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EXPERIMENTAL ARTICLES

Fossilization of the Cells of Natronophilic Endoevaporite Cyanobacterium *'Euhalothece natronophila'* in a Modelling System

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Abstract—Laboratory simulation of fossilization of cyanobacterial cells in the high-carbonate medium in the presence of calcium was carried out for the haloalkaliphilic natronophilic cyanobacterium *'Euhalothece natronophila'* Z-M001. This organism was isolated from the Magadi soda lake, where the bioherms consisting of mineralized coccoid cyanobacteria were found in the Quaternary sediments. The structural and chemical heterogeneity of the minerals produced during this process was established, with calcium carbonate and trona being the main products. The differences in the process of cyanobacterial cell carbonatization in soda lakes and marine or freshwater systems were determined. Initial precipitation of calcium carbonate was shown to occur due to a chemical reaction not involving cyanobacteria. At the subsequent stages, amorphous $CaCO_3$ is sorbed and crystallized on the surface of some of the cells within a cyanobacterial population, resulting in formation of a shell-like mineral layer. The cells embedded in trona in the same system were shown to undergo deformation and destruction. In both cases the mineralized cells were shown to lose their photosynthetic activity.

Keywords: extremely natronophilic cyanobacterium *'Euhalothece natronophila'*, Magadi, endoevaporite conditions, mineralization, calcium carbonate, trona, fossilization.

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Under modern conditions, carbonatization of cyanobacterial cells is a common phenomenon in freshwater environments [1]. Since at high pH values calcium as a macrocomponent disappears from solutions and accumulates as chemically precipitated $CaCO_3$, mineralization of bacteria in soda lakes has been considered unlikely [2]. However, under such conditions, mineralization is still possible at the boundary where the surface lake water mixes with the underground, calcium-containing water.

Inflow of the underground waters into a soda lake results in rapid precipitation of minerals. At the exposed steep banks of the alkaline Lake Magadi (Kenya) belonging to the Quaternary sediment layer of High Magadi Beds (9100–12000 years), several-meter-thick Green Beds were described [3]. They contain over 90% of the siliceous limestone of the lake. These layers contain mainly of SiO_2 (80–88%) and are enriched with calcite and biomorphic structures. Most of these structures are layered carbonate stromatolites and rounded bioherms up to 25 cm in diameter formed by mineralized coccoid cyanobacteria.

Identification of the Lake Magadi cyanobacterial microfossils revealed forms morphologically identical to the modern genera *Pleurocapsa, Gloeocapsa, Entophysalis, Chroococcus*, and *Synechococcus*. Scanning electron microscopy of cyanobacteria of the genera *Pleurocapsa* and *Gloeocapsa* showed that calcium carbonate precipitated on their mucous capsules, not penetrating into the cells and preserving them as negative structures. Calcium carbonate was then either substituted by opal (SiO_2) or overgrown by new $CaCO_3$ layers, which connected the envelopes of individual cells, resulting in formation of the spongy structures observable by electron microscopy [3].

The most widespread *Pleurocapsa* bioherms developed at the littoral, under continuous supply of silicon- and calcium-enriched surface waters. Under modern conditions, coastal and surface ephemeral flows also bear alkaline-earth cations (Ca, Mg) [4].

Unicellular cyanobacteria assigned to the *Euhalothece* group on the basis of their phylogenetic, physiological, and morphological characteristics [5] are typical members of the endoevaporite biota and may develop at the final stage of the evaporite process in a saturated brine between precipitating mineral crystals. *Euhalothece* spp. act as primary producers during the synsedimentory stage of evaporite formation from brines. Their presence as the dominant forms of cyanobacteria in halite sediments of saline thalassogenic] lagoons was determined by molecular techniques [6, 7]. Their presence in the sediments of the

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soda Lake Magadi was also confirmed by molecular techniques [8]. The culture of an extremely alkaliphilic cyanobacterium *'Euhalothece natronophila'* Z-M001, which developed above the soda sediment and between the soda crystals, was isolated from this lake [9].

Since the carbonatization stage determines the microfossil morphology, the goal of the present work was to investigate the process of mineral formation during carbonatization of *'Euhalothece natronophila'* Z-M001 cells in a model simulating the influx of Ca-bearing solutions in a soda lake.

MATERIALS AND METHODS

The test object was the unicellular haloalkaliphilic cyanobacterium *'Euhalothece natronophila'* Z-M001. In a normal state, the cells are spherical, 2.7–4 μm in diameter, single or in pairs. The ecology, physiology, and morphology of *'Euhalothece natronophila'* Z-M001 were described in [9].

Cultivation conditions. The cells were grown on a shaker at 30°C, under constant illumination by incandescent lamps (2000 lx) in M medium containing the following (mM) Na_2CO_3, 1000; NaCl, 800; KCl, 27; Na_2SO_4, 10; KNO_3, 20; $K_2HPO_4 \cdot 3H_2O$, 2, $FeCl_3$, 1.8×10^{-3}, A_5 trace element solution, 1 ml; pH 10–10.5.

Experimental procedures for investigation of calcification. A 3-day culture (live or killed by heating) was supplemented with $CaCl_2$ to the final concentration of 17 mM. The samples were collected after 10 min, 30 min, 4 h, 24 h, 4 days, and 14 days of incubation in light. For control experiments with dead cells, the suspension was heated at 80–100°C in the M medium to complete yellow coloration of the culture. The cell-free control was the sterile M medium supplemented with the same concentration of $CaCl_2$. The viability was assayed under light microscope in wet mounts stained with 0.5% aqueous erythrosine. The content of dead cells was stained red, while the live cells remained green.

Morphology and photosynthetic activity of *'Euhalothece natronophila'* was investigated in the following fractions of the suspension: precipitate (cells at the bottom), supernatant suspension (SS, the cells which did not precipitate and remained suspended in the medium), and total suspension (TS, the homogeneously mixed medium with the cells from both the precipitate and the supernatant suspension). The culture in the same M medium without $CaCl_2$ was used as the control.

Microscopy was carried out under a Jenaval light microscope equipped with a Zeiss Bundle Canon PS G9 camera (Germany). The results were processed using the AxioVision 4.7. software package.

The samples for scanning electron microscopy (SEM) were prepared by air-drying (in order to prevent reactions of the crystals with the fixative solutions). This was in fact evaporite concentration, similar to the naturally occurring process. Since the presence of all types of minerals was confirmed by light microscopy and on ultrathin sections prepared for transmission electron microscopy (TEM) without evaporite concentration [10], artifacts associated with precipitation of minerals during drying can be excluded. For investigation of morphology of the cells embedded in trona ($Na_2(CO_3 \cdot Na(HCO_3) \cdot 2H_2O$), the sample was washed several times with distilled water. After drying, the samples were contrasted with vacuum-deposited Au and Pt.

The mineralized cells and minerals were photographed at the Paleontological Institute, Russian Academy of Sciences, on an EVO-SOXVP-Zeiss SEM (Germany); for determination of the elemental composition of the minerals, a CamScan serie 4 SEM with a LINK 860 microanalyzer (United Kingdom) was used.

Photorelease of oxygen was determined in a polarographic cell with a Clark electrode and an Expert-001 pH-meter–ion meter (Russia). Aliquots of the supernatant and total suspensions from experimental samples and the homogeneously stirred suspension of the control cells were used.

Protein content in the cell suspension was determined as described previously [9].

Optical density (OD) of the supernatant suspension was determined at 683 nm (OD_{683}) in 1-ml cuvettes on a SPECOL spectrophotometer.

RESULTS

Immediately after addition of $CaCl_2$ into the M medium with 100 g/l (0.94 M) Na_2CO_3 and without cyanobacterial cells, chemical precipitation of calcium carbonate occurred. $CaCO_3$ initially precipitated as a loose white suspension (Fig. 1a), which slowly settled at the bottom of the vial. Dumbbell-shaped crystals of $CaCO_3$ (Fig. 2b) were formed only after 20 min. These crystals grew and formed agglomerations. Loose amorphous $CaCO_3$ was, however, present in the medium even after 2 h (Figs 2c–2e). Precipitation of the minerals was slow and was associated with transition of $CaCO_3$ from the loose state to a dense one, i.e., with the process of crystal formation.

Addition of $CaCl_2$ to the cell culture in the M medium ($OD_{683} \approx 0.4$) also resulted in formation of loose white flakes. No mineral layer was formed at this stage on either living or dead cells (Figs 1b, 1c). This stage of precipitation was therefore completely chemical.

After 10 min of incubation, two layers became visible in the suspension, namely, the upper green one (SS, supernatant suspension) and the white layer of $CaCO_3$ precipitate. Thus, unlike the cell-free

medium, crystallization of amorphous calcium carbonate in the medium with cyanobacteria (living or dead) commenced after 10 min.

In the variant with live cyanobacterial cells, 10 min after addition of $CaCl_2$ microscopy revealed large (~5 μm) spherical or oval globules (Fig. 3f), i.e., *'E. natronophila'* cells surrounded by a $CaCO_3$ envelope (Fig. 5b). After 30 min and 4 h, agglomerations of mineralized cells were observed (Figs 3g, 3h). The SS of the experimental culture contained nonmineralized cells (Figs 3a–3c, 5a).

In the suspension of the heat-killed cells, the same process occurred (Fig. 4): after 10 min of incubation, nonmineralized dead cells were found in the SS (Fig. 4a), while the carbonate-embedded cells were in the precipitate (Fig. 4b). No morphological differences were found between living and dead mineralized cells.

The crystal shape in the $CaCO_3$ precipitate formed in the cell-free medium and in the experimental culture was different. In the cell-free medium, the crystals were dovetail-shaped (Figs. 2d, 2e), while oval particles (single or in pairs) similar in shape to the cells were found in the medium with cyanobacteria (Figs. 3f–3i, 4b, 4c).

After 24 h, agglomerations of $CaCO_3$-mineralized cells persisted in the precipitate of the experimental culture, while large crystals of trona ($Na_2(CO_3) \cdot Na(HCO_3) \cdot 2H_2O$) with embedded cells of *'E. natronophila'* developed (Fig. 3i). Formation and growth of such crystals continued, so that after two weeks of incubation they were 90 μm or more in size (Fig. 3j). The cells embedded into trona crystals were compressed and lost their shape, as was determined by both light microscopy and by SEM of the partially washed cells (Fig. 5c). In the suspension of dead cells, similar formation of the trona minerals occurred (Fig. 4c).

The supernatant suspension remained green throughout the experiment. It contained nonmineralized cells (Figs 1b–1c, 3a–3e). Link analysis confirmed the absence of calcium on the surface of the SS cells (Fig. 5a).

The processes occurring in the M cell-free medium and in the suspensions of living and heat-killed cells are summarized in Fig. 6.

Dynamics of growth and oxygen evolutionL by the cells of *'E. natronophila'* after addition of $CaCl_2$ are presented in Fig. 7. It can be seen that the lag-phase continued for the first 24 h. On the fourth day, when the cells were in the exponential growth phase, the photosynthetic activity (PA) of both the control and experimental SS increased significantly. The photosynthetic activity of the total suspension (TS = SS + precipitate) was determined by the activity of the cells in the supernatant suspension for all experimental points (TS ≈ SS). Thus, our experiments did not

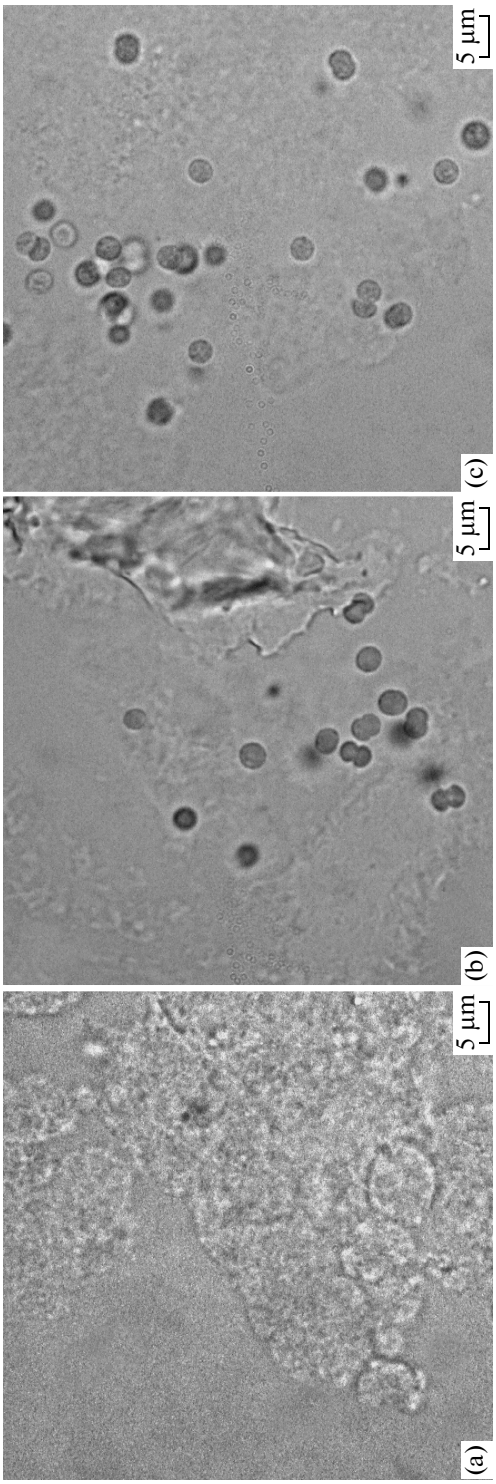


Fig. 1. Formation of amorphous calcium carbonate in the M medium immediately after addition of $CaCl_2$: in cell-free M medium (a), in *'E. natronophila'* culture (loose $CaCO_3$ precipitate and nonmineralized *'E. natronophila'* cells are visible) (b), and in the suspension of dead cells (c)

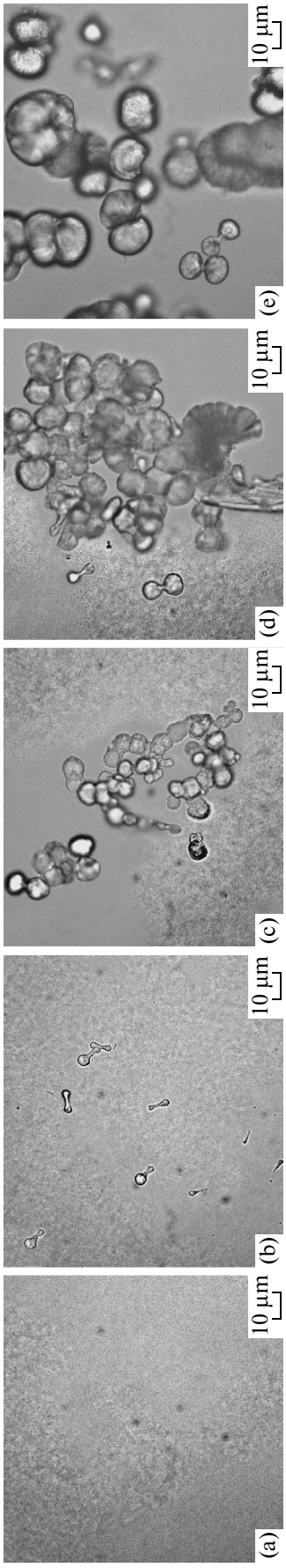


Fig. 2. Chemical precipitation of calcium carbonate in the cell-free M medium after addition of $CaCl_2$: after 10 min (a), 20 min (b), 1 h (c), 2 h (d), and 1 day (e).

reveal photosynthetic activity of *'E. natronophila'* cells embedded in the minerals.

DISCUSSION

Although calcification of cyanobacteria and cyano–bacterial mats has been a subject of extensive study during the last 15 years [see reviews 12–14], no consensus exists concerning the factors responsible for calcification in the mats.

It is presently generally accepted exopolysaccharides are important for $CaCO_3$ precipitation. Exopolysaccharides are able to adsorb Ca^{2+} cations by binding them to the deprotonated carboxyl groups of the C_6 monomers [14]. Thus, the amount of calcium in the mucus is higher than in the solution.

Saturation of the solution with carbonate ions is the major physicochemical factor responsible for $CaCO_3$ precipitation in freshwater environments. In the case of soda lakes, there is constant presence of high concentrations of carbonate ions, rather than temporary saturation.

In the present work, this problem was investigated in a laboratory system simulating the inflow of Ca-bearing solutions into a soda lake. The sequence of the mineralization events in saturated soda solutions was determined.

In the work [15], calcification of cyanobacterial films was investigated for three soda lakes: Pyramid Lake (United States, pH 9.3, alkalinity 22.08 mg-eq/l), Lake Nuoertu (China, pH 9.3, alkalinity 624.3 mg-eq/l), and Satonda Crater Lake (Indonesia, pH 8.5–8.58, alkalinity 3.97–4.17 mg-eq/l). In these environments, exopolysaccharides were shown to prevent immediate calcification, in spite of high concentrations of carbonate ions. The polysaccharides of the mucus act as a reservoir in which adsorbed Ca^{2+} is accumulated, so that precipitation of $CaCO_3$ becomes possible only after saturation of the exopolysaccharides with calcium.

Our data on the haloalkaliphilic cyanobacterium *'E. natronophila'* suggest another mechanism for carbonatization in extremely halophilic cyanobacteria (pH 10.5, alkalinity 1887 mg-eq/l). In a saturated soda solution, calcium does not exist in a dissolved form as a free cation. It forms complexes with various anions or precipitates chemically as $CaCO_3$. Thus, in the course of carbonatization of alkaliphilic cyanobacteria in the medium saturated with carbonate ions, sorption of amorphous $CaCO_3$ (not of the Ca^{2+} cations) with its subsequent crystallization occurs. This process occurs identically with living and dead cells. Cell carbonatization therefore does not depend on cell viability.

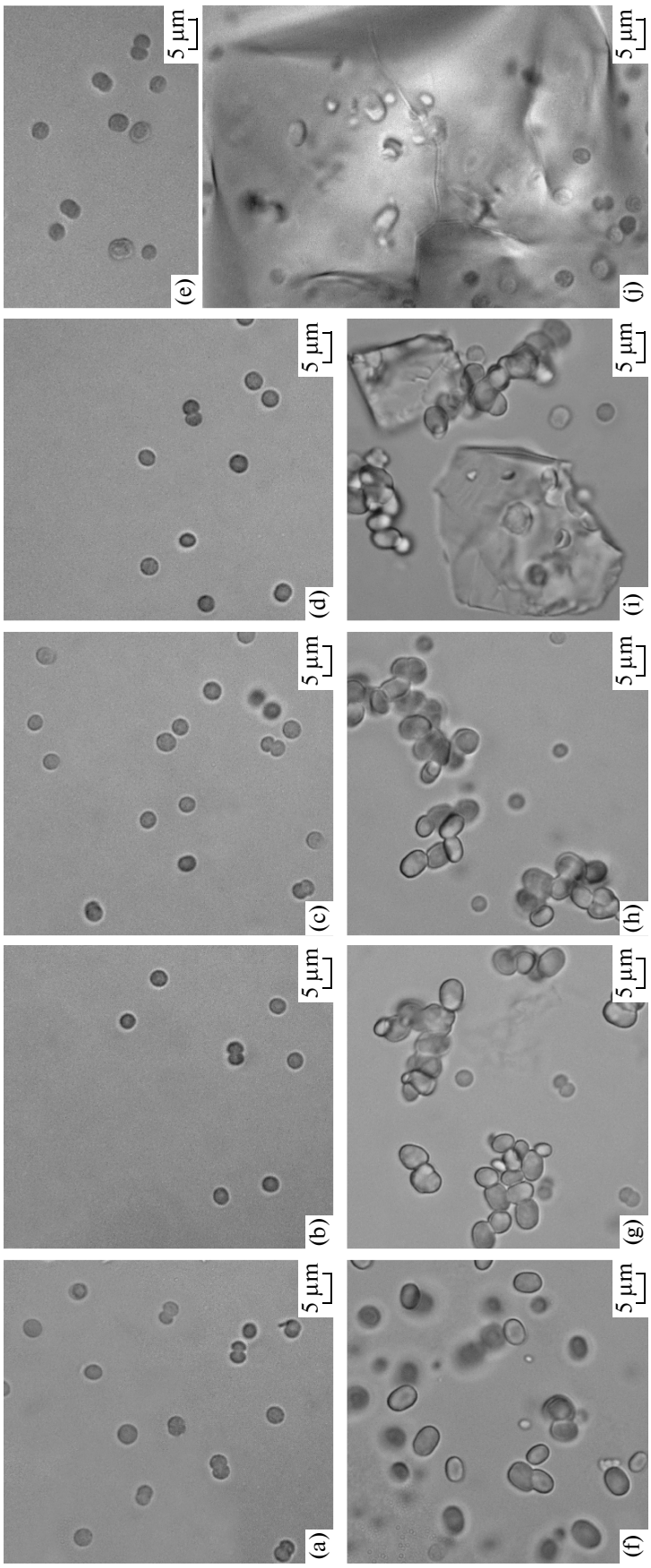


Fig. 3. Formation of minerals in '*E. natronophila*' culture: supernatant suspension (a–e), precipitate (f–h), and cells embedded in trona (j); after 10 min (a and f), 30 min (b and g), 4 h (c and h), 24 h (d and i) and 2 weeks (e and j).

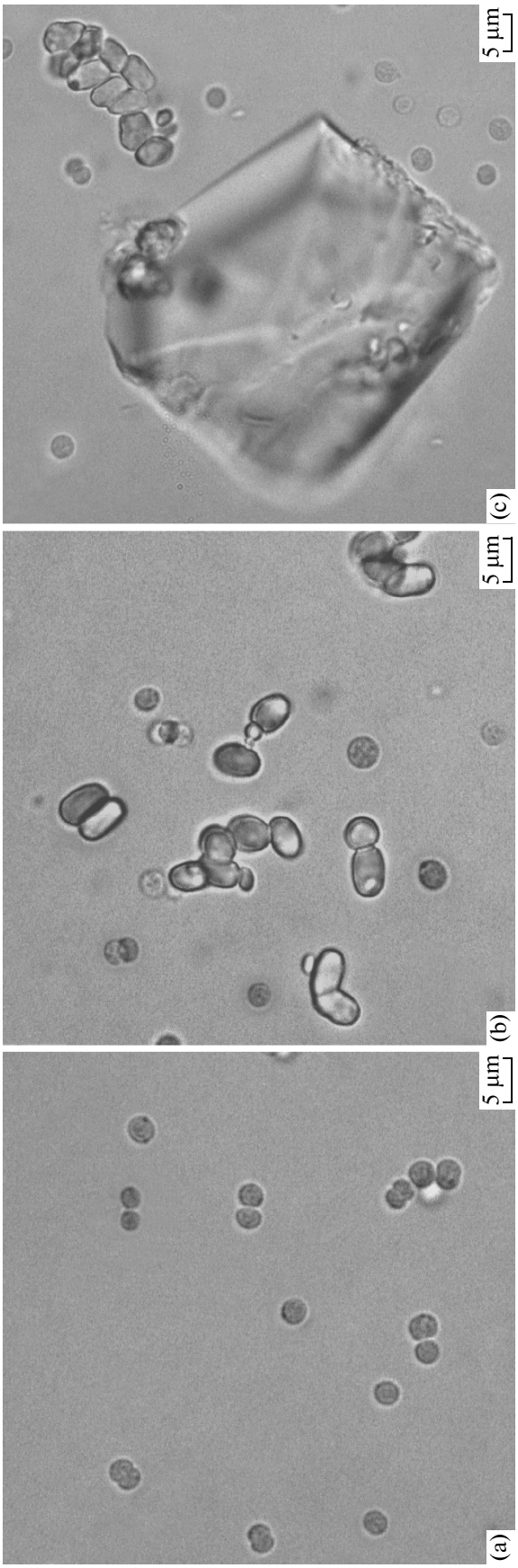


Fig. 4. Precipitation of calcium carbonate in the suspension of dead cells: supernatant suspension (a), precipitate 10 min after addition of $CaCl_2$ (b), and precipitate after 3 days (cells embedded in trona) (c).

Under the conditions of our experiment, a loose precipitate of amorphous calcium carbonate was formed immediately after addition of $CaCl_2$ to the carbonate-saturated medium. Importantly, formation of the amorphous sediment in a soda environment is a purely chemical process not associated with cyanobacterial cells.

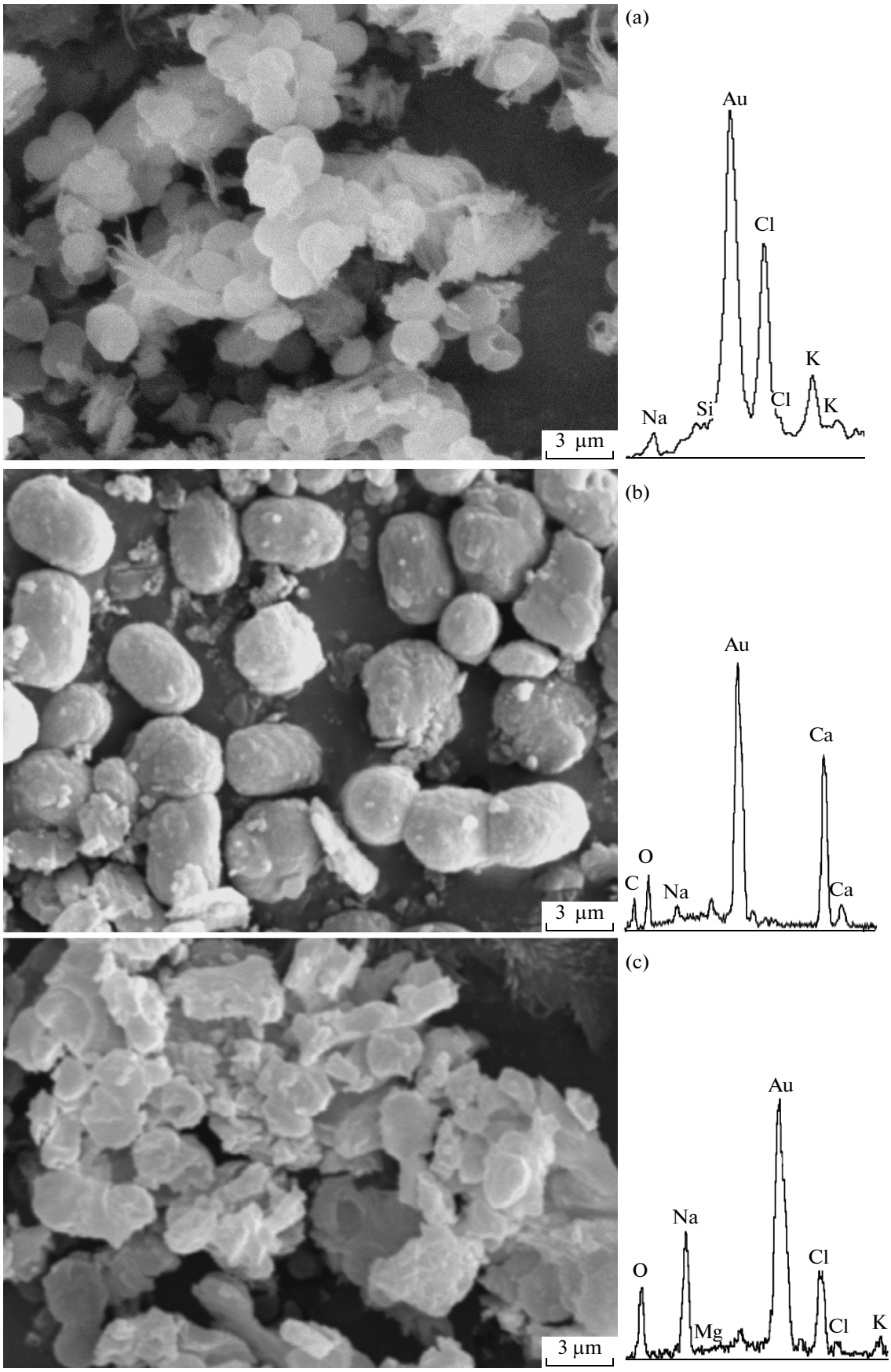
Amorphous $CaCO_3$ then transforms into crystals. This may also be a chemical process (Fig. 2), although cyanobacterial cells may be involved as crystallization centers (Figs 3, 4). Importantly, the crystal morphology was different for the cell-free medium and the cell suspension. In cell-free solutions, dumbbell-shaped crystals of calcium carbonate were formed, while the precipitate formed in the cell suspension consisted of oval globules of the mineralized cells.

Thus, carbonatization of cyanobacteria under high-soda conditions requires the presence of $CaCO_3$, rather than dissolved Ca^{2+}. The solid phase of calcium carbonate then interacts with the cells covering them with a shell.

'E. natronophila' is a member of the natronophilic endoevaporite microbiota, normally growing in saturated solutions [9], as well as among the minerals. The trona and halite crystals are transparent and may form an acceptable environment for photosynthesis by the endoevaporite microbiota. We have previously demonstrated the possibility of growth of unicellular cyanobacteria in the interstitial water below the precipitate of gypsum and halite (the cells were not embedded in the minerals) [16]. The results of our experiment demonstrate that only the *'E. natronophila'* cells free from the mineral shell are capable of photosynthesis, while the cells embedded in the mineral precipitate are not.

In the endoevaporite soda system, two pathways exist for interaction between the cells and the minerals. Part of the cell population resists embedding into the shell of a mineral ($CaCO_3$ or trona). These cells are therefore able to develop between the mineral crystals under soda endoevaporite conditions (Fig. 8) and are responsible for the primary production in this system. The other part of the population becomes overgrown by minerals, ceases photosynthesis, and probably gradually dies out. Since such fractionation of the cell

Fig. 5. SEM photographs and elemental analysis of *'E. natronophila'* cells: supernatant suspension and Link analysis demonstrating the absence of Ca precipitation on the cells and acicular crystals of potassium chloride (a), mineralized cells of *'E. natronophila'* in the shell of $CaCO_3$ (b), and mineralized cells in trona (14 days) (c).


(a)
Au
Cl
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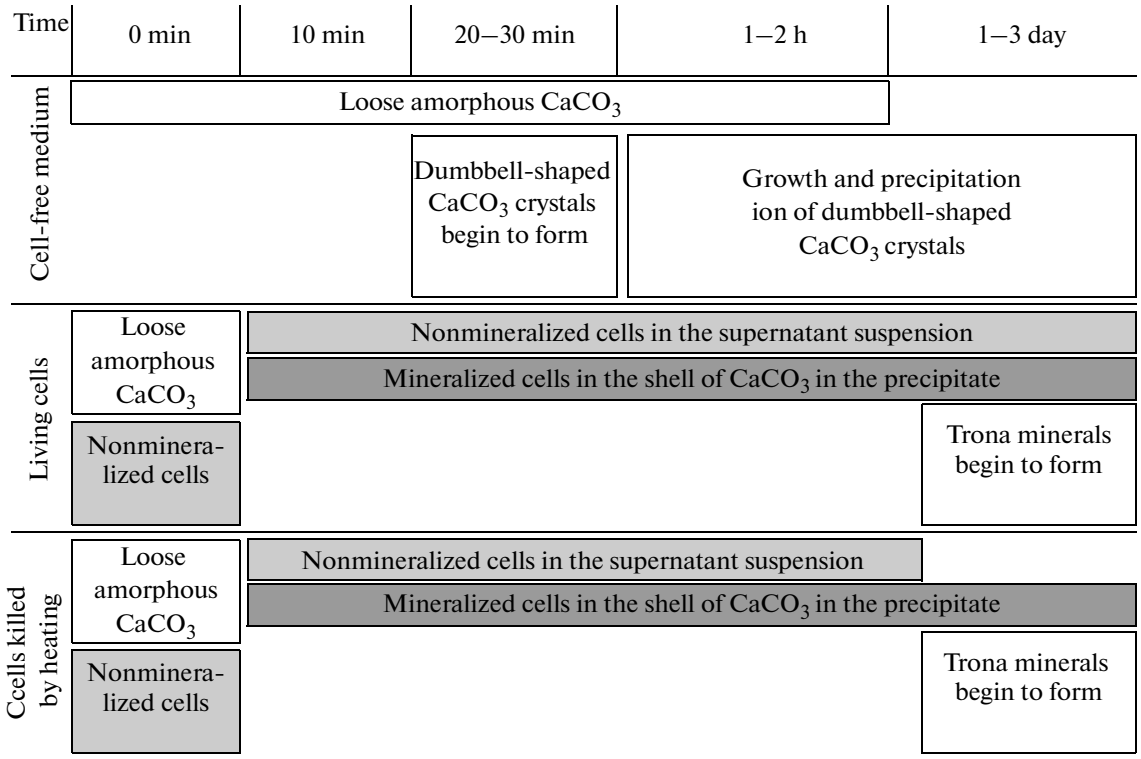

Fig. 6. Schematic representation of $CaCO_3$ precipitation in suspensions of living and cells killed by heating and in cell-free suspension.

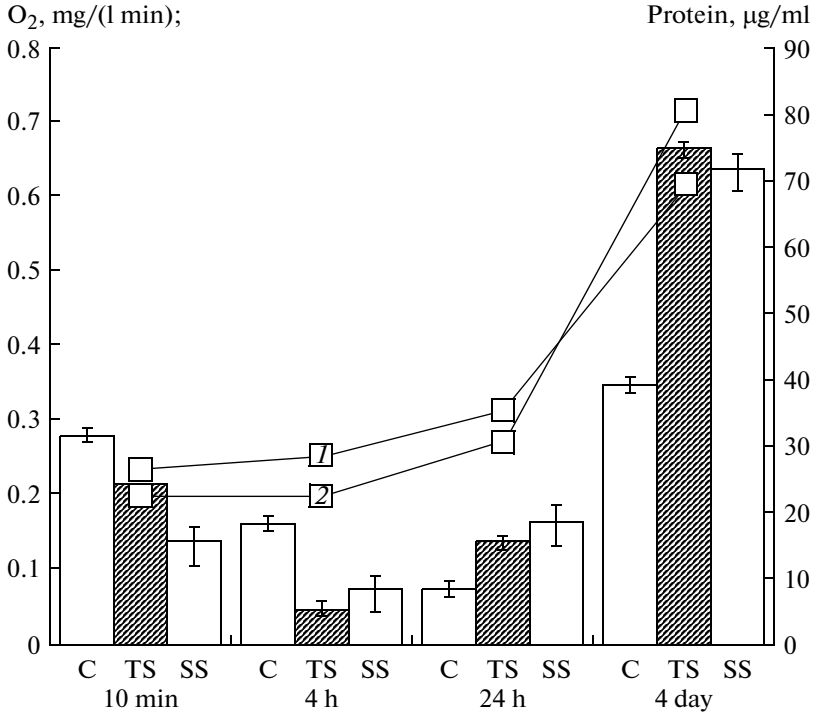

Fig. 7. Growth and photorelease of oxygen by *'E. natronophila'* cells. Columns: control (cells without $CaCl_2$ (C), total suspension (TS), supernatant suspension (SS). Curves: growth as protein accumulation in the control (*1*) and SS (*2*).

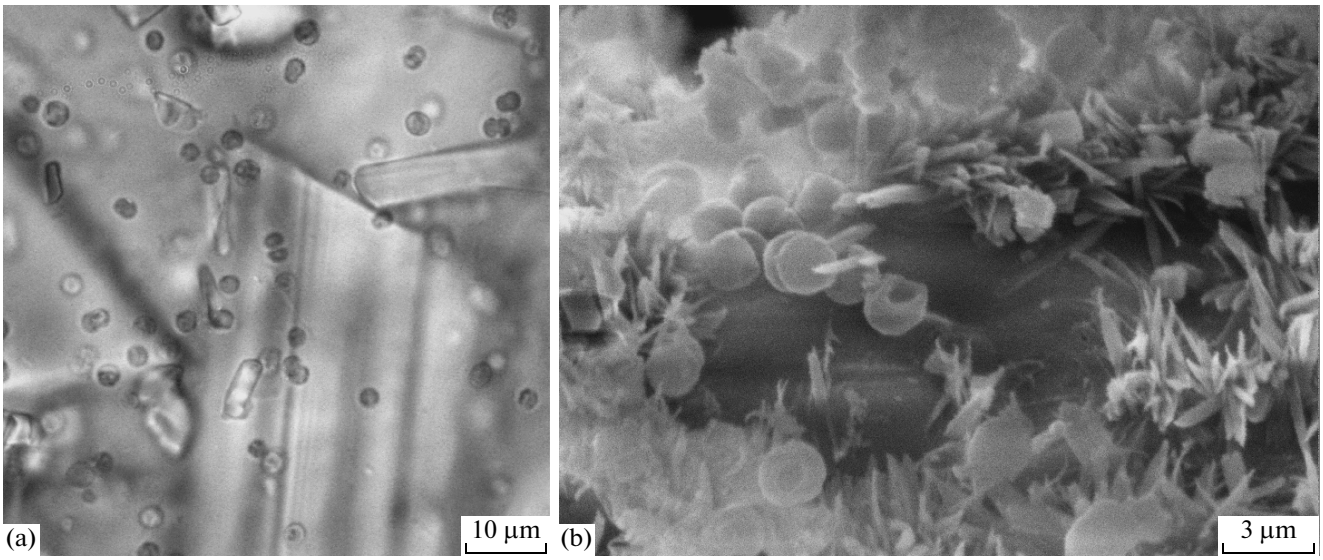

Fig. 8. Live cells of *'E. natronophila'* among rystals of trona: light microscopy (a) and SEM (b).

population occurs both in the living culture and in the suspension of heat-killed cells, the first stage of mineralization (accretion of minerals on the cell) therefore does not depend on the cell viability. The reasons for this fractionation are unclear. Cell envelopes probably play a certain role in the process. Their interaction with minerals and other structural changes in the cells embedded in minerals will be discussed in our next paper [10].

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