Characteristics of Vacuolar Inclusions in *Coelastrella rubescens* Namsu R1 Green Microalgae Cells in Low- and High-Intensity Light

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Abstract—*Coelastrella rubescens* Kaufnerová & Eliás (Chlorophyceae) is a green, single-celled algae that lives in the terrestrial-air environment. Under stress conditions, its cells go into a state characterized by low photosynthetic activity and high content of reserve lipids and secondary carotenoids. For the first time, a comparative morphological, ultrastructural, and elemental analysis of vacuolar inclusions in the *C. rubescens* NAMSU R1 strain when cultivated on a mineral medium under conditions of low and high (causing stress) light intensity. Microalgae cells stained with the fluorescent dye DAPI showed signs of the presence of polyphosphates. Polarization microscopy in cells of *C. rubescens* has identified structures capable of refracting polarized light, which is typical of crystals. Cell analysis of *C. rubescens* with the transmission electron microscopy (TEM) method revealed the presence of various vacuoles with heterogeneous contents (autophagic bodies, crystalloids, and rounded globules of inhomogeneous electron density). With the exception of autophagic bodies noted in cells only in bright light, these inclusions were characteristic of microalgae cells, regardless of the intensity of illumination. The elemental composition of vacuolar inclusions was characterized by TEM in combination with energy-dispersive X-ray spectroscopy: the predominant content of nitrogen, phosphorus, or both elements simultaneously was established in them. The potential physiological role of *C. rubescens* vacuolar inclusions is discussed.

Keywords: *Coelastrella rubescens*, carotenogenic microalgae, polyfunctional vacuoles, polyphosphates, nitrogen inclusions, analytical electron microscopy

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INTRODUCTION

Unicellular green algae of the genus *Coelastrella* (Chlorophyceae, Sphaeropleales) are typical inhabitants of the terrestrial-air environment. They occur as a component of fouling of rocks, buildings, and tree bark and are widespread in temperate and polar latitudes and high mountain regions [1–5]. The ground-air environment is characterized by a combination of a number of environmental factors that are stressful for photosynthetic microorganisms, such as a high level of insolation, sharp fluctuations in humidity and temperature, and a lack of mineral nutrition [6]. Therefore, they have an extensive arsenal of adaptations to adverse conditions. Among them, one can single out the accumulation of light filter compounds that screen radiation in the visible and UV ranges (carotenoids, mycosporin-like amino acids, etc.) [7–10], DNA repair systems [7], enzymes for the elimination of reactive oxygen species [7], effective nonphotochemical quenching of excited states of chlorophyll [10, 11], and reversible reduction of the photosynthetic apparatus [11, 12].

*Coelastrella rubescens* is a typical member of the genus *Coelastrella* [1]. In a number of works, it is considered as a producer of astaxanthin [13, 14], a carotenoid of great practical importance. It is a component of various cosmetics, functional nutrition, and feed for fish and crustaceans in aquaculture [15]. Astaxanthin accumulates in microalgae cells as a secondary carotenoid, i.e., structurally and functionally unrelated to the photosynthetic apparatus. For the NAMSU R1 strain studied in this work, the accumulation of a mixture of carotenoids, astaxanthin, and β-carotene was shown under stress [9]. This circumstance makes research of *C. rubescens*, in particular the NAMSU R1 strain, relevant.

A vacuole is a specific compartment of plant cells characterized by diversity both in terms of the composition of its components and the functions it performs.
They can store biogenic elements and (or) deposit end products of metabolism. Vacuoles may contain inclusions of a different nature, such as oxalates, polyphosphates [16–19], and derivatives of purine nucleotides [17, 20, 21].

A special place in the functioning of the algae cell occupy vacuoles involved in the process of autophagy and selective isolation and degradation of damaged or abnormally folded molecules and organelles as well as components of the latter [11, 22]. Autophagy plays an important role in the acclimation of photoautotrophs to unfavorable conditions [11, 23, 24]. In C. rubescens cells, vacuoles containing chloroplast fragments and autophagosomes fusing with them have previously been described [9]. Moreover, inclusions of different structure and electron density, presumably different chemical nature, have been found in vacuoles of C. rubescens.

The purpose of the work is to study the localization, structure, and elemental composition of vacuolar inclusions in C. rubescens strain NAMSU R1 green microalgae.

**MATERIALS AND METHODS**

**Strain and Cultivation Conditions**

NAMSU R1 strain of green microalgae *Coelastrella rubescens* Kaufnerová & Eliáš was isolated and described earlier [9]. The culture was grown in a periodic mode on the BG–11 medium [25] and under constant illumination of fluorescent lamps with cold white light 40 μmol photons/(m2s (hereinafter referred to as “low-light intensity”), temperature 24°C, and constant stirring at 85 rpm in a New Brunswick Innova 44 shaker-incubator (Eppendorf, Germany) for 15 days. During this period, samples were taken every 1–2 days to record the growth curve. The dry mass was measured gravimetrically according to [26]. Upon reaching the culture of the stationary phase of growth, the suspension was divided into two equal parts, one of which continued to be incubated under the same conditions without stirring. The other part of the suspension was incubated at high light intensity causing stress (150 μmol photons/(m2s). The total incubation period was 36 days.

**Light Microscopy**

The presence of inclusions in cells was detected by optical microscopy. Staining of polyphosphates in cells was performed using a fluorescent dye solution. 4',6-diamidino-2-phenylindole (4’,6-diamidino-2-phenylindole, DAPI) in dimethyl sulfoxide, concentration 1 mg/mL. We added 10 μL of DAPI stock solution to 90 μL of cell suspension and incubated it for 20 min in the dark. Polyphosphate fluorescence was visualized on a Leica DM2500 microscope. (Leica Microsystems, Germany) with a Leica DFC495 camera. Fluorescence was excited by radiation from an HXP 120 UV lamp. (Leica Microsystems, Germany) equipped with a filter D of the same manufacturer, in the range of 355–425 nm. Fluorescence emission was detected in the range of 455–700 nm. Visualization of crystalline inclusions in cells was carried out on a Leica DM2500 microscope with a Leica DFC495 camera in polarized light when the polarizer and analyzer are crossed at an angle 90°.

**Transmission Electron Microscopy (TEM)**

The cell ultrastructure and elemental composition of cell inclusions were studied by traditional transmission electron microscopy (TEM) and analytical TEM, respectively. Cell fixation and dehydration were performed according to the protocol described in Gorelova et al. [27]. Cells were first fixed in 2% (v/v) glutaraldehyde prepared in 0.1 mM sodium cacodylate buffer (pH 7.4) at room temperature for 30 min. Next, postfixation of the cells was carried out in a 1% (by mass) solution of osmium tetroxide prepared in the same buffer for 4 h. The samples were embedded in the Araldite epoxy mixture (Sigma-Aldrich, United States). To study the ultrastructure of cells and the elemental composition of cell inclusions, ultrathin sections and semithin sections were prepared, respectively. Sections were prepared using a Leica EM UC7 ultratome (Leica Microsystems, Germany) and an Ultra 45° diamond knife (DIATOME, Switzerland). The sections were mounted on copper grids for electron microscopy with an ultrathin formvar substrate (Ted Pella, United States).

To study the ultrastructure by TEM, the sections were additionally contrasted with lead citrate solution [28]. The images were obtained using JEM-1011 and JEM-1400 electron microscopes (JEOL, Japan). The dimensions of cell structures were measured using TEM images obtained on ultrathin sections using the Fiji (ImageJ) v. 20200708–1553 (NIH, United States).

The elemental analysis of inclusions by analytical TEM was performed using energy dispersive X-ray spectroscopy (EDX), as described earlier [16], on a JEOL-2100 electron microscope (JEOL, Japan) equipped with a bright-field detector for operation in the scanning TEM (STEM) mode (JEOL, Japan) and X-Max X-ray detector (Oxford Instruments, United Kingdom). For each sample, spectra were obtained from dotted areas of at least ten cells. The spectra were processed using the INKA program (Oxford Instruments, United Kingdom) and presented in the range 0.1–4 keV.

**RESULTS**

**Morphological Features of the Strain**

At low light intensity, culture of microalgae *C. rubescens* NAMSU R1 is represented by solitary coccoid cells and autosporangia 10–15 μm in diameter, stained...
bright green (Figs. 1a, 2a). Under high-intensity light, the cells acquired an orange color, which indicates the accumulation of secondary carotenoids in the cells (Figs. 1c, 2c). Both under standard and stress conditions, autosporangia with autospores were observed (Fig. 1). On the 12th day of growth, the culture reached the stationary phase (Fig. 2).

**Visualization of Cellular Inclusions using Light Microscopy**

In the cells of the strain *C. rubescens* NAMSU R1 cultivated at both variants of the PAR flux intensity, various inclusions were detected using light microscopy (Fig. 1). In cells cultured in low-intensity light, we noted a few rounded structures with a diameter of 0.1–1.3 μm (Figs. 1a, 1b). Similar inclusions were observed in cells cultivated in high-intensity light (Figs. 1c, 1d). To determine the potential chemical nature of the inclusions, the cells were stained with DAPI, a dye that has an affinity for a number of cellular components, in particular, for polyphosphates. In both variants of the experiment in the cytoplasm of cells, fluorescence in the yellow-green region of the spectrum characteristic of polyphosphate granules was observed. The data obtained indicated the accumulation by *C. rubescens* cells of reserves of inorganic phosphorus in the form of polyphosphates in the light of both low and high intensity.

In addition, microalgae cells contained inclusions that did not exhibit fluorescence characteristic of polyphosphates after staining with DAPI (Fig. 1). The

![Fig. 1. Visualization of polyphosphate inclusions after DAPI staining in *C. rubescens* NAMSU R1 cells under illumination with (a, b) low- and (c, d) high-intensity light; (a, c) bright-field microscopy; (b, d) fluorescence microscopy. The glow of chlorophyll has a red tint, colored polyphosphates are yellow-green. Arrows indicate polyphosphate inclusions. The length of the scale bar corresponds to 2 μm.](image)

![Fig. 2. Dynamics of dry mass in *C. rubescens* NAMSU R1 culture during cultivation. Means and standard deviations are shown.](image)
use of polarization microscopy made it possible to visualize these cell structures, which give a bright signal of polarized light (Fig. 3). By visual assessment, the occurrence and abundance of crystals in cells cultured under low intensity light was higher than in cells cultured under high-intensity light.

**Ultrastructural Features of Vacuolar Inclusions**

Cells of *C. rubescens* NAMSU R1 cultivated in low-intensity light had a relatively well-developed photosynthetic apparatus represented by a chloroplast (Fig. 4a) containing one (rarely two) pyrenoid and starch grains. The cells also contained one nucleus and vacuoles with different contents. In cells cultured in high-intensity light, the photosynthetic apparatus was less developed (Fig. 5a). A significant proportion of the area in the sections of such cells was occupied by lipid inclusions of low electron density (oleosomes).

In cells and autospores in sporangia in the case of cultivation in the light of both low (Fig. 4) and high (Fig. 4) intensity, vacuoles with inclusions of various ultrastructure, packing, and electron density were revealed. In the first case, rounded globules 0.1–1.3 μm in size with a material of uniformly high electron density or similar globules with angular, less electron-dense lamellar structures embedded in them, morphologically similar to crystals, were noted (Fig. 4a). In some vacuoles, such small (Fig. 4b) or larger (Fig. 4c) inclusions dominated. Inclusions were also noted that combined, in different proportions, parts of lamellar structures and ordered regions with alternating electron-dense and electron-transparent bands of equal width 4.1 ± 0.2 nm as well as randomly distributed granules (Figs. 4d, 4e). We also encountered vacuoles with electron-dense granules of different sizes, where there were no crystal-like structures (Fig. 4f).

Research of *C. rubescens* NAMSU R1 cells cultured in high light (Fig. 4a) showed the presence of vacuoles with inclusions similar to those found in cells cultured in low light. In particular, vacuoles were noted that contained lamellar crystal-like structures (Figs. 5a, 5b, 5c), vacuoles, in which, in addition to such inclusions, there were formations characterized by alternating bands of low and high electron density (Figs. 5d, 5e) and, in some cases, there were also small globules (from 15 to 50 nm) with an increased electron density (Figs. 5e, 5f). A distinctive feature of the heterogeneous content of vacuoles in cells grown in high-intensity light was the detection of autophagic bodies (Figs. 5a, 5b, 5d).

**Analysis of the Elemental Composition of the Detected Vacuolar Inclusions**

Research of *C. rubescens* NAMSU R1 cells cultivated in light of different intensity revealed the presence of vacuolar inclusions of different types. Figure 6
shows representative spectra for vacuolar structures characterized by the content of predominantly phosphorus (P) (the characteristic X-ray energy is 2.013 keV) (Fig. 6a), nitrogen (N) (0.392 keV) (Fig. 6b), and the joint presence of these chemical elements (Fig. 6c). The spectra of phosphorus-containing inclusions also included peaks of magnesium (Mg) (1.253 keV) and calcium (Ca) (main signal at 3.690 keV) (Fig. 6a), while only the Ca peak was present in the spectra of mixed-type inclusions (Fig. 6c). In the spectra of both phosphorus-containing and mixed-type inclusions, the P peak partially overlapped with the osmium (Os) peak. Osmium tetroxide was used to fix the samples (see Materials and Methods). Spectra with a predominant content of P (Fig. 6a) corresponded to inclusions of high electron density, namely, large vacuolar globules in cells exposed to low-intensity light (Fig. 4a), smaller globules in mixed inclusions (Figs. 4f, 5f), and areas of inclusions with alternating bands of high and low electron density (Figs. 4d, 4e; 5e, 5f) in the light of both illumination levels. The spectra corresponding to a high N content (Fig. 6b) were characteristic of inclu-

Fig. 4. Variety of vacuolar inclusions in C. rubescens NAMSU R1 cells grown in low-intensity light: (a) general view of cells, (b–f) images of vacuoles with inclusions of different structures; (a, d–f) ultrathin sections; (b, c) semithin sections. V—vacuoles; Os—oleosomes; P—pyrenoid; Chl—chloroplast; R—nucleus. Scale bars: (c) 100 nm, (b, c, f) 200 nm, (a) 500 nm.
sions of a crystalline type noted for both cultivation conditions (Figs. 4a–4e, 5). In this case, the point spectra of X-rays containing peaks N and P (Fig. 6c) were obtained from cellular inclusions of a mixed type in the region of boundaries of more electron-dense P-containing structures and less electron-dense (in the case of ultrathin sections) N-containing crystals (Figs. 4b, 4d, 4e, 5b, 5e, 5f).

**DISCUSSION**

In general, features of cell morphology and ultrastructure of *C. rubescens* NAMSU R1 under the described cultivation conditions were similar to those for representatives of the genus *Coelastrella* [1, 3, 9, 13, 14]. In the case of culturing in low-intensity light, the cells exhibited features characteristic of a culture with an active metabolism: a developed photosynthetic apparatus and the presence of a large number of dividing cells. Previously, it was shown that increasing the light intensity to 150 μmol photons/(m²s) (denoted in this work as high-intensity light) led to a decrease in the maximum photochemical efficiency FSII, the reduction of the photosynthetic apparatus, and the appearance of details of secondary carotenoids in the absorption spectra [9]. This is a typical response of terrestrial air microalgae to stress [6, 29]. An indirect indication that the *C. rubescens* NAMSU R1 cells cultivated in high-intensity light were under stress is that they had a characteristic orange color, indicating the accumulation of secondary carotenoids.

Regardless of light intensity, culture research of *C. rubescens* NAMSU R1 using a combination of microscopy methods revealed the presence of vacuolar inclusions of different structure and chemical composition. In particular, vacuolar inclusions were noted, which accumulated mainly one of the elements, P or N, and inclusions enriched both of these elements.

![Fig. 5. Variety of vacuolar inclusions in *C. rubescens* NAMSU R1 cells grown in high-intensity light: (a) general view of cells, (b–f) images of vacuoles with inclusions of different structures; (a, b, d–f) ultrathin sections; (c) semithin section. B—vacuoles; Os—oleosomes; Chl—chloroplast; N—nucleus. Scale bars: (d, f) 100 nm, (b, c, e) 200 nm, (a) 500 nm.](image-url)
represented by alternating bands with high and low electron density and characterized by a P peak in the EDRS spectra, were apparently organized according to the previously described type of “multicore cable” proposed for polyphosphate inclusions [16, 17]. The accumulation of the P reserve in this form is known for many microalgae [16, 17, 30, 31]. The formation of polyphosphates can occur under different conditions of the availability of this element in the medium, both in its deficiency and in its excess (as a result of excessive absorption of P) [30–33].

In cells incubated in low and high light intensity, vacuolar inclusions were noted containing predominantly N and having a crystalline structure (Fig. 2). It is known that the reserve of this biogenic element in microalgal cells can be represented by various derivatives of purine bases, such as microcrystalline inclusions of uric acid [34] or guanine [20, 21]. In recent years, interest has been growing in the deposition of N in the cells of eukaryotic microalgae in the form of crystals of purine bases. Moreover, this form of N storage is found in different taxonomic groups of microalgae [20, 21]. In this work, crystals with a high content of N in the composition of vacuolar inclusions were first discovered in *Coelastrella*. Among the representatives of the Scenedesmaceae family, to which this genus belongs, the formation of N-containing crystals in the cells of microalgae of the genera *Desmodesmus* and *Tetradesmus* was observed [16, 18–21]. At the same time, it was shown that the formation of these vacuolar inclusions occurs under conditions of sufficient or excess N content in the medium for subsequent use under conditions of a deficiency of this element [16, 19, 21]. In this work, the formation of N-containing crystals was noted under conditions of the late stationary phase of *C. rubescens* culture growth at different light intensities. Since an increase in biomass by less than two times was recorded during the cultivation

**Fig. 6.** EDRS spectra of vacuolar inclusions in *C. rubescens* NAMSU R1 cells: (a) phosphorus-containing inclusions, (b) nitrogen inclusions with a crystal-like structure, (c) mixed-type inclusions containing nitrogen and phosphorus.
period, which preceded the transfer of cell biomass under conditions of different levels of illumination, it can be assumed that there is an excess content of nutrients in the medium due to the low cell density. Excessive absorption of nitrates and phosphates from the environment could be toxic to cells without converting them into poorly soluble compounds deposited in their isolating compartment (vacuoles). In addition, it is assumed that guanine crystals have a wider range of functions than N depot, in particular, modulation of the intensity and spectral composition of light available for photosynthesis [21].

Among the representatives of the genus Coelastrella, carotenogenic microalgae [3, 13, 14] capable of accumulating high amounts of secondary carotenoids are known [15]. The presence of rounded electron-dense inclusions in cell vacuoles was shown previously for another carotenogenic algae, *Haematococcus lacustris* (Gir.-Chantar.) Rostaf. (Volvocales, Chlorophyceae) [35]. The authors of the cited publication suggested that they corresponded to inclusions of astaxanthin (English astaxanthin granules) and were involved in the metabolism of secondary carotenoids. Inclusions similar in localization, structure, and electron density were also found in *C. rubescens* in the present work. However, based on the TEM-EDRS analysis of the elemental composition, it can be concluded that they are polyphosphates rather than carotenoid inclusions. Thus, we can conclude that it is necessary to use analytical TEM to characterize vacuolar inclusions and clarify the existing ideas about the nature of certain types of intracellular inclusions in carotenogenic microalgae.

This work characterizes the vacuoles that form in the *C. rubescens* NAMSU R1 cells cultured under normal- and high-light stress conditions. The polyfunctionality of these organelles is shown in connection with the accumulation in them of inclusions differing in structure, functions, and elemental composition. Probably, the vacuoles in this species of microalgae are involved in acclimation to unfavorable environmental conditions, i.e., play the role of “adaptive organelles.” Such organelles provide intracellular homeostasis due to the formation and consumption of intracellular reserves of biogenic elements and also implement emergency rearrangements in the cell according to the mechanism of stress-induced autophagy.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare they have no conflicts of interest. This article does not contain any research involving humans and animals as research objects.

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