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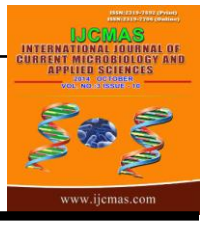

Original Research Article

Adaptation of *Dunaliella viridis* Teodor. to copper sulfate is related with increase of genetic instability and formation of Qwasi-stable states

A.I.Bozhkov[1], N.G.Menzyanova[1], V.Yu.Kizilova[1], Y. A.Kuznetsova[1] and I.F.Kovalenko[2]

[1]Biology Research Institute of the V. N. Karazin Kharkov National University Sq. Svobody, 4, 61022 Kharkov
[2]Institute for Problems of Cryobiology and Cryomedicine 23 Pereyaslavska St., 61015, Kharkiv, Ukraine
**Corresponding author*

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A B S T R A C T

It has been investigated the cellular and biochemical parameters (content and rate of DNA synthesis and nuclear proteins, some indicators of prooxidant and antioxidant system) in two cell cultures *Dunaliella viridis* – sensitive (*CuS D. viridis*) and resistant (CuR_{75}*D. viridis*) to copper ions. It is shown that the most rapid reaction of the cell cultures on introduction of 75 mg / l of copper sulfate is the loss of cell motility, which was accompanied by the formation of cell aggregates and specific epigenotype (pattern of metabolic systems). It was found that the formation resistance to toxic concentrations of copper ions accompanied by an increase of DNA content of 2.2 times, and the nuclear proteins 3 times more compared *CuS D. viridis*. However, the rate of DNA synthesis and nuclear proteins in cells CuR_{75} *D. viridis* was 3 and 6 times lower respectively compared with *CuS D. viridis*.

Introduction

Among stability of metal-ligand complexes series (Irving-Williams series) copper ions occupy the first place, i.e. copper is characterized by higher level of toxicity (Irving and Williams, 1953). Given the steady growth of the world's copper production (in 2013 it amounted to 16.1 million tons), it is the most dangerous biota pollutant. Investigation of mechanisms of toxicity and adaptation to copper ions is relevant.

Toxicity of copper ions is associated with the activation of free radical processes (Pinto et al., 2003; Schwarz et al., 2013), inhibition of the function of the electron-transport chain, respiration, activity of enzymes and other metabolic processes (Yruela et al., 2000; Nikookar et al., 2005; Navari-Izzo et al., 2006; Russo et al., 2008). However, Dunaliella cells are able to adapt to a sufficiently high and toxic concentrations of copper ions (Bozhkov and

Mogilyanskaya, 1996; Bozhkov and Goltvyanskiy, 1998; Levy et al., 2007). Investigation of the mechanisms of adaptation to toxic concentrations of copper ions is of great interest. In this respect, great progress is achieved.

It is shown that in response to copper ion the following processes are induced: copper ions induce the synthesis of phytochelatins (Le Faucheur et al., 2006; Kalinowska and Pawlik-Skowronska, 2010; Machado-Estrada et al., 2013), stress proteins (Torres et al., 2008; Guo et al., 2013), and change the activity of antioxidants (Morelli and Scarano, 2004; Sharma et al., 2012; Fidalgo et al., 2013). We can conclude that there are systemic metabolic changes that are manifested in the morphofunctional changes in cell culture (Bozhkov et al., 2010).

Along with this, it is not clear which of these changes are "primary" and induce a cascade of adaptive responses. One can assume that in present concrete conditions of experiment an abrupt change in the concentration of copper sulphate in the medium induces cooperative cellular changes: loss of mobility, the formation of cell aggregates and the transition to sexual reproduction in *Dunaliella*.

Such changes in the cell population are multiplying the original heterogeneity of culture, which may be accompanied by increased genetic instability. It is known that the population of *Dunaliella* is characterized by morphological and functional, biochemical and genetic heterogeneity (Bozhkov et al., 2014a).

It can be assumed that the expressed cellular heterogeneity provides a wide range of options for future adaptation strategies created to response to extreme environmental factors and genetic instability may play a decisive role in this.

Instability of the genome is not only to be understood as increasing the mutability and as a consequence as functioning unstably. In the base of this instability lay genomic instability can – result the change of the DNA content by conjugation (targeted transfer of DNA from one organism to another), transformation (capture of "foreign" DNA from the environment), transduction (DNA transfer by viruses and plasmids) during sexual process.

In this regard, it can be assumed that highly heterogeneous cell population may use different strategies to adapt and clarify this is of fundamental importance in adaptiogenesis.

In this paper we investigated cell responses (change in mobility, the formation of cell aggregates, respiration rate) from 1 minute to 72 h after the addition of copper in a *D. viridis* culture resistant and susceptible to copper ions; pattern of metabolic changes in these cultures; content and rate of synthesis of DNA, RNA and nuclear proteins in them to identify possible adaptation strategies of *Dunaliella viridis* to toxic concentrations of copper ions.

Materials and Methods

Algological pure culture of *D. viridis* was cultured in Artari medium in the modification of Massjuk (Massjuk, 1973) under the constant light (6.5 klx lamps BL-40) and constant temperature (26-28°C) in a 250 ml Erlenmeyer flat-bottomed conical flasks (20 ml of culture volume). This is a standard cell culture named *CuS D. viridis*, i.e. sensitive to copper ions.

D. viridis resistant to copper ions culture (*CuR_{75} D. viridis*) was obtained in our laboratory and maintained for more than 14 years.

Culturing of *CuR*$_{75}$ *D.viridis* was carried out under the same conditions as *CuS D. viridis*, with the only difference that the $CuSO_4 \times 5H_2O$ was added to a final concentration 75 mg/l.

The dynamics of growth *CuS* and *CuR*$_{75}$ *D. viridis*, specific growth rate, mobility and formation of cell aggregates were determined.

To characterize the metabolic patterns (epigenotype) of sensitive and adapted *D. viridis* cultures on the 21st day of growth the indicators of primary metabolism (DNA, RNA, protein, and protein contents in the nucleus in the cytosol of cells) were used, as well as indicators of antioxidant systems activity: the content of free proline, β-carotene, and prooxidant system – the content of carbonylated protein.

Determining the cell concentration, their mobility and cell aggregates of *CuS* and *CuR*$_{75}$ *D. viridis*

Cell counts were performed in Goryaev chamber. The concentration of cells was expressed in 10^6 cells/ml.

The specific growth rate was calculated from the formula: $\mu=(\ln C_1 - \ln C_0)/(t_1 - t_0)$, where C_1 и C_0– cell concentration at the points of time t_1 and t_0, correspondingly.

CuS- and *CuR*$_{75}$-cultures of *D. viridis* on the stationary growth phase (the 21 day of growth) were transferred on the fresh Artari medium (initial cell concentration is 1,3-1,4 million of cells/ml) and water solution of $CuSO_4 \times 5H_2O$ to final concentration 75 mg/l. After 1 minute, 3, 24, 48 and 72 hours the aliquots for determination of single motionless cells quantity, common quantity of single cells and quantity of aggregated cells were thieved.

For microscopial investigation microalga cells were immobilized by 0,05 % iod alcoholic solution and were snapshoot with digital camera Canon "Digital IXUS 750" through the ocular of light microscope «Biomed-5».

Dying of cells by DAPI

The microalga cells were fixed in 3 % glutar aldehyde in K-phospahte buffer (0,05 M, pH=6,8) with addition of NaCl in concentration by 1 % lower than in cultivation medium (Borowitzka and Siva, 2007). The fixed cells were dyed by DAPI (AppliChem, Germany) during 5 min, the fluorescence was observed by confocal microscope LSM 510 META Carl Zeiss (Germany) with filter set 49 (excitation 445-450 nm, emisstion 365 nm).

Determination of the rate of DNA synthesis and protein in the nuclei, and the protein in the cytosol of cells *CuS* and *CuR*$_{75}$ *D. viridis*

Radioactive label was introduced into the culture of microalgae with cell concentration - 20 million/ml. ^{14}C-leucine and ^{3}H-thymidine (Russia) at 1 MBq dose was added into the culture: 200 μl of the isotope label solution was added to 20 ml of a cell suspension with a concentration of cells 20 million/ml. Exposure time with radioactive label was 45 min. During exposure, the microalgae were under standard culture conditions: at 6.5 klx light, 26 °C. After completion of the incubation, cell suspension was diluted by cool Artari medium (at 4 °C) and the cells were pelleted by centrifugation at 5000 g, 15 min, at 4 ºC. The resulting cell pellets were resuspended in cold Artari medium and centrifuged under the same conditions to remove unbound radiolabel.

Isolation of cell nuclei and determination specific radioactivity of DNA and protein

Isolation of nuclei was performed according to the method (Rizzo and Nooden, 1973) with minor modifications.

After washing the microalgae cell pellets were resuspended in medium buffer (25 mM Tris-HCl buffer, pH 7.5, 0.25 M sucrose, 3 mM EDTA, 5 mM $MgCl_2 \times 6H_2O$, 40 mM KCl, pH = 7.5, at 4 °C. 20 % Triton X-100 to a final concentration 0.4 % was added in the cell suspension and was incubated for 10 minutes at 4 °C. After completion of the incubation, the cell suspension was diluted with buffer to a final concentration of Triton X-100 0.1 % and homogenized in a Potter-Elvehaim electric homogenizer at 4 °C. The homogenate was centrifuged at 3000 g, 10 min, at 4 °C. The supernatant was collected to obtain the cytosol fraction. Pellets of crude nuclei were suspended in buffer and Triton X-100 was added to a final concentration of 0.5 % (for the removal of outer nucleus membrane and fragments of EPR) and was incubated 15 min, at 4 °C. After completion of incubation, the nuclei suspension is centrifuged at 3000 g, 10 min, at 4 °C. After washing the pallets of nuclei were suspended by buffer and chloroform - methanol (1:2) was added. Incubation for 12 h, at 4°C. Samples were centrifuged 3000 g, 15 min. Pellets of the cell nuclei were washed 3 times with 2 ml of 5 % $HClO_4$, centrifuged at 3000 g, 10 min, at 4 °C. In pellets the content of RNA and DNA were determined by Spirin (Spirin, 1958). DNA and RNA content was expressed as μg / million cells.

Aliquots of DNA hydrolysates were neutralized 20 % NaOH (final pH=7.0-7.4) and added to scintillation vials. Radioactivity DNA hydrolysates were determined in dioxane scintillator in radioactivity counter "BETA" (Russia). The specific radioactivity (SRA) of DNA is expressed in cpm/min per 1 mg of DNA.
After hydrolysis of RNA and DNA of nucleus the total protein of the cell was dissolved in 1 N NaOH and the protein content was determined by Lowry (Lowry et al., 1957).

Contents of DNA, RNA, and protein were expressed in μg/million cells.

Aliquots of the alkaline solution of the protein nuclear fraction were added to scintillation vials. The radioactivity of the protein was determined in dioxane scintillator in radioactivity counter "BETA" (Russia). Nuclear protein SRA was expressed in cpm/min per 1 mg of protein.

DNA specific radioactivity was expressed in cpm/min per 1 mg of DNA, specific radioactivity of proteins - in cpm/min per 1 mg protein.

Preparation of cytosol fraction and assessment of the pool of labeled precursors

The supernatant after precipitation of nuclei was centrifuged at 18 000 g, 1.5 h, at 4 °C. The supernatant was collected as cytosol fraction. To determine pool of C^{14}-leucine and ^{3}H-thymidine incorporation in an aliquot of cytosol 60.4 % $HClO_4$ was added to a final concentration of 5 % and incubated at 4 °C, 12 h. The samples were centrifuged at 3000 g, 15 min. The supernatant was used for determining of the pool of ^{14}C-leucine and ^{3}H-thymidine. Aliquots of the supernatant were neutralized with 20 % NaOH (final pH = 7.0-7.4) and added to scintillation vials. Radioactivity was determined in aliquots of dioxane scintillator with a counter radioactivity "BETA" (Russia). Pool ^{14}C-leucine and ^{3}H-thymidine

incorporation was expressed in cpm/10^8 cells. The proteins precipitated from cytosol by 5 % HClO4 were dissolved in 1 N NaOH and the protein content was determined by Lowry (Lowry et al., 1957).

Determination of free proline in the cells *CuS* and *CuR_{75} D. viridis*

Cells *D. viridis* were washed 2 times with a fresh Artari medium by centrifugation at 5 000 g, 15 min. Cell pellets were resuspended in 8 ml of distilled cold H_2O and incubated for 30 minutes at 4 °C. The cell suspension is homogenized, at 4 °C. Homogenates were incubated in a boiling water bath, 10 min, t=100 °C. After incubation, samples were cooled and centrifuged at 3000 g, 10 min. In the supernatant the content of free proline was determined (Bates et al., 1973). The proline content was expressed in μg/million cells.

Determination of carbonylated protein in the cells *CuS* and *CuR_{75} D. viridis*

The cells of *CuS* and *CuR_{75} D. viridis* were washed 2 times with a fresh Artari medium by centrifugation at 5000 g, 15 min. The cell suspension was transferred to plastic centrifuge tubes of 10 ml and centrifuged at 3000 g, 10 min. Carbonylated protein content was determined as described previously (Bozhkov et al, 2011b).

Determination of β-carotene in the cells *CuS* and *CuR_{75} D. viridis*

The cells of *CuS* and *CuR_{75} D.viridis* were washed 2 times with a fresh Artari medium by centrifugation at 5000 g, 15 min. Cell pellets after washing was successively processed with mixtures of organic solvents, as previously described (Bozhkov and Menzyanova, 1999). The chloroform extracts were analyzed by TLC in hexane-diethyl ether (4:1 by volume) on plates Sorbfil (Russia). Fraction of β-carotene fraction eluted with chloroform-methanol (2:1 by volume) was assessed with a spectrophotometer SF-28 (Russia) at λ=440 nm. Content of β-carotene was assessed and expressed in μg/million cells.

All the experiments were repeated not less than 3 times and there were 3 analytical repeats in each experiment.

The results obtained were statistically processed using the Student's t-test.

Results and Discussion

Characteristic of copper ions resistant (75 mg/l $CuSO_4 \times 5H_2O$) cells of *Dunaliella viridis*

From the 1st to the 21st day of growth the number of cells in the control culture, sensitive to copper ions, *CuS D. viridis* increased 20-fold and reached 25-28 millions of cells/ml (Fig. 1A).

Determination of the growth rate of culture resistant to 75 mg/l of copper sulphate (CuR_{75} D. viridis) showed that the number of cells increased 15-fold during the same period (Fig. 1A).

Such delay in the accumulation of biomass is associated with long, up to 3 days, lag period after the transfer of culture to the medium with 75 mg/l of copper sulfate (Fig. 1). The specific growth rate suggested the difference in the duration of the lag time between *CuS* and *CuR_{75} D. viridis* (Fig. 1B). We can assume that such "irregular" specific growth is associated with the change of sexual and asexual reproduction in *CuR_{75} D. viridis* or other factors affecting the proliferative potential of culture and genetic instability.

Consequently, adapted for growth at 75 mg/l concentration of copper sulfate *D. viridis* culture is characterized by delay of start of growth followed by the decrease of the growth rate.

The mobility and size of cells are indicators of the state of *D. viridis* culture. In standard culture 85-95% of the cells are moving and always there is a small number of fixed cells. In the case of sudden changes in the conditions of cultivation the cells lose their mobility and the number of fixed cells can reach up to 100 % (Bozhkov et al., 2010).

Transfer of *CuS D. viridis* on fresh standard medium is also accompanied by the loss of mobility of the part of cells. So, after 1 min in culture there were 20-22 % of fixed cells and their number peaked at 48 o'clock and thereafter decreased and could be from 5 to 10% (Fig. 2A).

In that case, when 75 mg/l of copper sulfate was added in the *CuS D. viridis* culture the 50 % of cells were immobilized after 72 hour (Fig. 2A).

If the same quantity of copper ions was added to the resistant culture – CuR_{75}, the quantity of immobilized cells was the same as in the *CuS*-culture, however the mobility of cells in the case of CuR_{75} after 24 hours backed and didn't differ from the control without copper ions significantly (Fig. 2A). Another important morphological indicator of Dunaliella culture is formation of induced aggregates (Bozhkov et al., 2010).

In *CuS* culture there were no induced aggregates (Fig. 2B). In the case when 75 mg/l of copper sulfate was added to *CuS*-culture, 50% of cells were aggregated after 24 hours (Fig. 2B).

In the case if copper sulfate was added to CuR_{75}, there was twice less of aggregates after 24 hours compared to *CuS*, received copper ions. The quantity of cells in aggregates in the case of CuR_{75} decreased quickly compared to *CuS*-culture (Fig. 2B).
These results suggest: 1 - one of the primary reactions to the addition of high concentrations of copper in medium is a loss of mobility, followed by cell aggregation; 2 – such a stress-response was less pronounced in CuR_{75} *D. viridis*, which is maintained on such medium for many years; 3 - this explains the increase in the lag period in CuR_{75} *D. viridis* compared with *CuS D. viridis*.
It can be assumed that such a primary cellular response of *D. viridis* will be accompanied by the restructuring of metabolism.

Epigenotype characteristics of *Dunaliella viridis* cultures sensitive and resistant to copper ions

To characterize the metabolic pattern - epigenotype of *CuS* and CuR_{75} *D. viridis* cultures the following characteristics of antioxidant systems were determined: the content of free proline and content of β-carotene. To characterize the prooxidant system the content of carbonylated proteins was determined.

It was found that the content of free proline in the cells CuR_{75} *D. viridis* was reduced by 2.8 times, and β-carotene by 5.7 times, compared with *CuS D. viridis* (Fig. 3). It can be assumed that in cells of *D. viridis* CuR_{75} other indicators of antioxidant defense system were reduced. This is supported by an increased content of carbonylated proteins in CuR_{75} *D. viridis* by 40 %, compared with the control (Fig. 3).

Consequently, the formation of resistance of cells to high concentrations of copper

sulphate (75 mg/l) at cell concentration of $1.3x10^6$ cell/ml was followed by inactivation of antioxidative components and increasing products of free radical reactions, i.e. ratio antioxidants/pro-oxidants in the system in changed favor of the second.

Along with this, other metabolic parameters also changed in cells after the addition of copper sulphate (75 mg/l) to CuR_{75}-culture. Thus, the content of DNA in cell nuclei of CuR_{75} *D. viridis* increased 2.2-fold (Fig. 3), RNA increased 2.2-fold in the nuclei of the resistant cells as compared to controls (Fig. 3).

These results suggest that the process of adaptation to high concentrations of copper sulphate accompanied by the formation of the specific adaptive epigenotype and deep restructuring of structural and functional organization of the cell nucleus.

It is known that at the formation of extreme conditions *Dunaliella* cells can switch from asexual to sexual reproduction, which is accompanied by the formation of diploid and tetraploid cells (Oren, 2005). We can assume that in the culture CuR_{75} *D. viridis* the polyploid cells appear (Fig. 4).

Increase of nuclear DNA content by 2.2 times is accompanied by an increase in protein content, which is part of chromatin. The protein content in the cell nuclei of CuR_{75} *D. viridis* culture increased 3.6 times as compared with the control (Fig. 3), i.e. not equivalent as compared with DNA increase.

We cannot exclude other strategies of adaptation to copper ions - reprogramming of metabolism and characteristics of the genome not only due to the formation of polyploid cells, but also due to the changes in the haploid gametophyte. It is important to determine a cause of such a significant effect of increased nuclear protein content: is it a specific answer at the level of cell nucleus or manifestation of the general stress response of cell.

Determination of protein content in the cell cytosol fraction of *Dunaliella* showed that its contents in CuR_{75} *D. viridis* cells was increased only 1.8-fold compared to control (Fig. 3).

Consequently, one of the most pronounced changes in quantitative adapted to copper ions *D. viridis* cells was increase of the protein content in the cell nuclei. One can assume that such a significant increase in nuclear protein is associated with increased synthesis and transport of them from the cytoplasm to the nucleus. In the next series of experiments the specific radioactivity of proteins which were synthesized after 45 min of incubation in 21 day old culture of cells of *CuS* and CuR_{75} *D. viridis* was assessed.

Assessment of the specific radioactivity in the proteins and DNA in CuR_{75} *D. viridis*

Since *Dunaliella* is autotrophic organism, in the first series of experiments the ability of *CuS* and CuR_{75} *D. viridis* to absorb exogenous ^{14}C-leucine and to incorporate it into metabolism was determined. It was found that during 45 min of incubation with exogenous leucine it penetrated into the cells quite well and in an equal amount both to resistant and to sensitive to copper ions cells (Fig. 5).

After 45 min labeled leucine was actively included in the composition of proteins in cells *CuS D. viridis* and the relative radioactivity of nuclear proteins in them was 5.4. (relative units).

Consequently, in control culture the nuclear proteins were synthesized fast as compared to *CuR$_{75}$ D. viridis* and can be referred to metabolically active proteins in 21-day-old culture. It should be noted that 45-minutes exposition with ^{14}C-leucine is sufficient for labeling of total protein in *Dunaliella* cells.

Determination of specific radioactivity of nuclear proteins in *CuR$_{75}$ D. viridis* in the same conditions showed that it was by 21 times lower than in *CuS D. viridis* (Fig. 5).

Such a significant difference in specific radioactivity of nuclear proteins between *CuS* and *CuR$_{75}$ D. viridis* can be explained by that the nuclear proteins in *CuR$_{75}$* were synthesized before radioactive label addition i.e. are the precursor proteins and time character of synthesis and transport in *CuS* and *CuR$_{75}$ D. viridis* was different. To understand this complex results the specific radioactivity of cytosol proteins i.e. protein of compartment where the synthesis of all the cellular proteins occurs was determined. The specific radioactivity of cytosol proteins in *CuR$_{75}$ D. viridis* cells was found to be by 2.7 times more (Fig. 5) than specific radioactivity in *CuS D. viridis* cells, and at the same time the relative radioactivity of proteins in them was 3.2 times higher compared to *CuS*-culture.

It should be noted the different relations of specific radioactivity of cytosol proteins and cell nuclei. So, the specific radioactivity of cytosol proteins in *CuS D. viridis* cells was by 8 times less than specific radioactivity of nuclear proteins suggesting the high speed of transport of newly synthesized proteins in cell nuclei in control culture. At the same time the specific radioactivity of cytosol proteins in *CuR$_{75}$ D. viridis* cells was by 6 times higher compared to nuclear proteins of these cells suggesting the newly synthesized proteins were not transported in nuclei of *CuR$_{75}$ D. viridis* cells.

Consequently in cell nuclei of *CuR$_{75}$ D. viridis* there are a lot of not metabolically active proteins. Such "discordance" of content and synthesis speed of nuclear proteins in *CuR$_{75}$ D. viridis* allows to suppose that protein content increase is caused not only by DNA content increase but also by "reconstruction" of nuclear organization possibly by means of relative protein contents increase in nucleoplasma and other nuclear compartments.

The data obtained allow to suppose that metabolic processes in cells adapted to copper ions are slower as compared to control culture or the temporal character of synthesis and transport of nuclear proteins in *CuS* and *CuR$_{75}$ D. viridis* differs widely. It was of interest to determine the rate of synthesis of DNA in *CuR$_{75}$ D. viridis* cells against its twofold increase compared to *CuS* (Fig. 6A).

The increase of nuclear DNA content in cells of CuR75 culture is suggested conclusively by the DAPI fluorescence in cell nuclei of CuR$_{75}$, compared with CuS (Fig. 6B).

To determine the DNA synthesis rate the radioactive ^{3}H-timidine with 45 minutes exposition was used. The specific radioactivity of DNA in *CuR$_{75}$ D. viridis* cells was found to be 3.7 times lower compared to the control (Fig. 6). These results suggest the rate of synthesis both nuclear proteins and DNA is lower in cells resistant to copper ions. Consequently, in the nuclei of CuR$_{75}$ of *D. viridis* the same discordance on the increase of the content and specific radioactivity both of protein and of DNA was observed.

Results of the present work allow us to conclude that *Dunaliella* culture can adapt to lethal concentrations of copper sulphate.

Such adaptation provided resistance of *Dunaliella* culture to a high temperature (Bozhkov et al., 2011a) and had a character of hormesis (Kovaleva et al., 2012). Consequently, the CuR_{75} culture *D. viridis* is characterized by new functional properties.

The central issue of the problem of adaptation to extreme conditions is to understand the mechanisms of resistance. Numerous recent studies have shown that the induction of resistance was accompanied by the synthesis of specific stress proteins (Torres et al., 2008), phytochelatins (Machado-Estrada et al., 2013), alteration of membrane systems (Janicka-Russak et al., 2012). Due to these works a modern molecular biology paradigm has developed. The backbone of it is that the primary response to external influence is the extreme response of molecular systems - changes in gene expression (Chen et al., 2011), enzyme activity (Lozano et al., 2014), the protein-synthesizing apparatus (Li et al., 2013), activation of pro- and antioxidant systems (Morelli and Scarano, 2004; Sharma et al., 2012), which trigger processes and hierarchical changes in the cell.

In addition, changes in molecular systems cells can not provide adaptation to extreme influences by themselves or, in other words, adaptation is not a simple sum of the changed parameters. It has been suggested that resistance to high concentrations of copper sulphate in *Dunaliella* was the result of manifestation of emergent properties of different functional systems (Kovaleva et al., 2012).

Numerous observations of the primary response of microalgae cells to extreme exposure revealed that the earliest primary response is an integral cellular response. Or in other words, the cell responds to an extreme factor as supramolecular system, and already on the background of changing physical and chemical characteristics changes in molecular systems implemented, which form a new adaptive metabolic pattern (Bozhkov et al., 2014b).

Thus, already in the first few minutes after replacing the culture medium or the addition of toxicants in medium or environmental temperature changes the part of cells lose mobility. Loss of mobility is accompanied by changes in the integral metabolic rate and physical and chemical characteristics of the intracellular medium, and already on this background molecular restructuring and forming of a specific epigenotype are realizing (Rostama et al., 2012).

Consequently, the primary response of cell cultures to extreme impact is realizing on the cellular level, i.e. on intermolecular level, which is manifested as a single cooperative system response.

The choice of the adaptation strategy is carried out on the first step of the response of cell cultures. So, for *Dunaliella* cultures the following adaptation strategies can be implemented: reprogramming of the metabolic system; transition to sexual reproduction path; quasi-stable transition in critical condition and finally mixed version of adaptation strategies.

The results obtained suggest that the abrupt increase of copper ions content in the culture medium, which is added immediately after transplanting of culture to fresh medium, is accompanied by the manifestation of stress reaction, which is caused by the change of environment and a high content of copper ions. Thus, some cells are destroyed and their contents are released to the environment, as are proteins, nucleic acids and other cellular components. Most of cell loose mobility, they changed the plasmolemma permeability at least for dyes

and cell aggregates were formed in the presence of copper ions. These quasi-stable states are characterized by high genetic instability.

Part of the cells enters into the sexual process which is accompanied by the formation of polyploid and tetraploid cells. Consequently, the stress-response, a change due to the culture medium and the presence of high concentrations of copper ions leads to formation of a high degree of cell heterogeneity. In such culture the destroyed cells, cells with reprogrammed adaptive metabolism, polyploid cells after the conjugation of gametophytes and cells in quasistable state with partially broken plasma membrane emerge. At the moment, we cannot say what proportion of each possible cell variant is. But it can be argued that in the process of further long-term culturing such cell heterogeneity will decrease - the effect of selective adaptation. Culture "chooses" one of the possible adaptation strategies, or one attractor, which provides its functioning in the changed conditions.

We can assume that in the *Dunaliella* culture, which is supported on a medium containing 75 mg/l $CuSO_4x5H_2O$ there is periodic triggering between stable cell states and quasi-stable-critical states. The quasi-stable-critical states are characterized by high cell heterogeneity, genomic instability and the formation of specific epigenotype.
In our experiments to be understood the source of genome instability is it only the mutability, but also the change in DNA content. In microalgae cells the DNA content may vary due to amplification or endoreplication or diminution, change of asexual reproduction to sexual.

Increasing of the DNA content in cells CuR_{75} *D. viridis* at low DNA specific radioactivity, i.e. without its synthesis, can be explained by the conjugation of two haploid vegetative cells to form a diploid or tetraploid cells, i.e. with the transition to sexual reproduction or horizontal transfer of DNA, i.e. penetration of the DNA of microorganisms present in the medium with *Dunaliella* cells, when they are in critical quasistable state condition or epigenetic changes induced genomic instability.

It is known that under extreme environmental changes, particularly at high concentrations of heavy metal ions gene expression of HSP70 increased. This leads to an increase in the frequency of transposition of mobile elements, consequently the expression of HSP70 stimulates recombination and transposition, increasing genetic diversity of the population (Kolchanov et al., 2003). This, in turn, will contribute to the selection of new genetic variants.

Consequently the formation of quasi-stable cell state play an important role in the choice of adaptation strategies. Change of asexual reproduction to sexual also increases the genetic variability in populations. It is difficult to explain the contradiction revealed in the content and the rate of synthesis of proteins of cell nuclei. Low rate of synthesis of nuclear proteins on the background of increase in their quantity can be provided by several mechanisms: transport of proteins prior to radiolabeling; transport of nonspecific prior existing proteins to the nucleus and the formation of the 'specific' nuclei in the sexual process or quasi-stable states. The solution of this issue requires special studies. However, regardless of the mechanisms of transformation of *Dunaliella* cells, adapted to life in extreme conditions, it can be suggested that the restructuring of the genome, its instability is an important factor in their adaptiogenesis and evolution.

Fig. 1 The quantity of cells in *D. viridis* culture from the zero to the 21st day of cultivation (A) and the specific growth rate (B) in the case of sensitive – *CuS* (1) and resistant – CuR_{75} (2) cultures

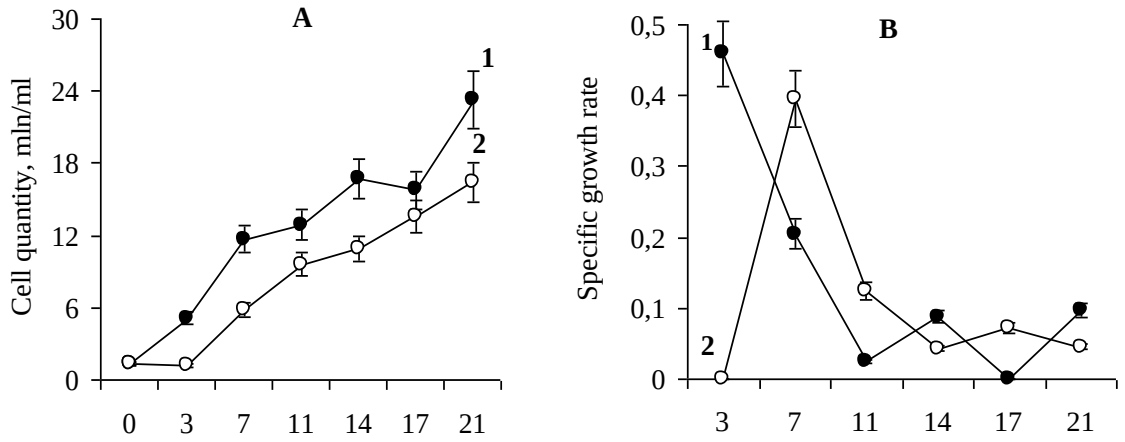


Fig.2 The quantity of immobilized cells (A) and quantity of cells in aggregates (B) in control culture – *CuS* from the 1st minute to 72 hours after transfer to the new standard medium of cultivation (1), in control culture after addition of copper sulfate to the medium to 75 mg/l (2) and in adopted to copper ions culture – CuR_{75} after addition of the new portion of copper sulfate to 75 mg/l (3). On the photos the control culture *CuS* without copper ions addition (a) and 24 hours after 75 mg/l of copper sulfate addition (b) are presented

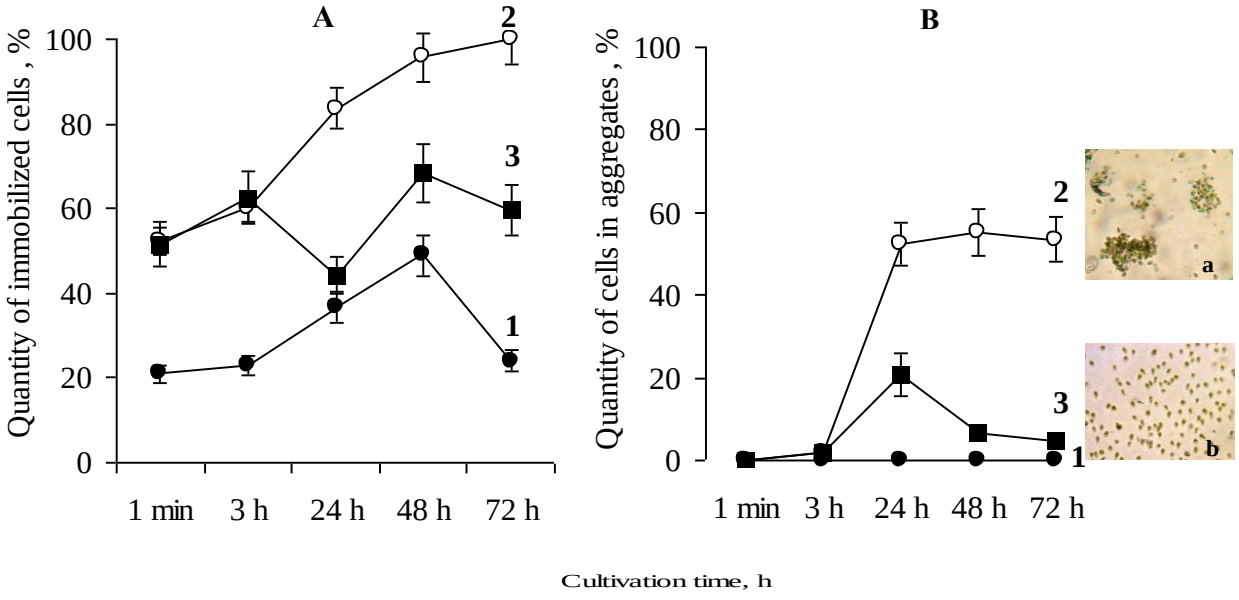


Fig.3 The pattern of indicators (epygenotype) of primary metabolism: content of nuclear DNA (μg/mln of cells, ÷2), nuclear RNA (μg/mln of cells), nuclear protein and cell cytosol protein (μg/mln of cells, x4); products of free-radical reactions: carbonylated proteins (nM/mg of protein); components of antioxidant protection: proline (μg/mln of cells, ÷2) and β-carotene (μg/mln of cells) in cells sensitive to copper ions – *CuS* and resistant to it – *CuR*

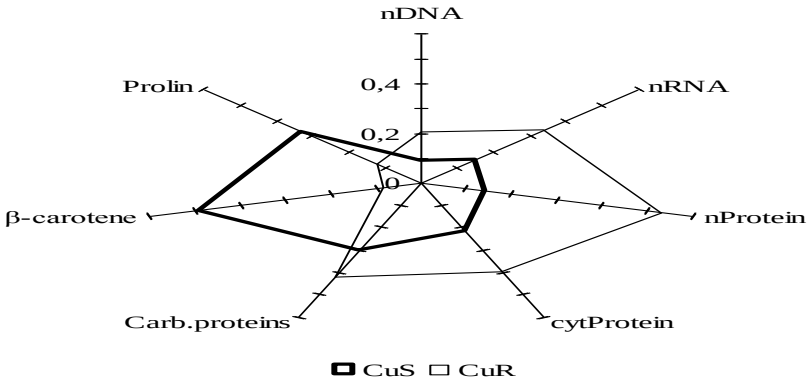


Fig.4 The cyst formation in CuR_{75} culture of *D. viridis*

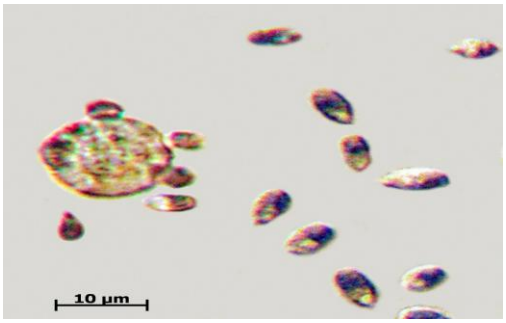


Fig.5 The specific radioactivity (SR) of aminoacids pool in cytosol 45 minutes after administration of radioactive leucine (A), the specific radioactivity of nuclear proteins (B) and specific radioactivity of cytosol proteins (C) in cells of CuS and CuR_{75}-cultures of *D. viridis*

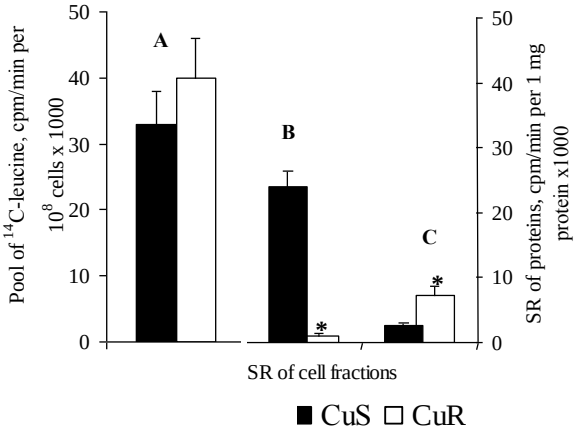
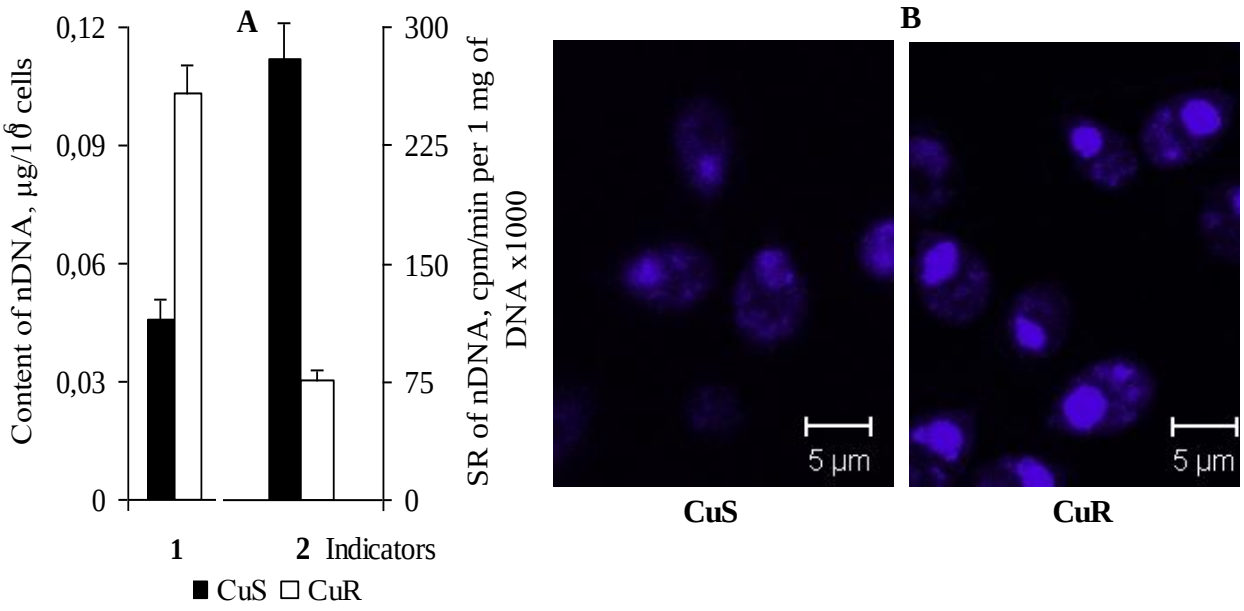


Fig.6 The content of nuclear DNA (1) and specific radioactivity of nuclear DNA (2) in cells of CuS and CuR *D. viridis* after 45 minutes after radioactive thimidin administration and DNA fluorescence in cells of CuS and CuR dyed by DAPI



References

Bates, L. S., Waldren, R. P., Teare, I. D. 1973. Rapid determination of free proline for water-stress studies. Plant Soil. 39: 205-207.

Borowitzka, M. A., Siva, C. J. 2007. The taxonomy of the genus *Dunaliella* (Chlorophyta, Dunaliellales) with emphasis on the marine and halophilic species. J. Appl. Phycol. 19: 567–590.

Bozhkov, A. I., Mogilyanskaya, S. M. 1996. Adaptation of *Dunaliella viridis* Teod. to different concentration of the copper sulfate. The role of systems of copper ions excretion in medium. Algologia, 6 (2): 122–132 (in Russian).

Bozhkov, A. I., Goltvyanskiy A. V. 1998. Induction of resistance to copper sulfate in *Dunaliella viridis* Teod. Algologia. 8(2): 162-169 (in Russian)..

Bozhkov, A. I., Menzyanova, N. G. 1999. Growth dynamics, lipid composition, and (β-carotene content in cells of *Dunaliella viridis* Teod. under cultivation in different types of photobioreactors. International Journal on Algae.1(3): 31-39.

Bozhkov, A. I., Goltvyanskiy, A. V., Rostama, Sh. 2010. Induced aggregation of cells *Dunaliella viridis* Teod. by half-lethal concentration of copper ions in the culture. Algologia. 20 (2): 151-166 (in Russian).

Bozhkov, A. I., Menzyanova, N. G., Sedova, K. V., Goltvyanskiy, A. V. 2011a. The influence of High Temperature on Cells of *Dunaliella viridis* (Teod.) (*Chlorophyta*) Sensitive and Resistant to Copper Ions. International Journal on Algae.13(3): 203- 222.

Bozhkov, A. I., Kovaleva, M. K., Menzyanova, N. G. 2011b. Is it possible to "cancel" the aging process of cell cultures by creating optimal conditions of cultivation? Advances in Gerontology. 24(1): 26–37 (in Russian).

Bozhkov, A. I., Menzyanova, N. G., Kovaleva, M. K., and Pyatak N. I. 2014a. Growth of Dunaliella in "ideal" conditions retains annual variability of biochemical features. Universal Journal of Plant Science. 2(2): 31-39.

Bozhkov, A. I., Kovaleva, M. K., Menzyanova, N. G., Kuznetsova, Y. A. 2014b. The Rate of Aging of the *Dunaliella viridis* Teodor. Culture Depends on the Algae Pre-Adaptation to Copper Sulfate Toxic Effect. Advances in Aging Research. 3: 187-198.

Chen, H., Lao, Y. M., Jiang, J. G. 2011. Effects of salinities on the gene expression of a (NAD+)-dependent glycerol-3-phosphate dehydrogenase in *Dunaliella salina*. Science of the Total Environment. 409:1291–1297.

Fidalgo, F., Azenha, M., Silva, A. F., Sousa, A., Santiago, A., Ferraz, P., Teixeira, P. 2013. Copper-induced stress in Solanum nigrum L. and antioxidant defense system responses. Food and Energy Security. 2(1): 70-80.

Guo, R., Lee, M. A., Ki, J. S. 2013. Different transcriptional responses of heat shock protein 70/90 in the marine diatom *Ditylum brightwellii* exposed to metal compounds and endocrine-disrupting chemicals. Chemosphere. 92 (5): P. 535-543.

Irving, H., Williams, R. J. P. 1953. The stability of transition-metal complexes. J. Chem. Soc.: 3192–3210. doi:10.1039/JR9530003192.

Janicka-Russak, M., Kabala, K., Burzynski, M. 2012. Different effect of cadmium and copper on H^+-ATPase activity in plasma membrane vesicles from *Cucumis sativus* roots. J. Exp. Bot. 63 (11): 4133-4142.

Kalinowska, R., Pawlik-Skowronska, B. 2010. Response of two terrestrial green microalgae (Chlorophyta, Trebouxiophyceae) isolated from Cu-rich and unpolluted soils to copper stress. Environmental Pollution. 158: 2778-2785.

Kolchanov, N. A., Suslov, V. V., Shumniy V. K. 2003. Molecular evolution of genetic systems. Journal of Paleontology. 6: 58-71 (in Russian).

Kovaleva, M. K., Menzyanova, N. G., Jain, Anshu, Yadav, Abhishek, Flora, S. J. S., Bozhkov, A. I. 2012. Effect of hormesis in *Dunaliella viridis* Teodor. (*Chlorophyta*) under the influence of copper sulfate. International Journal on Algae. 14(1): 44-61.

Levy, J. L., Stauber, J. L., Jolley, D. F. 2007. Sensitivity of marine microalgae to copper: The effect of biotic factors on copper adsorption and toxicity. Science of the Total Environment. 387: 141–154.

Le Faucheur, S., Schildknecht, F., Behra, R., Sigg, L. 2006. Thiols in Scenedesmus vacuolatus upon exposure to metals and metalloids. Aquatic Toxicology. 80: 355–361.

Li, G., Peng, X., Xuan, H., Wei, L., Yang, Y., Guo, T., Kang, G. 2013. Proteomic analysis of leaves and roots of common wheat (*Triticum aestivum L.*) under copper-stress conditions. *J. Proteome Res.* 12(11): 4846–4861.

Lowry, O. B., Rosebrough, N. J., Farr, A. L., and Randall, B. J. 1957. Protein measurement with Folin phenol reagent. Biol. chem. 193: 265–273.

Lozano, P., Trombini, C., Crespo, E. Blasco, J., Moreno-Garrido, I. 2014. ROI-scavenging enzyme activities as toxicity biomarkers in three species of marine microalgae exposed to model contaminants (copper, Irgarol and atrazine). Ecotoxicology and Environmental Safety. 104: 294–301.

Machado-Estrada, B., Calderon, J., Moreno-

Sanchez, R., Rodriguez-Zavala, J. S. 2013. Accumulation of arsenic, lead, copper, and zinc, and synthesis of phytochelatins by indigenous plants of a mining impacted area. Environ. Sci. Pollut. Res. 20: 3946–3955.

Massjuk, N. P. 1973. Morphology, systematics, ecology, geographical distribution of the genus Dunaliella Teod. and the prospects for its practical use. Kiev, Naukova Dumka press, p. 243 (in Russian).

Morelli, E., Scarano, G. 2004. Copper-induced changes of non-protein thiols and antioxidant enzymes in the marine microalga *Phaeodactylum tricornutum*. Plant Science. 167(2): 289–296.

Navari-Izzo, F., Cestone, B., Cavallini, A. et al. 2006. Copper excess triggers phospholipase D activity in wheat roots. Phytochemistry. 67: 1232–1242.

Nikookar, K., Moradshahi, A., and Hosseini, L. 2005. Physiological responses of Dunaliella salina and Dunaliella tertiolecta to copper toxicity. Biomolecular Engineering. 22: 141–146.

Oren, A. 2005. A hundred years of Dunaliella research: 1905–2005. Saline Systems. 1(2): 1-14.

Pinto, E., Sigaud-Kutner, T. C. S., Leitao, M. A. S., Okamoto, O. K., Morse, D., Colepicolo, P. 2003. Heavy metal-induced oxidative stress in algae. J. Phycol., 39: 1008–1018.

Rizzo, P. J., Nooden, L. D. 1973. Isolation and chemical composition of Dinoflagellate nuclei. J. Protozool. 20 (5): 666-672.

Rostama, Sh., Bozhkov, A. I., Goltvyanskiy, A. V. 2012. The effect of copper, lead and cadmium ions on induced aggregation in cells of Dunaliella viridis (Teodor.) (Chlorophyta). International Journal on Algae. 14(2): 136-150. DOI: 10.1615/ Inter J Algae. V14.i2.30.

Russo, M., Sgherri, C., Izzo, R., and Navari-Izzo, F. 2008. Brassica napus subjected to copper excess: Phospholipases C and D and glutathione system in signaling. Environmental and Experimental Botany. 62: 238–246.

Schwarz, J. A., Mitchelmore, C. L., Jones R., O'Dea, A., and Seymour, S. 2013. Exposure to copper induces oxidative and stress responses and DNA damage in the coral *Montastraea franksi*. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology. 157(3):272–279.

Sharma, P., Jha, A. B., Dubey, R. S., and Pessarakli, M. 2012. Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. Journal of Botany. 2012. Article ID 217037, 26 pages. DOI: 10.1155/2012/217037.

Spirin, A. S. 1958. Spectrophotometric determination of total nucleic acids. Biokhimiya. 23: 656-662 (in Russian).

Torres, M. A., Barros, M. B., Sara C. G., Pinto, E., Rajamani, S., Sayre, R. T., Colepicolo, P. 2008. Biochemical biomarkers in algae and marine pollution: A review. Ecotoxicology and Environmental Safety. 71: 1-15.

Yruela, I., Alfonso, M., Baron, M., and Picorel, R. 2000. Copper effect on the protein composition of photosystem II. Physiol. Plant. 110: 551–570.