

Formation of Calcium Carbonate Polymorphs Induced by Living Microalgae

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ABSTRACT

Calcium carbonate (CaCO_3) occurs in the three polymorphs calcite, aragonite and vaterite. The formation of these crystals in inorganic solutions is influenced by parameters like pH, temperature or impurities. Living freshwater microalgae can also induce the formation of CaCO_3 when they live in a suitable environment containing saturated amounts of Ca^{2+} . Through this *biologically induced* biomineralization only the formation of the polymorph calcite has been reported yet. We investigated the precipitates which have been formed in solutions containing the freshwater microalgae *Scenedesmus obliquus* and different zinc amounts (0, 3.27 and 6.53 mg Zn^{2+}/l) by XRD and SEM. As references precipitates from the same solutions but without algae were investigated. We could show that the presence of living microalgae has a great influence on the precipitation of calcium carbonate crystals. In algae-containing media without or with a low zinc amount always calcite and aragonite are formed. In the corresponding medium with 6.53 mg Zn^{2+}/l pure aragonite crystals were built. In contrast, in the inorganic, algae-free solutions without zinc, pure calcite is precipitated. Both inorganic solutions with zinc show major calcite precipitation and weak aragonite precipitation. Thus the algae cells advance significantly the formation of aragonite, which is enhanced by the presence of zinc cations in the media. Possible mechanisms are discussed.

Keywords: CaCO_3 Polymorphs; Biomineralization; Zinc; Microalgae; XRD

1. Introduction

The formation of the three calcium carbonate (CaCO_3) polymorphs calcite, aragonite and vaterite in inorganic solutions has been the topic of many studies [1-4]. It was proposed that first an unstable amorphous phase is built which transforms to the metastable phase vaterite or aragonite, followed by the transformation to the stable phase calcite [1]. The precipitation of the polymorphs can be affected by different factors like temperature, pH of the medium, concentration ratio of individual components, supersaturation, ionic strength or impurities [3]. The investigations of Ogino *et al.* (1987) [1] revealed that aragonite formation is favored at higher temperatures ($>40^\circ\text{C}$) in aqueous solution, which has also been supported in other experiments [4]. At room temperature, the pH of the solution in a constant-composition environment is the most important factor for the formation of the polymorphs [3]. At 24°C , vaterite is the major product in the pH range between 8.5 and 10. Aragonite preferably formed at pH 11; when the pH is higher than 12, calcite is the dominant product. The precipitation of the different polymorphs can also be modified by impurities in the

solution. When the ionic radius of the impurities is smaller than that of the Ca^{2+} cations, what is the case for Zn^{2+} cations [5], aragonite is deposited [6]. This influence is explained by adsorption phenomena on the crystalline faces of the nuclei. Kitano *et al.* (1976) [7] examined specifically the adsorption behavior of Zn^{2+} on CaCO_3 polymorphs in aqueous solutions. They showed that Zn^{2+} is strongly adsorbed on aragonite crystals in comparison to other polymorphs. It was assumed that the zinc ions inhibit the transformation of aragonite to calcite [1,7]. The Zn^{2+} adsorption on the calcite surface occurs via an exchange of Ca^{2+} in a surface-adsorbed layer [8].

Also organic additives show an effect on the mineralization of calcium carbonate (reviews in: Meldrum, 2003 [9]; Ren *et al.*, 2011 [10]). Amino acids like glycine, aspartic acid and glutamic acid or polysaccharides like cellulose influence the precipitation and morphology of calcite and vaterite [11-13].

Living organisms like photosynthetic microalgae can induce the precipitation of CaCO_3 through biomineralization. It is known that many freshwater algae build calcite crystals when they live in a supersaturated environment regarding calcium [14,15]. This is a result of the

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influence of the algae's metabolism on their aqueous environment [16]. Through photosynthesis the algae assimilate CO_2 and/or HCO_3^- , and release OH^- [16,17]. This leads to an increase of the pH and the concentration of carbonate anions within the solution. It is also largely accepted that the pH affects both number of cell surface binding sites and metal chemistry within aqueous solution: in the acidic range, the functional groups of the cell wall are closely protonated, which limits the approach of metal cations. The cell walls of green algae consist of polysaccharides and proteins [18,19]. With increasing pH, more functional groups, such as carboxyl or amino groups, are exposed, which increases the biosorption of metal ions on the negatively charged cell walls. In the first step, Ca^{2+} is bound on the cell wall, and afterwards CO_3^{2-} anions are accumulated and CaCO_3 is precipitated extracellular. This mechanism is named *biologically induced* [16], because the calcification is not actively controlled by the organisms. In the *biologically controlled* biomineralization, different types of biominerals are produced under the genetic control of the organisms. Up to now, in the presence of freshwater microalgae only the extracellular *biologically induced* formation of calcite is reported.

Positively charged metal ions can also be bound on the cell surfaces of living organisms [e.g. 20-27]. Zinc is an essential trace metal for all organisms, but can be toxic when present in high amounts. Exposure of microalgae to sublethal Zn^{2+} concentrations caused inhibition of e.g. photosynthesis [28]. When the algae are confronted to toxic elements like Zn^{2+} in their environment, they detoxify it by taking up the ions. The uptake is split in two steps: the first is a rapid biosorption onto the cell wall, during the second step the ions are transported into the cells [29]. Subsequently, the interior of the cells can be detoxified by chelating the Zn^{2+} cations to e.g. nontoxic zinc-phosphate-based nano needles [30].

The aim of this study was to investigate the role of living algae on the precipitation of different polymorphs of calcium carbonate. For the experiments the unicellular alga species *Scenedesmus obliquus* (Chlorophyta) was cultivated in media with and without Zn^{2+} and the results were compared to the inorganic media without algae. This alga species was chosen because preliminary experiments have shown that it calcifies readily when cultured in supersaturated media regarding Ca^{2+} .

2. Materials and Methods

2.1. Organisms and Cultures

The experiments were conducted with living *Scenedesmus obliquus* (strain 276-1), obtained from the SAG culture collection Göttingen. The algae were cultivated in a modified calcification medium [17], where Na_2EDTA ,

KH_2PO_4 , $(\text{NH}_4)_2\text{Mo}_7\text{O}_{24}$ and MnCl_2 were omitted and FeCl_3 was reduced to 0.01 mg/l to prevent chelate formation with Zn^{2+} . The solution was saturated regarding Ca^{2+} . The media contained different amounts of zinc (0 mg Zn^{2+} /l, 3.27 mg Zn^{2+} /l or 6.53 mg Zn^{2+} /l), added as $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$ (Roth, Karlsruhe). Preparation of the media: all stock solutions beside Na_2CO_3 were sterilized (autoclave *Systec V-75*) and combined, the pH was adjusted to 6.3. The algae were washed in demineralized water (Millipore), counted with a hemocytometer (*Marienfeld, Lauda-Königshofen*) to ensure the same cell concentration in all cultures and added to the medium (about 4.0×10^8 cells/l). The culture was held in a rotary shaker (*Infors HT Multitron II*, 100 rpm) at 26°C and permanent illumination (*FL tubes Gro-Lux* 15W, 3500 lx). Due to photosynthesis, the pH increased to a value over 9 during several days. Not till then the Na_2CO_3 was added to prevent loss of CO_3^{2-} anions and the pH was adjusted to 8.5. At this point the experiment started. The inorganic, algae-free solution was prepared identically; the pH was adjusted daily according to the organic, algae-containing solution with 0.1 M NaOH. The duration of the experiment was 72 h. After this time all cultures were living as revealed by their green color.

2.2. SEM Measurement

Precipitation of crystals was investigated after 2, 4, 6, 24 and 72 h duration of the experiment. For observation in a scanning electron microscope (*Zeiss DSM 982 GEMINI*) the specimens were washed in demineralized water (*Millipore*) using a glass vacuum filter system with 0.2 $\mu\text{m}/2 \mu\text{m}$ pore size filter membrane (*Millipore*) and then air-dried. To obtain electron conductivity, the membranes were coated with graphite.

2.3. Powder X-Ray Diffraction (XRD)

XRD was used to identify the CaCO_3 polymorphs after 72 h duration of the experiment. The XRD patterns were recorded with a D500 diffractometer (Siemens). Measurements were made in Bragg-Brentano geometry, using $\text{CuK}\alpha_1$ radiation (X-ray tubes setting of 30 mA and 40 kV) employing a primary-beam Johansson monochromator in a 2θ range of 20° to 50°.

3. Results & Discussion

3.1. pH Trend

At the beginning of all experiments the pH of each culture was adjusted to 8.5. Due to photosynthesis of the algae, the pH rose up to 10.8 after 48 h in the culture without zinc; afterwards it stayed nearly constant (**Figure 1**). In presence of zinc, the pH-shift was retarded and did not reach the high values of the culture without zinc. This

is due to the fact, that Zn^{2+} cations restrain photosynthesis of algae. The pH-shift is correlated to Zn^{2+} concentration within the solution: the culture with 6.53 mg Zn^{2+}/l only reached pH 10.1 after 72 h, the culture with 3.27 mg Zn^{2+}/l reached pH 10.5 after 48 h and remained at this value. According to the observed pH values in cultures containing algae, the pH in the media without algae were adjusted daily with a 0.1 M NaOH solution.

3.2. Precipitation of Crystals and Identification of Polymorphs

The formation of precipitates was investigated at the starting point of the experiments and after 2 h, 4 h, 6 h, 24 h and 72 h in solutions with algae.

At the starting point of the experiments, there was no precipitation on the algae (**Figure 2(a)**). After 2 h of incubation, crystals with embedded algae could be detected

in the cultures without zinc (**Figure 2(b)**). In the next hours the crystals grew bigger (data not shown). In the cultures with zinc, no crystallization occurred during the first 6 h (data not shown).

After 24 h of incubation, in all solutions precipitates could be found (data not shown). In the media with algae and with zinc, big crystals occurred with diameters of about 50 μm . Always many algae were embedded in these crystals. In the inorganic solutions with Zn^{2+} , only very small precipitates were found (\varnothing below 100 nm; REM image, data not shown).

After 72 h of incubation, in all solutions crystals were found which differed greatly in their dimensions and morphology (**Figure 3**). The precipitates formed in the organic solutions contained several (**Figures 3(a) and (c)**) or many algal cells (**Figure 3(b)**). The largest precipitate, where always many algal cells were incorporated, were

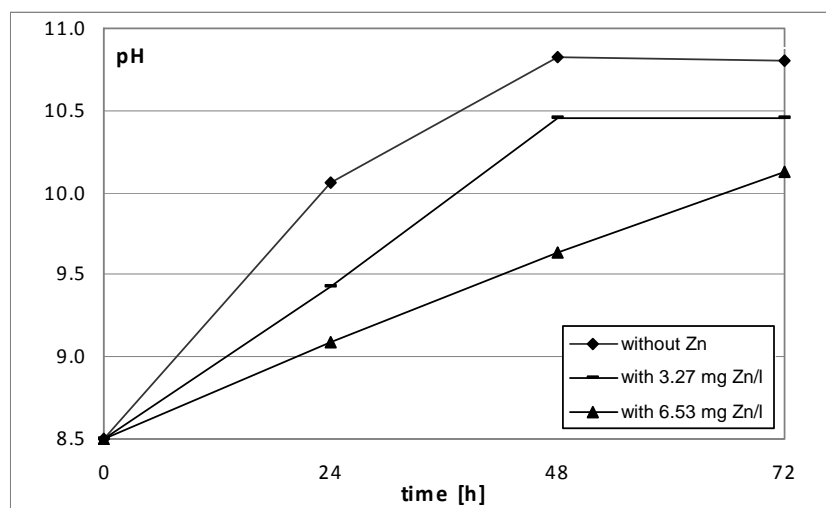


Figure 1. pH shift in the cultures with algae and different zinc amounts during the experimental time of 72 h.

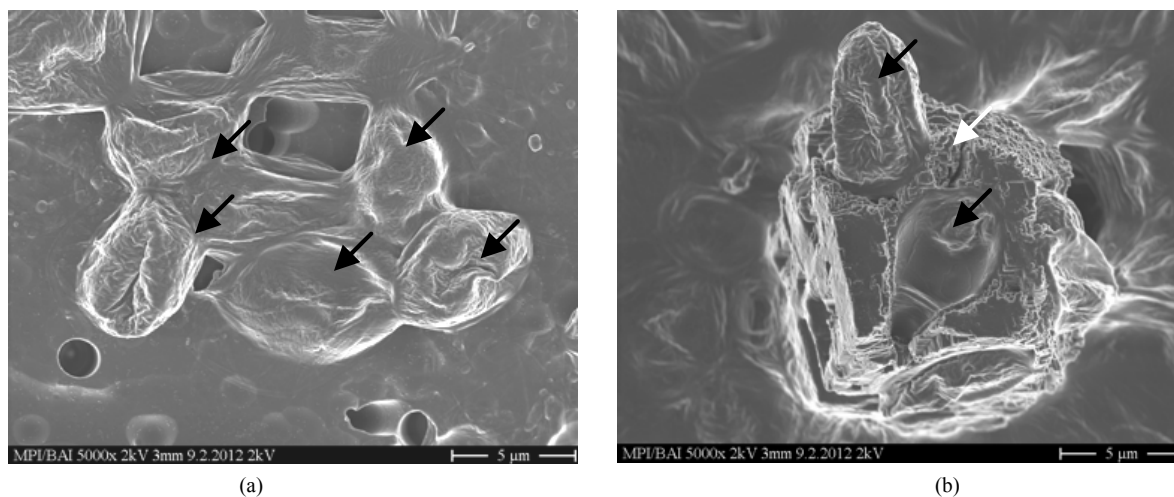


Figure 2. SEM images. (a) Algae (black arrows) without precipitates at the beginning of the experiment; (b) Crystals (white arrow) with embedded algae were built after 2 h in the medium without zinc.

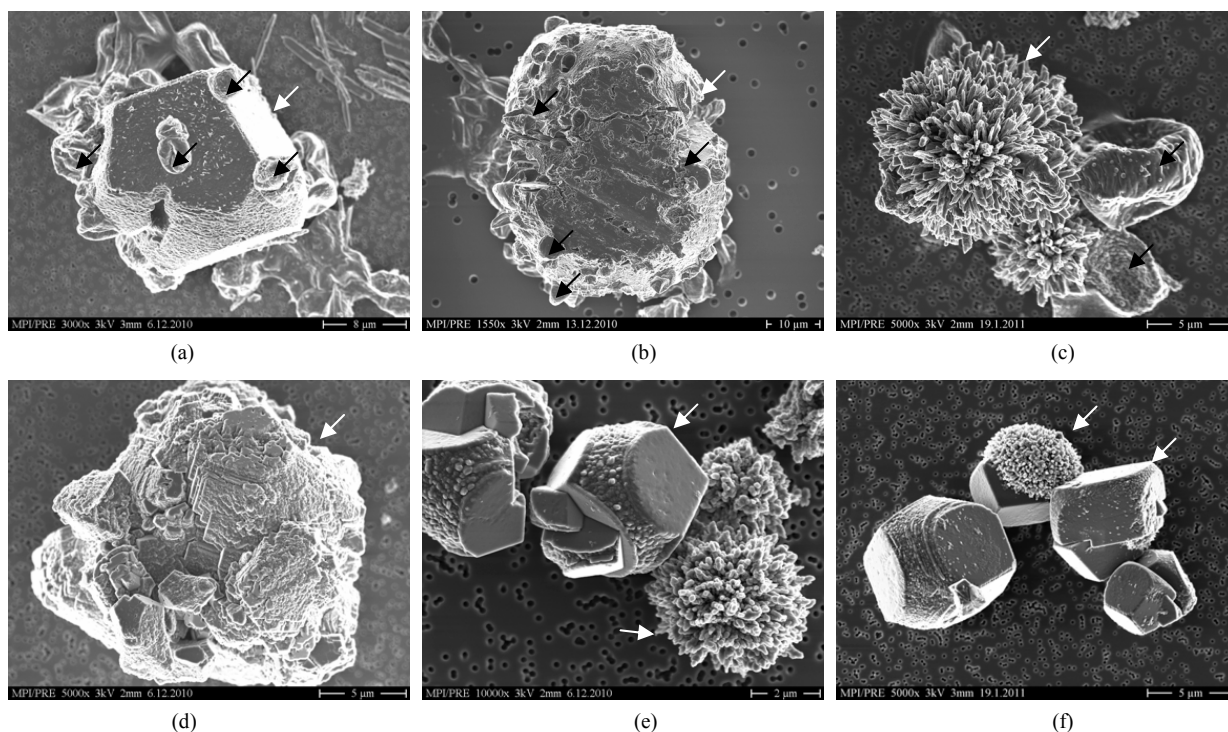


Figure 3. Crystals after 72 h of incubation. (a)-(c) Cultures with algae and different amounts of zinc: (a) Without zinc; (b) With 3.27 mg Zn^{2+}/l ; (c) With 6.53 mg Zn^{2+}/l . (d)-(f) Inorganic solutions with different amounts of zinc: (d) Without zinc; (e) With 3.27 mg Zn^{2+}/l ; (f) With 6.53 mg Zn^{2+}/l . Black arrows—algae, white arrows—crystals.

found in the organic solution with 3.27 mg Zn^{2+}/l (**Figure 3(b)**).

These precipitates appear in the SEM images as big single-crystals, but could also be formed as many smaller crystals and afterwards baked together. In the inorganic solution with zinc, two different crystal types were found (**Figures 3(e)** and **(f)**). Due to the crystal morphology we assume that the needle-like crystals refer to aragonite and the rhombohedral to calcite [3].

The crystals found after 72 h in the experimental solutions were washed and then analyzed regarding their modification by XRD. Representative XRD diagrams are shown in **Figures 4** and **5**. In the case of solutions without algae cells the dominant (104) reflection of calcite (JCPDS card No. 01-072-1650) can be found in all samples at 29.5° (3.02 \AA) (**Figure 4**). Furthermore, the weaker (012)-, (110)-, (113)-, (202)-, (024)-, (018)-, and (116)-reflection of calcite are present in the sample, which contains no Zn^{2+} cations within solution. In former studies about precipitation in inorganic solutions, vaterite was found to be the major product in the pH range between 8.5 and 10 and at 24°C [3]. In these experiments, the experimental solution only contained calcium chloride and sodium carbonate. In our studies, a culture medium for algae with a more complex composition was used for all experiments, also for preparing the solutions without algae. This was probably the reason, why calcite

was built and not vaterite.

In the inorganic sample which has been formed in medium with 3.27 mg Zn^{2+}/l weak intensities (near the detection limit) can also be detected at 26.3° , 27.2° , and 46.0° which can be attributed to the characteristic (111)-, (021)- and (221)-reflections of aragonite (JCPDS card No. 00-003-1067). The reflections of aragonite correspond with SEM observation of a small amount of a second acerosus phase (cf. **Figure 3(e)**). The precipitate of the sample, which has the highest amount of Zn^{2+} cations within the media, shows only the dominant (104) reflection of calcite. A small amount of aragonite crystals could be observed in this medium by SEM observations (**Figure 3(f)**), which is below of detection limit of XRD.

The presence of algae cells has a significant influence on the $CaCO_3$ precipitation within media in comparison to media without algal cells, which is demonstrated in **Figure 5**. In the case of the Zn^{2+} free media the precipitate is dominated by the reflections of calcite, but small intensities of aragonite are also present (Please note that the strong (104) reflection of calcite at about 29.5° is cut at about 1600 a.u. within **Figure 5(a)**). With increasing zinc concentration in the solutions the amount of calcite decreases dramatically. In fact, in the case of media with 6.53 mg Zn^{2+}/l no calcite reflections can be determined within the corresponding XRD diagram. Only the reflections of aragonite are present (**Figure 5(c)**). Thus, the

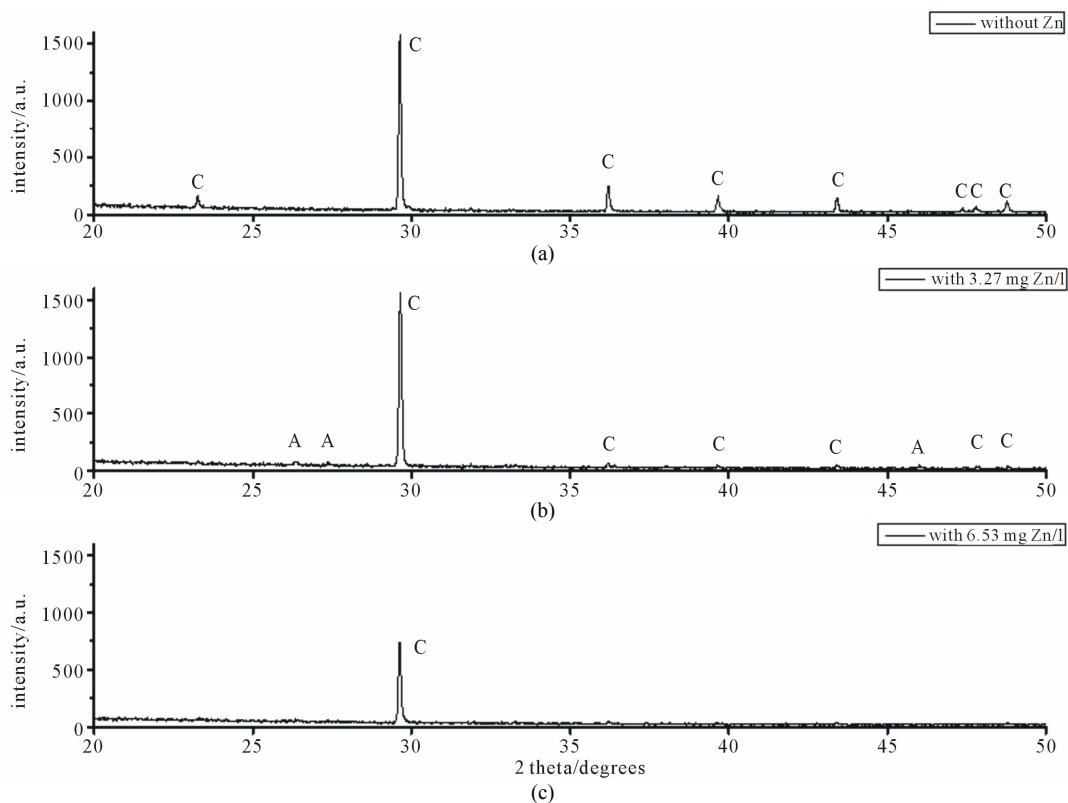


Figure 4. XRD diagrams of precipitates in absence of algae (72 h of incubation). (a) Without zinc; (b) With 3.27 mg Zn²⁺/l; (c) With 6.53 mg Zn²⁺/l.

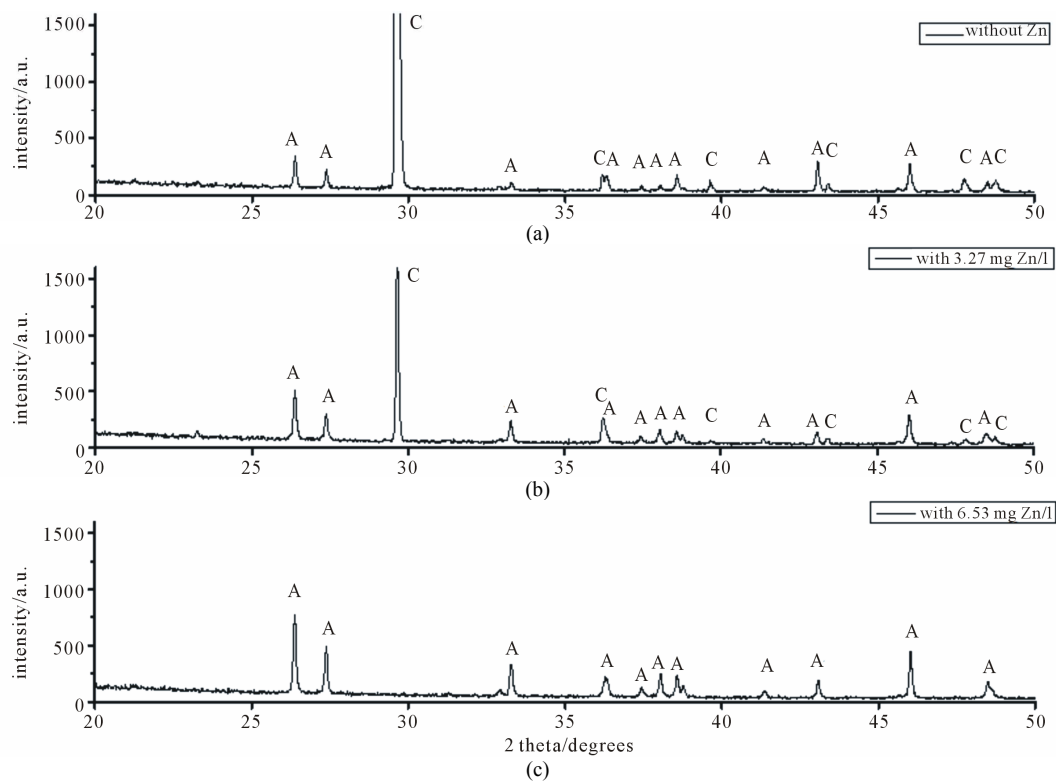


Figure 5. XRD diagrams of precipitates in presence of algae (72 h of incubation). (a) Without zinc; (b) With 3.27 mg Zn²⁺/l; (c) With 6.53 mg Zn²⁺/l.

algae cells advance significantly the formation of aragonite, which is enhanced by the presence of zinc ions in the media.

The algae function obviously as heterogeneous nucleation sites. Since only crystals associated with algae have been found in the solutions, the cell walls are evidently directly involved in nucleation. The binding sites of the cell walls have a great affinity for Ca^{2+} cations, the bound Ca^{2+} attract anions like CO_3^{2-} [16]. Our preliminary experiments have shown that *Scenedesmus obliquus* does not calcify when cultivated in the dark, and the pH reaches only the maximum of 9. This was also observed by other authors with different microalga species [17,31]. This shows that the algae do not only act as nucleation sites for CaCO_3 precipitation but are actively involved through light-dependent photosynthesis and thus alkalinization of the medium. The increase of pH was measurable in the bulk solution but was probably higher close to the cells. This was also supposed by other authors [14,17]. In presence of zinc in the medium the aragonite modification is favored. In this case, obviously the transformation into the thermodynamically stable calcite modification is hindered. A possible mechanism that occurs in micro-environments created by the cells is suggested in **Figure 6**. A part of the absorbed Ca^{2+} on the cell wall can be exchanged by Zn^{2+} [18]. The interaction of Zn^{2+} with the cell wall enables a local enrichment of the ions which are supposed to stabilize the metastable aragonite modification [7] and thus prevents its transformation into thermodynamically stable calcite. Furthermore, Zn^{2+} ions restrain photosynthesis [28], which is connected with a lower pH within the micro-environments compared to the algae-containing zinc-free medium. As mentioned above, the formation of aragonite is favoured at lower pH compared to the one of calcite [3]. Accordingly, only aragonite crystals in the media containing algae and high amounts of zinc are detected. On the contrary, calcite is produced nearly exclusively in the

media without algae and high amounts of zinc. Only traces of aragonite are produced, as revealed by SEM observations.

4. Conclusion

We compared the precipitates which have been formed in solutions with and without algae and with and without different zinc amounts. We could show that the presence of living microalgae has a great influence on the precipitation of calcium carbonate crystals. Due to photosynthesis, the pH in the algae-containing organic media increased and CaCO_3 crystals are precipitated. In organic media without zinc or with 3.27 mg Zn^{2+}/l always calcite and aragonite are formed. In the organic media with 6.53 mg Zn^{2+}/l aragonite is obtained even exclusively. In contrast, in the inorganic solutions without zinc, pure calcite is precipitated. Both inorganic solutions with zinc show major calcite precipitation and weak aragonite precipitation as revealed by XRD measurements and SEM observations. Thus, the algae cells advance significantly the formation of aragonite, which is further enhanced by the presence of zinc cations in the media. The following effects of algae and zinc in the solution were supposed: first, as the algae accumulate, a “micro-environment” around the clusters is formed, where the CaCO_3 is precipitated (**Figure 6**). When high Zn^{2+} amounts are present in the solution, photosynthesis is inhibited and the pH in this “micro-environment” is lower compared to the “micro-environment” around the algae in solution without Zn. The lower pH prefers the building of aragonite [3]. Second, the algae function as heterogeneous nucleation sites where in the first step Ca^{2+} and Zn^{2+} ions are absorbed on the negatively charged cell walls and in the second step anions like CO_3^{2-} are bound (**Figure 6**). The presence of Zn^{2+} leads to the precipitation of aragonite [6]. In the “micro-environment” around the cells more Zn^{2+} ions are present than in the corresponding in-

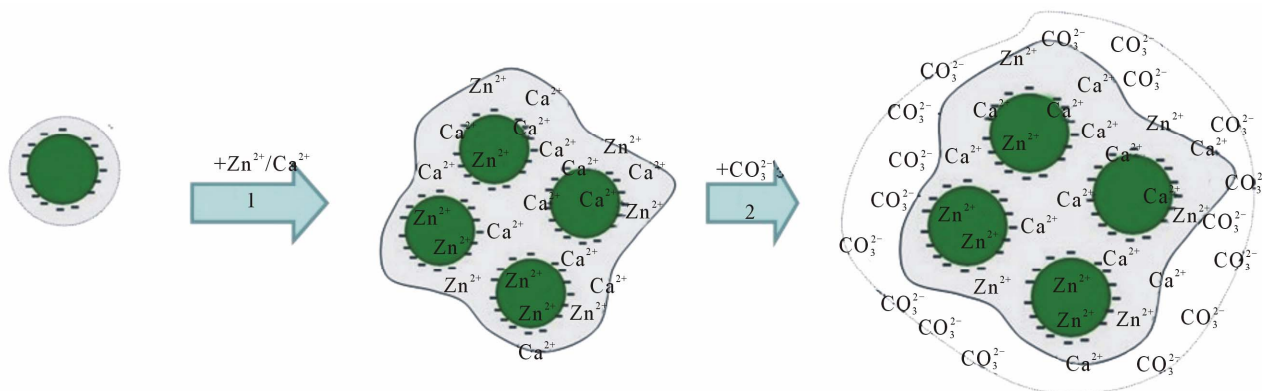


Figure 6. Scheme of possible heterogeneous nucleation of aragonite crystals in presence of algae and zinc. Between several aggregated cells a “micro-environment” is built leading to a local increase of the pH and Zn^{2+} ions.

organic bulk solution, since these ions are attracted from the cell walls and bound on them. Zn^{2+} ions inhibit the transformation from aragonite to calcite [1]. Hence, aragonite is precipitated in the algae-containing media with high amount of Zn whereas in the corresponding inorganic solution mainly calcite is found.

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