). The black lines display the results of the uptake-exchange model utilized in the (Mehta *et al.*, 2023a) study with varying ratios (0.25—dotted, 0.05—dashed, 0.01—solid) for the rate constants of exchange ( $k_{ex}$ ) versus the rate constant for uptake ( $k_{up}$ ).

Briefly this approach models the rate of Ca uptake into the cell versus the rate of exchange of Ca from the cell. The uptake process corresponds to the incorporation of Ca from the solution into the

bacteria and is associated with a certain Ca isotope fractionation factor. The exchange process contains two equal reactions (which have an identical rate): (a) a release reaction of Ca from the bacteria to the solution (with no isotopic fractionation) and, (b) re-uptake reaction of Ca (also with no isotopic fractionation) from the solution. The uptake reaction impacts both the isotopic composition and extracellular concentration of Ca, while the exchange reaction only impacts the isotope composition of Ca (but not the concentration of Ca as the release and re-uptake reactions balance each other). There is no isotope fractionation of Ca within the exchange term. Both the uptake and exchange reactions are modelled using first order rate laws based on the concentration of calcium in the solution (for uptake) or cell (for exchange). For a full description of the model approach, see (Mehta *et al.*, 2023a).

Figure 3 shows the evolution of the Ca (this study, A) and Ba and Sr (from previous study; Mehta *et al.*, 2023a) isotope compositions of the solution and cells as a function of the fraction of remaining element in the solution using various, yet constant-with-time ratios of uptake rate constant ( $k_{up}$ ) over exchange rate constant ( $k_{ex}$ ). The model with  $k_{ex}/k_{up} = 0.05$  provided a reasonable fit between the data and model for both Sr and Ba in our previous study (Mehta *et al.*, 2023a). The fitting of the Ca isotope data with the same ratio  $k_{ex}/k_{up}=0.050$  also agreed with the data. However, we note that, in the case of Ca, we cannot decipher if this ratio is indeed 0.05 or is lower as the variation between these different cases would only appear for  $f(Ca)_{sol} < 0.26$ , while  $f(Ca)_{sol}$  remained greater than 0.26 in the present experiments. Overall, the kinetic isotope effects on Ca isotope fractionation by *Cyanothece* sp. PCC 7425 was well modeled based on the assumption of a Ca uptake that was mostly unidirectional, i.e. with no or very little (<5%) back reaction of Ca isotopes from the cells to the solution phase over the course of the experiments.

Whether such a unidirectional Ca isotope flux would be observed when *Cyanothece* sp. PCC 7425 is cultured in presence of Ba, Sr and Ca remains an intriguing topic for future work.

Unlike Ca, dissolved Mg concentrations remained constant over the time of the incubation, which shows that in our experiment, Mg incorporation in the cell was negligible (Figure S2). Moreover, this is supported by previous observations showing that ACC in *Cyanothece* PCC 7425 contains less than 4 % of Mg (defined as the  $\text{Mg}/(\text{Mg}+\text{Ca})$  molar ratio in ACC inclusion) (Cam *et al.*, 2018; Mehta *et al.*, 2023b). This sharply contrasts with several stable biogenic ACC in eukaryotes that contain 20-40 mol % Mg in ACC, and where Mg incorporation promotes stabilization of amorphous carbonate (Levi-Kalishman *et al.*, 2002). It also contrasts with the case of abiotic precipitation experiments, where in a solution with a high Mg:Ca ratio as, e.g., in the growth medium of *Cyanothece* PCC 7425, amorphous calcium magnesium carbonate should precipitate (Purgstaller *et al.*, 2021). This is not observed within cyanobacteria, likely due to some sort of chemical fractionation occurring between the extracellular solution and the site of ACC formation within the cells. Overall, this suggests that intracellular ACC formed in *Cyanothece* PCC 7425 are not similar to those formed abiotically under similar conditions if one considers extracellular solutions. What factors control the Ca:Mg ratio within cyanobacterial ACC should be investigated by future studies.

### 3.2 Comparison between the Ca fractionation factor associated with *Cyanothece* sp. PCC 7425 and that of other biotic and abiotic carbonates

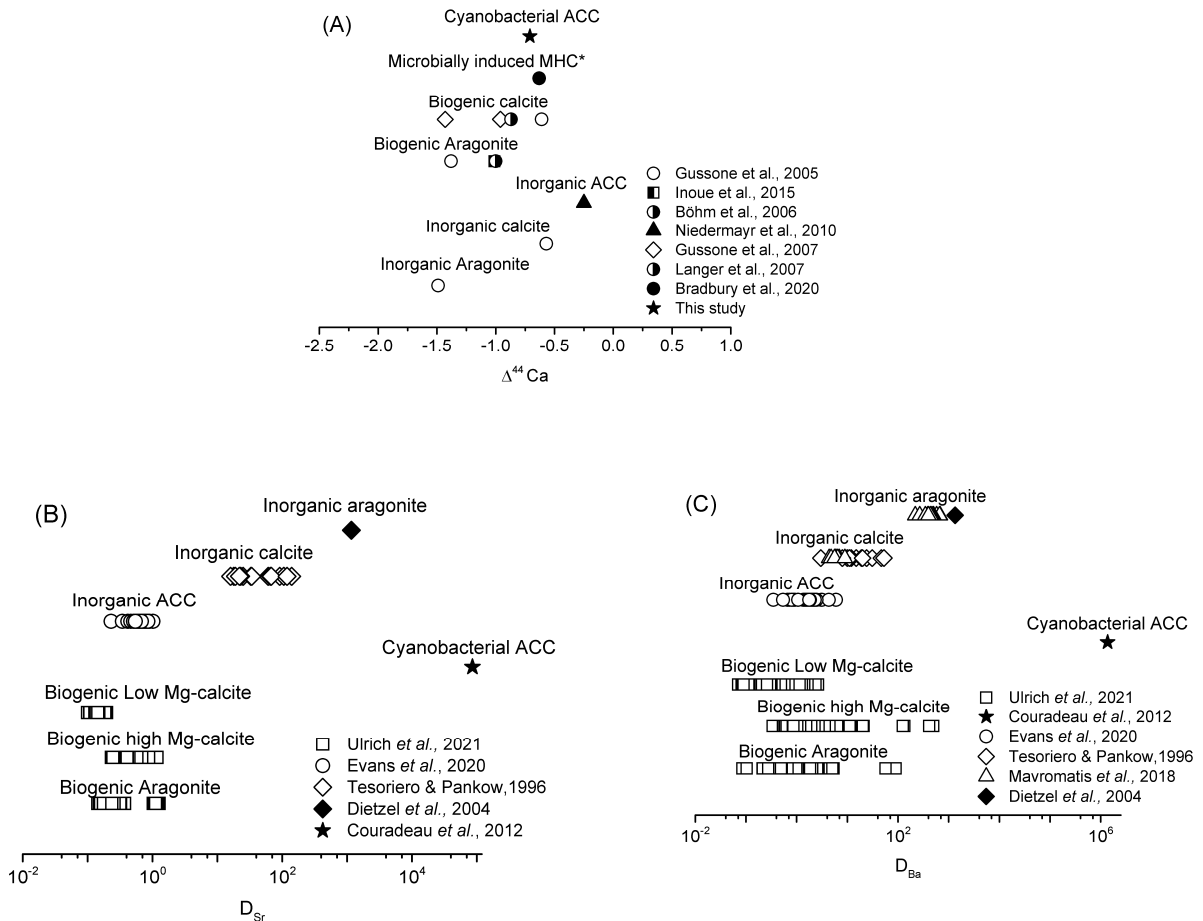


Figure 4: (A) Representative compilation of the  $\Delta^{44}\text{Ca}$  of relevant biogenic and abiotic carbonates. The references for the published values reported in the figure are listed in the legend. Studies reporting the temperature dependency of Ca isotope fractionation were included with the exception for inorganic ACC. For inorganic ACC, the temperature was not specified in the study. All the  $\Delta^{44}\text{Ca}$  values are extrapolated to 30°C. \*MHC stands for Monohydrocalcite. The numeric values are listed in Table S4. (B) Partitioning coefficient of Sr ( $D_{\text{Sr}} = [\text{Sr}/\text{Ca}]_{\text{carbonate}}/[\text{Sr}/\text{Ca}]_{\text{solution}}$ ) in biogenic and abiotic carbonates. (C) Partitioning coefficient of Ba ( $D_{\text{Ba}} = [\text{Ba}/\text{Ca}]_{\text{carbonate}}/[\text{Ba}/\text{Ca}]_{\text{solution}}$ ) in biogenic and abiotic carbonates. The  $D_{\text{Ba}}$  and  $D_{\text{Sr}}$  values are reported at 22 °C for (Evans *et al.*, 2020), 25 °C for (Tesoriero & Pankow, 1996; Mavromatis *et al.*, 2018; Ulrich *et al.*, 2021), and 30 °C for (Dietzel *et al.*, 2004; Couradeau *et al.*, 2012). The data on  $D_{\text{Ba}}$  and  $D_{\text{Sr}}$  is not exhaustive but representative of the reported ranges in the literature.

Figure 4A summarizes the  $\Delta^{44}\text{Ca}$  for relevant biogenic carbonates and abiotic carbonates, recalculated at 30 °C using the relationship between Ca isotope fractionation and temperature

reported for biogenic carbonates and abiotic carbonates, unless noted otherwise (Gussone *et al.*, 2005, 2007; Böhm *et al.*, 2006; Langer *et al.*, 2007; Niedermayr *et al.*, 2010; Inoue *et al.*, 2015; Bradbury *et al.*, 2020). While the Ca isotope fractionation factor reported for *Cyanothece* sp. PCC 7425 is distinctly different from those reported for biogenic aragonite, it bears resemblance with those reported for biogenic calcite. These differences can be interpreted in different ways as described below. The role of CaCO<sub>3</sub> polymorph has been shown to impact  $\Delta^{44}\text{Ca}$  in abiotic systems, but such impacts may not be as pronounced in biotic systems (e.g. Gussone *et al.*, 2020). Instead, it has been suggested that the dominant source of fractionation in biogenic systems occurs during the cellular uptake of Ca as it is assumed that all the Ca entering the cell is quantitatively precipitated to carbonate mineral, and thus there is no Ca isotope fractionation during precipitation (Gussone *et al.*, 2006; Inoue *et al.*, 2015). In this model, the type of polymorph precipitated by the organism has negligible impact on Ca isotope fractionation provided that Ca cellular uptake pathways remains same between the organisms (Gussone *et al.*, 2006; Inoue *et al.*, 2015). Thus resemblance between organisms forming biogenic calcite formation and *Cyanothece* sp. PCC 7425 and other organisms that form calcite, may suggest similarity in Ca cellular uptake pathways. Alternatively, this similarity should be inferred carefully as the uncertainty on the  $\Delta^{44}\text{Ca}$  values reported in our study is very small in comparison to that reported in other studies (Table S4). Moreover, we do not know how the Ca isotope fractionation varies across different ACC-forming cyanobacteria and the impact of rate of ACC precipitation. At present we do not know the relationship between intracellular precipitation rates and Ca uptake rates. Different strains of ACC-forming cyanobacteria may precipitate ACC at varying rates due to differences in extracellular chemistry and intracellular Ca regulation mechanisms but at this point, this idea is purely speculative. While the precipitation rate dependence of  $\Delta^{44}\text{Ca}$  of abiotic and biotic calcium carbonate minerals is well documented (Gussone *et al.*, 2020 and references therein), similar



relationships are undocumented in the case of ACC-forming cyanobacteria. Further studies are needed to investigate how the rate of ACC precipitation compares between different strains and its relationship to Ca isotope fractionation associated with ACC forming cyanobacteria.

One motivating question for this study was to test whether the Ca isotope fractionation factor associated with ACC-forming cyanobacteria may serve as a tracer of these microorganisms in the modern and past environments. The similarity between the Ca isotope fractionation produced by ACC-forming cyanobacteria and that of the precipitation of biogenic calcite makes the application of this approach challenging. However, in the future, the use of  $\Delta^{44}\text{Ca}$  in combination with other proposed tracers of ACC-forming cyanobacteria may provide a more reliable approach (Cam *et al.*, 2016; Mehta *et al.*, 2023a). For example, as shown in Figure 4A-C, comparing  $\Delta^{44}\text{Ca}$  and distribution co-efficient of Ba ( $D_{\text{Ba}}$ ) and Sr ( $D_{\text{Sr}}$ ) between different biogenic carbonates, abiotic carbonates, and ACC forming cyanobacteria, it becomes clear that the  $\Delta^{44}\text{Ca}+D_{\text{Ba}}+D_{\text{Sr}}$  of ACC forming cyanobacteria creates a unique fingerprint of ACC forming cyanobacteria, that may be used as a tracer to search for evidence of ACC forming cyanobacteria in the geologic rock record.

#### **4. Conclusions**

This study measured Ca isotope fractionation during Ca uptake by the ACC-forming cyanobacterium *Cyanothece* PCC 7425. The light isotope enrichment of Ca in *Cyanothece* results in a  $\Delta^{44}\text{Ca}$  equal to  $-0.72\%$ . Monitoring the Ca isotope composition of *Cyanothece* sp. PCC 7425 cells reveals that the Ca uptake by *Cyanothece* sp. PCC 7425 is mostly a unidirectional process. These findings could offer valuable insights into how Ca is cycled intracellularly in ACC forming cyanobacteria. While Ca is a biologically essential elements, high intracellular concentration of Ca are known to have toxic effects on the cellular functions (Barrán-Berdón *et al.*, 2011). As a consequence, intracellular Ca concentration is tightly regulated within a range of  $\sim 100\text{nM}$  in the

cells (Dominguez, 2004; Clapham, 2007). Yet, in ACC forming cyanobacteria, if Ca stored in ACC inclusions was virtually dispersed in the cells, this would represent a total Ca cytoplasmic concentration of  $\sim 0.8\text{--}1.2 \text{ mol}\cdot\text{L}^{-1}$  (Li *et al.*, 2016). The variations in Ca isotope compositions of the ACC forming cyanobacteria cells during the formation of ACC may provide a traceable record of how Ca moves from the surrounding extracellular solution to the specific site where ACC precipitates, and therefore shedding light into the factors governing Ca regulation, uptake and storage.

Numerical modelling suggested that any back reaction that was occurring between the cells and solution had little to no impact on the observed Ca isotope values as the amount of Ca in the solution remained above 0.26. The Ca isotope fractionation factor of *Cyanothece* sp. PCC 7425 is different from some organisms forming biogenic aragonite but has some similarities with several organisms forming biogenic calcite. Our results suggest that Ca isotope offsets associated with ACC formation by cyanobacteria may not be unambiguous tracers of ACC-forming cyanobacteria in past and modern environments and that the combination of this proxy together with additional ones such as Sr/Ca, Ba/Ca and/or Ba and Sr isotopic compositions would be needed to recognize former cyanobacterial ACC in the geological record.

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### Data availability statement

The data reported in this manuscript are available in the supplementary information.

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## Supplementary Information

### Calcium isotope fractionation by intracellular amorphous calcium carbonate (ACC) forming cyanobacteria

Table S1: Chemical composition of the initial BG-11 growth medium

Parameter	Value	Unit
pH	7.8	unit of pH
[NaHCO <sub>3</sub> ]	0.48	mM
[Ca <sup>2+</sup> ]	0.3	mM
[Na <sup>+</sup> ]	18.1	mM
[Mg <sup>2+</sup> ]	0.302	mM
[K <sup>+</sup> ]	0.365	mM
[NO <sub>3</sub> <sup>-</sup> ]	17.7	mM
[Cl <sup>-</sup> ]	1.5	mM
[CO <sub>3</sub> <sup>-</sup> ]	0.380	mM
[SO <sub>4</sub> <sup>2-</sup> ]	0.301	mM
[PO <sub>4</sub> <sup>3-</sup> ]	0.180	mM
[NH <sub>4</sub> <sup>+</sup> ]	90.0	μM
[Fe <sup>3+</sup> ]	30.0	μM
[Mn <sup>2+</sup> ]	9.1	μM
[Cu <sup>2+</sup> ]	0.3	μM
[Zn <sup>2+</sup> ]	0.8	μM
[Co <sup>2+</sup> ]	0.2	μM
[BO <sub>3</sub> <sup>-</sup> ]	46.3	μM
[MoO <sub>4</sub> <sup>4-</sup> ]	1.6	μM

Table S2: Numeric data on time evolution of OD, pH, extracellular concentration of Ca and Mg, and Ca isotope composition of the filtrate and cell fraction in inoculated controls. This data corresponds to figure 1-2 in the main text and Figure S2 is SI .

Days	OD	pH <sup>[1]</sup>	Ca (μM) <sup>[2]</sup>	Mg (uM) <sup>[3]</sup>	Solution*			Bacteria*		
					δ <sup>44</sup> Ca <sub>BSE</sub>	δ <sup>44</sup> Ca <sub>915A</sub>	δ <sup>44</sup> *Ca	δ <sup>44</sup> Ca <sub>BSE</sub>	δ <sup>44</sup> Ca <sub>915A</sub>	δ <sup>44</sup> *Ca
0.0	0.141	6.87	300	302.00	-0.01	0.9782	0.0000	-	-	-
1.0	0.162	7.19	-	-	-	1.0756	0.0974	-	-	-
3.7	0.227	7.69	284.94	329.51	0.1390	1.1290	0.1508	-	-	-
4.0	-	-	285.21	329.15	0.1073	1.0973	0.1191	-0.7360	0.2540	-0.7242
4.9	0.287	7.81	280.06	336.65	0.1267	1.1167	0.1385	-0.6864	0.3036	-0.6746
7.0	0.441	9.27	252.12	323.74	0.1133	1.1033	0.1251	-0.6005	0.3895	-0.5886
7.1	-	-	253.57	322.98	0.1184	1.1084	0.1302	-0.6656	0.3244	-0.6538
7.8	0.498	8.63	235.62	324.73	0.1607	1.1507	0.1725	-0.5245	0.4655	-0.5127
8.7	0.624	9.08	206.35	314.22	0.3296	1.3196	0.3414	-0.4170	0.5730	-0.4052
9.1	-	-	190.51	313.05	0.3805	1.3705	0.3923	-0.5453	0.4447	-0.5335
9.8	0.762	9.46	169.24	313.49	0.5413	1.5313	0.5532	-0.3724	0.6176	-0.3606
10.1	-	-	148.13	301.66	0.6026	1.5926	0.6144	-0.3495	0.6405	-0.3377
10.8	0.953	-	123.6	323.81	0.6167	1.6067	0.6286	-0.4066	0.5834	-0.3948
11.1	-	-	117.61	325.97	0.6903	1.6803	0.7021	-0.3403	0.6497	-0.3285
11.8	1.116	-	80.13	318.15	0.8102	1.8002	0.8220	-0.2927	0.6973	-0.2809

[1] standard deviation less than 4 %

[2] standard deviation less than 8 %

[3] standard deviation less than 6%

\* measurement error is 2 SD



1 Table S3: Saturation Index of possible carbonate minerals that may form in extracellular solution.  
 2 The calculations are done using Visual Minteq, using the measured the time evolution dataset on  
 3 pH, [Ca<sup>2+</sup>], [Mg<sup>2+</sup>], with system open to atmosphere at 30 °C.

pH	Saturation Index	
	Calcite	Aragonite
6.87	-3.116	-3.26
7.69	-1.482	-1.626
7.81	-1.243	-1.387
9.27	-0.007	-0.151
8.63	-0.007	-0.151
9.08	-0.007	-0.151
9.46	-0.007	-0.151

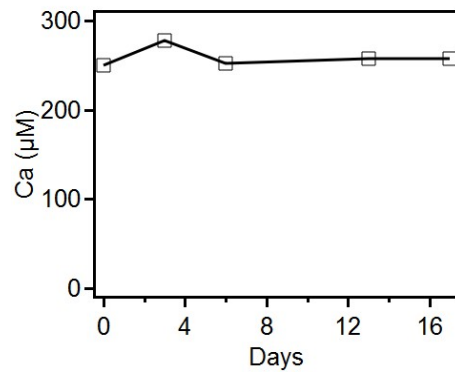
4  
 5 Table S4: The  $\Delta^{44}\text{Ca}$  of relevant biogenic and abiotic carbonates. Only studies reporting the  
 6 temperature dependency of Ca isotope fractionation were included. For abiotic ACC, the  
 7 temperature was not specified in the study.

Type of CaCO <sub>3</sub>	$\Delta^{44}\text{Ca}$
Biogenic Aragonite (corals)	$-1.38 \pm 0.11\text{‰}$ (Gussone et al., 2005)
	$-1.01 \pm 0.48\text{‰}$ (Inoue et al., 2015)
	$-1.0 \pm 0.63\text{‰}$ (Böhm et al., 2006)
Biogenic Calcite (sclerosponges, planktic foraminifera, brachiopods, coralline red alga)	$-0.61 \pm 0.41\text{‰}$ (Gussone et al., 2005)
Biogenic Calcite (coccolithophores)	$-0.87\text{‰}^*$ (Langer et al., 2007)
	$-0.96 \pm 0.47\text{‰}$ (Gussone et al., 2007)
	$-1.43 \pm 0.32\text{‰}$ (Gussone et al., 2007)
Biogenic ACC (cyanobacteria)	$-0.72 \pm 0.05\text{‰}$ (this study)
Abiotic ACC	$-0.25\text{‰}^*$ (Niedermayr et al., 2010)
Inorganic Aragonite	$-1.49 \pm 0.24\text{‰}$ (Gussone et al., 2005)
Inorganic Calcite	$-0.57 \pm 0.5\text{‰}$ (Gussone et al., 2005)
Microbially mediated Monohydrocalcite	$-0.63 \pm 0.18\text{‰}$ (Bradbury et al., 2020)

\* no data on standard deviation reported in the study

9

10 Figure S1: Time evolution of the concentration of Ca in a non-inoculated control. No replicate of  
11 the non-inoculated control was performed, but the observations were consistent with previous



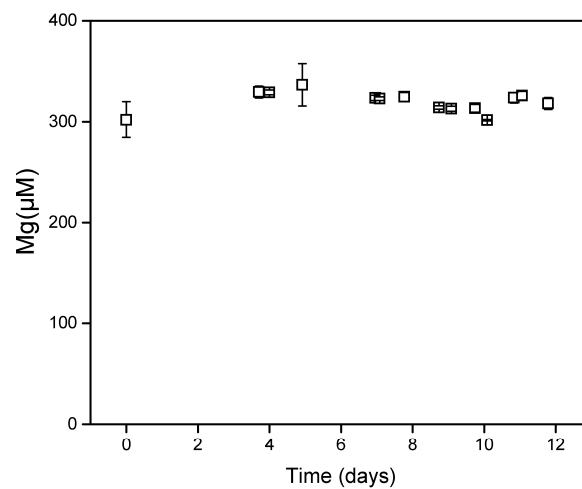
12 studies ((e.g. Cam et al., 2018)

13

14

15

16 Figure S2: Time evolution of the concentration of Mg in cultures. The error bars indicate the  
17 standard deviation of triplicate measurements.



18

19

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