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### OXIDATIVE STRESS IN PHYCOBIOTECHNOLOGY -MECHANISMS AND REGULATION PROCESSES

### 167.01 – BIOTECHNOLOGY, BIONANOTECHNOLOGY

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### **CONCEPTUAL LANDMARKS OF THE RESEARCH**

**Relevance and Importance of Research:** Microalgae and cyanobacteria are attractive for various applications due to their diversity, adaptability, and valuable composition of their biomass, [9, 18]. Production of phycological biomass for various purposes aligns well with the major global shift towards the environmentally and human-friendly bioeconomy. Microalgae and cyanobacteria are a valuable source of proteins, vitamins, microelements, lipids, and carbohydrates [16]. Several species, such as *Arthrospira platensis* Gomont 1892, *Nostoc linckia* Bornet ex Bornet & Flahault 1886, *Porphyridium cruentum* (S.F.Gray) Nägeli 1849, *Dunaliella salina* (Dunal) Teodoresco 1905, *Haematococcus pluvialis* Flotow 1844 are recognized as safe for human and animal consumption [4, 11, 17, 26, 28, 29]. Some, like *Arthrospira platensis*, are evaluated from the nutritional point of view as complete foods.

Microalgae and cyanobacteria are suitable raw materials for obtaining various food and pharmaceutical products [15, 23, 27]. The productivity of phycological objects significantly surpasses the productivity of crop plants and can be obtained from significantly reduced areas of cultivation. Additionally, there are numerous biotechnological tools (various specific stimulants, including nanomaterials, sets of physical conditions determining specific responses, advanced genetic engineering, etc.) that are available for elaborating phycobiotechnologies of a much higher efficiency than in the traditional cases [3, 14, 24]. Even though all these tools are meant to improve the biotechnological properties of microalgae and cyanobacteria, they can induce adverse reactions too. Of the primary concern here is the oxidative stress that can be generated by overaccumulation of the reactive oxygen species (ROS) [5, 22]. The primary radicals' development is an inevitable and necessary process for the living organisms. These radicals play a crucial role in signal transduction, cell growth and differentiation, apoptosis, etc. [25]. The cell antioxidant systems are adapted to certain ROS concentration levels within which they can efficiently maintain the redox balance of the normal physiological conditions [20, 21]. Oxidative stress occurs when various factors cause induce secondary and tertiary radicals in quantities that overwhelm the antioxidant system, making the latter unable to eliminate the radicals. The effects of the oxidative stress depend on its intensity. In some cases, the oxidative stress can be used for biotechnological purposes. The technological conditions of phycological biomass production can also initiate stress and cause imbalances between the free radicals and antioxidants in the biomass of cyanobacteria and microalgae. When that happens, the produced biomass accumulates (besides various valuable compounds) potentially harmful free radicals and products of oxidative degradation of organic compounds [10, 12, 30]. Understanding and effective management of potential hazards are essential for the safe application of biotechnologies and their products.

Cyanobacteria and microalgae in their natural habitats are often exposed to changing external conditions, such as drastic fluctuations in light intensity and temperature. To survive in such environment, they use intrinsic mechanisms that allow ROS detection and to rapid initiation of the antioxidant defense. These mechanisms can be used in the intensive technologies of the microalgal and cyanobacterial biomass production – to ensure its safety for humans and animals in terms of the presence of products of the oxidative degradation of macromolecules.

Cyanobacteria and microalgae can suffer from stress of various intensity after exposure to xenobiotics. In such cases the organisms use different ways to reduce the xenobiotic toxicity, such as transformation of aggressive ions into their zero-valent forms (e.g., via biosynthesis of metal nanoparticles), sequestration of the toxicants within specific structures, cellular efflux, production of substances that neutralize the free radicals and reactive molecules appearing because of the stress factors [1, 6, 19]. Understanding the mechanisms underlying these processes will open perspectives to use the phycological objects for remediation of polluted environments and for nanobiosynthesis.

**Purpose of the thesis:** Identification the common responses of microalgae and cyanobacteria to various types of the induced oxidative stress, and grounding the possibility of using the oxidative stress as a phycobiotechnology tool.

### **Objectives:**

• To identify the peculiarities of the oxidative stress induced by physical and chemical factors in cyanobacteria and microalgae of biotechnological interest.

• To estimate the possibility of using the response to the induced oxidative stress for obtaining phycological biomass with predicted composition.

• To estimate the possibility of using microalgae, cyanobacteria, and the oxidative stress in remediation of water contaminated by heavy metals and in the iterative systems.

• To ground the principles of nanobiosynthesis and biofunctionalization of nanoparticles based on the protective mechanisms against the oxidative stress in microalgae and cyanobacteria.

• To assess the possibilities and limitations of using the response to the oxidative stress as a tool in phycobiotechnology.

• To elaborate phycological procedures based on the application of the response to the induced oxidative stress.

**Research hypothesis:** The response of phycological cultures to the induced oxidative stress can be a useful tool for obtaining valuable biomass with controlled composition, for nanoparticle biosynthesis and biofunctionalization, and for remediation of polluted environments. The

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application of this tool is conditioned by the intensity of the antioxidant protection processes, as well as by maintaining a balance between beneficial effects and the accumulation of reactive species.

**Synthesis of the research methodology and justification of selected methods:** Three cyanobacteria and three eukaryotic microalgae strains were used as the study objects of the thesis. The common responses of these two domains of living organisms to the oxidative stress were identified. The selected strains are characterized by biotechnological importance and flexibility for the biotechnological workflows. They are currently used in the local/regional biotechnological production (based on the preceding research results).

The research was done by standard methods in the field of phycobiotechnology that were carefully adapted for the selected strains. The methods were chosen based on rigorous scientific argumentation. They corresponded well to state of art in the field, and to the current methodological standards.

Scientific novelty and originality: The originality of the work is using the response of microalgae and cyanobacteria of the biotechnological interest to the oxidative stress as a tool for directing the phycobiotechnological processes. This response was used for elaborating technologies of obtaining phycological biomass with targeted valuable content. The antioxidant protection reactions of microalgae and cyanobacteria cultures to stress conditions were used for biosynthesis of silver and selenium nanoparticles, as well as for biofunctionalization of gold and silver nanoparticles. For the first time it was demonstrated that water polluted by heavy metals can be decontaminated in the process of iterative cycles of cyanobacteria cultivation, using the efficiency of their antioxidant protection systems as a reference. There were identified new indicators of low-intensity oxidative stress, including the  $\alpha$ -chlorophyll/ $\beta$ -carotene ratio and the strong negative correlation between antiradical capacity and the level of malondialdehyde (MDA). There were established criteria for distinguishing between the low-intensity and the mediumintensity oxidative stress based on the ratio between the intensity of a factor or the time of its action on the one hand and the phycological culture response on the other. Thus, the obtained results confirmed the research hypothesis that the induced oxidative stress can be used as an efficient tool for controlling the phycobiotechnological processes.

The main results that contribute to address the significant scientific issue provide evidence-based argumentation for application of the response of cyanobacteria and microalgae to the induced oxidative stress as a biotechnological tool for development of original procedures in: biosynthesis of nanoparticles, including their biofunctionalization; obtaining of high-quality phycological biomass with a controlled composition of bioactive compounds that are safe for humans and animals; bioremediation of the effluents contaminated by heavy metals. Thus, a new research direction was founded: *the oxidative stress as a tool in ficobiotechnology*.

**Theoretical significance:** Conceptual framework for using the oxidative stresses of varying intensity as a tool for directing the phycobiotechnological processes was established. New indicators and criteria were elaborated to determine the possibilities of application of the oxidative stress in the domain of phycobiotechnology. The mechanisms to induce the oxidative stress, as well as the common response reactions of microalgae and cyanobacteria to the stress (such as the imbalance in the antioxidant protection system generated by the uncoordinated activity of first-line antioxidant enzymes and the alteration of gene expression associated with stress) were identified. New safety and quality control indicators were offered for the applications of the low-intensity oxidative stress as a phycobiotechnological tool.

**Applied value of the thesis:** The obtained conceptual knowledge of the response of microalgae and cyanobacteria to the induced oxidative stress permitted to develop new biotechnological processes to produce valuable phycological biomass (8 processes), biosynthesis and biofunctionalization of nanoparticles (14 and 1 processes respectively), decontamination of the environments polluted by heavy metals and other elements (4 processes). These elaborations are important for the biotechnological and pharmaceutical enterprises. Three of them are implemented at FICOTEHFARM (a pharmaceutical and biotechnological enterprise). The fundamental results obtained in the study are used in the training of personnel with high qualifications in the fields of Ecology, Microbiology, and Biotechnology (Cycle III, PhD).

**Approval of scientific results:** The thesis results were presented and approved at various international and national (with international participation) scientific events, including: The International Conference *Advances in Modern Phycology*, 6th edition, 2019, (Kyiv, Ukraine); The European Workshop on the Molecular Biology of Cyanobacteria, 10th edition, 2017, (Cluj-Napoca, Romania); The International Symposium EuroAliment, 8th edition, 2017, (Galați, Romania); The International Conference on Nanotechnologies and Biomedical Engineering – ICNMBE, 2nd edition, 2013, and 9th edition, 2021, (Chişinău, Moldova); The International Congress of Geneticists and Breeders from the Republic of Moldova, 11th edition, 2021, (Chişinău, Moldova); The International Scientific Conference on Microbial Biotechnology, 2nd edition, 2014; 3rd edition, 2016; 4th edition, 2018; 5th edition, 2022, (Chişinău, Moldova); The National Conference with International Participation *Life Sciences in the Dialogue of Generations: Connections between Universities, Academia, and the Business Community*, editions 2019 and 2022, (Chisinau, Moldova); The National Symposium with International Participation *Modern* 

Biotechnologies – Solutions to the Challenges of the Contemporary World, 2021, (Chisinau, Moldova).

**Relevant publications:** The thesis results are reflected in 75 scientific works on the corresponding topic, including: 1 monograph as the sole author; 1 collective monograph; 3 chapters in monographs published by *Springer* and *Elsevier* indexed in WoS/Scopus; 36 articles in scientific journals and collections, including 17 articles in WoS/Scopus indexed journals (of which 11 as written as the first author); 2 articles in the recognized international journals; 14 articles in the national registry journals (3 without co-authors); 3 articles in collections of scientific papers; 24 conference papers (of which 21 were for the international conferences in the country and abroad); 4 presentations in the plenary sessions of conferences (2 international); 7 invention patents.

**Volume and structure of the thesis:** The thesis consists of an introduction, seven chapters, conclusions and recommendations, and a bibliography with 502 titles and 8 annexes. It is written on 268 pages of the main text and includes 98 figures and 16 tables.

### 1. OXIDATIVE STRESS IN MICROALGAE AND CYANOBACTERIA

The topic of oxidative stress and the response of the living organisms to this phenomenon is a problem of general concern, not only in biology, but also in agricultural and medical sciences. Stress is generated and developed according to the same common principles for all forms of life and their physiological or pathological states. At all systematic levels, the formation of free radicals occurs in the same cellular sites and through the same mechanisms. Similarly, all cellular life forms possess efficient protection systems against ROS, functioning based on general principles. In this context, studying the phenomenon itself and the peculiarities of oxidative stress in cyanobacteria and microalgae contributes to accumulating data aimed at a profound understanding of oxidative stress and the possibility of its management.

The formation of free radicals and ROS is a normal phenomenon for all living cells. Moreover, primary radicals play essential vital functions, ensuring growth, differentiation, adaptation to environmental conditions, and protection of the structural and functional integrity of all living organisms. The ability of cells to maintain a dynamic balance between the formation and neutralization of reactive species is a fundamental characteristic of life. At the same time, responses to oxidative stress can be applied for practical purposes to achieve certain benefits through intelligent management.

A critical study of scientific publications on the thesis topic highlighted the main sources of ROS formation in cyanobacteria and microalgae - two basic vital processes: respiration and photosynthesis. These processes are influenced by physical, chemical, and biological factors acting on algal cultures. Accelerating vital processes through various stimulation procedures inevitably leads to an increase in the level of primary radicals, which can induce the formation of secondary radicals in the absence of efficient protection mechanisms in microalgal and cyanobacterial cells. Both the stimulation of biosynthetic processes in microalgae and cyanobacteria and oxidative stress conditions generate significant changes in the quality of algal biomass. Oxidative stress is associated with positive technological changes, and under certain conditions can be approached as a tool for regulating specific biosynthetic processes in phycological objects.

### 2. EXPERIMENTAL DESIGN, STUDY OBJECTS, AND RESEARCH METHODS

The researches presented in this paper, were conducted in the Phycobiotechnology Laboratory of the Institute of Microbiology and Biotechnology at the Technical University of Moldova during 2012-2022. The overall design included 6 main stages, organized according to the scheme in Figure 1.



### Fig. 1. The general design of the conducted study

The first stage involved identifying physiological fluctuations in productive, biochemical, and antioxidant activity parameters in the studied strains of cyanobacteria and microalgae throughout a growth cycle in a closed biotechnological system, while adhering to optimal conditions for each culture. Biomass samples were collected at 24-hour intervals throughout the entire cycle. Stages II, III, and IV present independent studies reflecting research results in three biotechnological stress application domains: 1) technological stress in the biomass production

process for direct consumption or further processing; 2) stress in nanobiotechnology; 3) stress in bioremediation processes. Stage V was integrative, identifying general mechanisms of oxidative stress onset and propagation in phycological cultures and highlighting practical applications of stress conditions as biotechnological tools. Stage VI is focused on new phycological technologies developed using stress as a biotechnological tool.

Three strains of cyanobacteria (Arthrospira platensis CNMN-CB-02; Arthrospira platensis CNMN-CB-11; Nostoc linckia CNMN-CB-03) and three strains of microalgae (Porphyridium cruentum CNMN-AR-01; Dunaliella salina CNMN-AV-02; Haematococcus pluvialis CNMN-AV-05) served as objects. All these objects are characterized by biotechnological importance, stable productivity and safety parameters, and are the focus of researchers in fundamental studies and the technological transfer of innovative developments into industrial production. The research applied a set of methods accepted in the field of phycobiotechnology and fundamental research: biomass quantity determination method, quantitative methods for biomass components: proteins, phycobiliproteins, carbohydrates, lipids, photosynthetic pigments, phenols; antioxidant activity determination; malondialdehyde determination method, antioxidant enzyme activity determination methods, scanning and transmission electron microscopy, neutron activation analysis, relative gene expression level determination method for stress-associated genes, and statistical analysis of experimental data.

### 3. DYNAMICS OF ANTIOXIDANT ACTIVITY CHANGES IN PHYCOLOGICAL CULTURES BIOMASS DURING THE GROWTH CYCLE UNDER OPTIMAL CONDITIONS

Cyanobacteria and microalgae cultures grow in closed systems following general laws common for microorganisms, encompassing all growth phases. Each phase of the cycle is characterized by distinct features and presents various possibilities for intervention to achieve desired effects in cultivation technologies. To develop phycological technologies based on innovative features of controlled synthesis, it is crucial to understand the peculiarities of the development of cyanobacteria and microalgae cultures under conditions closest to their specific optimum.

Typically, the parameter studied during growth cycles is the biomass quantity [2, 13, 31]. Meanwhile, other biochemical parameters also are changed during the cultivation of phycological cultures. For example, if the total protein quantity in biomass is a relatively constant parameter, the quantity of lipids and carbohydrates, as well as the quantity and ratio of pigments, vary significantly, depending on the development phase. Additionally, antioxidant activity significantly

changes during the growth cycle. At the current stage of the study, physiological fluctuations of productive, biochemical, and antioxidant activity parameters were identified in the studied strains of cyanobacteria and microalgae throughout a growth cycle in a closed biotechnological system, while adhering to optimal conditions for each culture. Biomass samples were collected at 24-hour intervals throughout the entire cycle. Some of the results of the monitorng of the growth and quality parameters during the growth cycles of the studied cultures are presented below.

### 3.1. Growth cycles of phycological cultures in optimal conditions of closed systems

We observed classic cycles of biomass accumulation for all studied phycological cultures, with the visible presence of specific growth phases. Figure 2 illustrates the biomass accumulation for four studied phycological cultures.



# Fig. 2. The growth curves of phycological cultures in a closed system under optimal conditions: (a) – *Nostoc linckia* CNMN -CB-03, (b) – *Arthrospira platensis* CNMN-CB-02, (c) – *Porphyridium cruentum* CNMN-AR-01, (d) – *Haematococcus pluvialis* CNMN-AV-05

The duration of such cycle (examined until the cultures enter the stationary phase) varies from one culture to another, and ranges between 10 and 18 days. For phycological cultures with slower growth rate compared to other microbial cultures, the cycle can last from 10 to 30 days, or even longer, depending on the culture's characteristics and applied conditions. For cyanobacterial cultures, the differences have been observed between cycles specific to laboratory and production conditions.

### 3.2-3.5. Antioxidant activity of phycological biomass throughout the life cycle

The analysis of biomass antioxidant activity, monitored using various methods relevant to phycological culture and the type of substances with antioxidant activity prevalent in the biomass, allowed identification of sensitive temporal points where significant changes occur in the cells' defense capacity against oxidative stress occur. Figure 3 presents the results of determination of the antioxidant activity and the quantity of malondialdehyde in the Nostoc biomass grown in the laboratory under optimal conditions, as an example.



Fig.3. Antioxidant activity and the quantity of malondialdehyde in Nostoc biomass over the course of a growth cycle under laboratory conditions: (a) –ABTS and DPPH testes of water soluble components, (b) ABTS and DPPH testes of lipid soluble components, (c) – the reducing capacity of the phospho-molybdenum reagent by lipid soluble conponents, (d) – TBARS test of biomass

The obtained results point to high antioxidant activity, determined by various components of the biomass. Different applied methods (based on highlighting different groups of compounds) delivered different values of activity. Simultaneously, for all applied methods, the notable jumps in values are highlighted in specific phases of the culture's growth cycle. The most evident fluctuations occur in the lag phase and at the beginning of the exponential growth phase. The end of the exponential growth phase is also emphasized. In the same time intervals, the values of the TBARS test are informative. In the lag phase and the first day of the exponential growth phase, the test values are the highest, indicating a stress condition that culture adapts in the new cycle. The same sensitive phases - the lag phase and the latter part of the exponential growth phase - were also expressed in spirulina (Figure 4).



Fig.4. Antioxidant activity and the quantity of malondialdehyde in Spirulina biomass during a growth cycle under laboratory conditions: (a) – ABTS test of water soluble components, (b) TBARS test of biomass

Thus, the latency phase in all studied species is characterized by a low capacity for antioxidant protection. Another noteworthy aspect resulted from the analyzed data is that during the transition of the culture from one growth phase to another, certain jumps in the values of the monitored parameters are observed, that reflects the functional restructuring of the microalgae and cyanobacteria cells. From a technological standpoint, these time intervals may be suitable for implementing manipulations to achive controlled biosynthetic processes in phycological cultures, and obtain biomass valuable and safe for consumption.

### 4. TECHNOLOGICAL STRESS IN INDUSTRIALLY RELEVANT CYANOBACTERIA AND MICROALGAE

Stress conditions of any nature impose significant changes in the vital activity of microalgae and cyanobacteria, and physiological processes are oriented towards culture adaptions to specific conditions. As a result, both the biochemical composition and the antioxidant activity of the obtained biomass are altered compared to conditions, considered optimal for each culture. Temperature variations, light intensity and duration, levels of nutrient medium salinity, and the application of stimulators – are the main challenges that cyanobacteria and microalgae cultures cultured under industrial conditions must respond to. This chapter analyzes the response reactions of cultures of *Arthrospira platensis*, *Nostoc linckia*, and *Porphyridium cruentum* to different types of stress, associated with the technological process. The research was conducted following the scheme represented in Figure 5.



Fig. 5. The study design of the effects of technological stress on phycological cultures

The influence of hyperthermic and light stress on Spirulina culture was studied under both laboratory and production conditions. Salinity stress was induced in the laboratory for Spirulina and Nostoc cultures. This type of stress was modeled in several variants of intensity, depending on the sodium chloride concentration. Metal stress was induced by the presence of Cu(II) and Zn(II) salts, which were added to the media for cultivating Spirulina, Nostoc, and Porphyridium. In the case of copper-induced stress, the salt was added in two variants: during the latency phase and in the exponential growth phase. The monitored parameters are presented in Fig. 5.

# 4.1. - 4.2. Influence of thermal and light stress on the technological strain *Arthrospira* platensis CNMN-CB-11

In these subsections, the effect of hyperthermal stress and photoperiodicity on Spirulina in both laboratory and industrial production conditions is presented. The antioxidant activity of Spirulina biomass and the quantity of malondialdehyde under temperature stress are depicted in Figure 6. From the presented results, it is evident that high temperature (40°C) is a stress factor for *Arthrospira platensis* culture, both in laboratory and industrial production conditions. Under laboratory conditions, the quantity of malondialdehyde in the biomass grown at high temperatures is three times higher, than in biomass grown at optimal temperature. In case of industrial production conditions, the increase in MDA quantity is 37%. The ABTS test values in extracts, obtained from biomass grown at the optimal temperature of 30°C under laboratory conditions vary throughout the life cycle within the range of 28.0% and 54.7% inhibition, while under production conditions, they range between 31.0% and 58.0% (Figure 6). We can highlight the beginning of

the exponential growth phase, where a pronounced decrease in the antioxidant activity of aqueous extracts from biomass grown at  $40^{\circ}$ C compared to that grown at the optimal temperature is recorded. Under laboratory conditions, the activity of the aqueous extract from biomass grown at  $30^{\circ}$ C is 1.8 times higher compared to biomass growth at  $40^{\circ}$ C.



Fig. 6. Antioxidant activity and the quantity of malondialdehyde in the biomass of Arthrospira platensis under hyperthermal stress throughout the life cycle. (a) – laboratory, (b) - industrial conditions

Figure 7 reflects the results obtained when applying light stress under laboratory and production conditions. The application of a periodic illumination regime to Spirulina under laboratory conditions was associated with a very pronounced increase in MDA.



Fig. 7. Antioxidant activity and the quantity of malondialdehyde in the biomass of *Arthrospira platensis* under illumination stress throughout the life cycle. (a) - laboratory conditions; (b) - production conditions. ((24) - continuous illumination; (12/12) - periodic illumination 12 hours light:12 hours dark)

Thus, throughout the life cycle under periodic illumination conditions, the MDA level in Spirulina progressively increases compared to continuous illumination conditions, with a 2.2 timesdifference at the beginning of the cycle and 10 times at the end. In production conditions, photoperiodicity causes an increase in MDA quantity by a maximum of 55.0% compared to the respective values obtained for continuous illumination conditions (p<0.001). During the first 6 days of Spirulina cultivation under periodic illumination conditions, antioxidant activity is by 36.8-

55.5% lower compared to continuous illumination conditions. In the following days (except for the day 7th), antioxidant activity values in both illumination variants are very close. In production conditions, the difference in antioxidant activity levels of aqueous extracts from Spirulina biomass grown under continuous illumination conditions and under photoperiodism is not as evident as in the laboratory ones. The activity of antioxidant enzymes in Spirulina grown under thermal stress and photoperiodicity in laboratory conditions is presented in Figure 8.



Fig. 8. Antioxidant enzyme activity in Spirulina biomass grown in the laboratory under stress conditions throughout the life cycle: (a) - thermal stress, (b) - light stress.

Under optimal temperature conditions, the highest level of primary antioxidant enzyme activity is recorded during the lag phase and the exponential growth phase. At 40°C, the highest activity of SOD and POD is in the lag phase and at the beginning of the exponential growth phase. The activity of these two enzymes in Spirulina biomass throughout the life cycle at 40°C is higher than at 30°C, with POD activity being 2.2 times higher at the beginning of the exponential growth phase. In laboratory conditions, both under continuous illumination and photoperiodism, the highest activity of primary antioxidant enzymes SOD and POD is characteristic for lag phase and the exponential growth phase. Under photoperiodism, during the mentioned stages of the life cycle, the activity of the studied enzymes is higher (over 1.4 times for POD and over 2 times for SOD). Although under photoperiodism, some technological parameters are characterized by attractive values (increased accumulated biomass, as well as the quantity of phycobiliproteins and carbohydrates in biomass), this situation is to be treated very carefully to avoid compromising the safety of the products obtained due to the accumulation of free radicals.

# 4.3. Influence of salinity stress on strains *Arthrospira platensis* CNMN-CB-11 and *Nostoc linckia* CNMN-CB-03 under laboratory conditions

The quantity of sodium chloride in the growth media for the strains *Arthrospira platensis* CNMN-CB-11 and *Nostoc linckia* CNMN-CB-03 is <1 g/L. Stress induced by this compound in both strains is associated with a reduction in the quantity of obtained biomass; therefore, in

controlled industrial conditions, such situations are avoided. In the conducted study, five concentrations of NaCl (10, 20, 30, 40, and 50 g/L) were tested. The quantity of malondialdehyde in biomass was used as a marker of oxidative stress, as in the previous examples. Measurements were taken three times throughout the life cycle. The results are presented in Figure 9.



# Fig. 9. Antioxidant activity and the quantity of malondialdehyde in the biomass of cyanobacteria at different NaCl concentrations in the medium. (C/3, C/6, and C/10 - control on days 3, 6, and 10); 10, 20, 30, 40, 50 - NaCl concentration, g/L: (a) - Arthrospira platensis, (b) - Nostoc linckia.

The results obtained for Spirulina indicate, that high salinity is a stress factor for Spirulina culture. Oxidative stress develops slowly, so on the 3rd day of the life cycle, the level of the oxidative stress marker in biomass is at the control level, and in the subsequent two monitoring periods, it increases significantly. At the same time, during the second monitoring and partially during the third one, there is an increase in the antioxidant activity of the biomass, which is evidence of the mobilization of the cell's antioxidant capacities to neutralize the formed radicals. For Nostoc, the stress induced by the elevated level of sodium chloride is more pronounced, as evidenced by the increase of the MDA level and the decrease of antioxidant activity in the biomass.

# 4.4. Influence of metal ions on *Arthrospira platensis* CNMN-CB-11, *Nostoc linckia* CNMN-CB-03, and *Porphyridium crientum* CNMN-AR-01

Two different situations were analyzed: the action of a metal with high toxic potential - copper, and a less toxic one - zinc, on three industrially important strains – A. platensis CNMN-

CB-11, *N. linckia* CNMN-CB-03, and *P. cruentum* CNMN-AR-01. The contact of the algal cultures with copper occurred in two variants: during the lag phase and during the exponential growth phase. Figure 10 presents some of the results, obtained for the culture of *P. cruentum*.



Fig.10. Antioxidant activity and malondialdehyde content in Porphyridium cruentum biomass in the presence of copper and zinc. ((C) - control; (CuSO4) - 5 mg/l CuSO4 added on the first day; (CuSO4+) - 5 mg/l CuSO4 added on 3rd day; (Zn acetate) - 5 mg/l ZnC4H10O6 added on the first day)

As was seen, copper induces oxidative stress in all strains, whether added at inoculation or on the third day of growth. Under these conditions, the quantity of malondialdehyde in algal biomass significantly increases, the antioxidant activity of aqueous extracts decreases, and the activity of primary antioxidant enzymes undergoes significant changes. The quantity of biomass for all three strains significantly decreases when copper is added at inoculation. When copper is added on the third day of cultivation, the biomass loss is less pronounced, as is the loss of protein and other valuable compounds. Zinc acetate at a concentration of 5 mg/L did not act as an inducer of stress in the studied strains. Thus, the quantity of MDA, the antioxidant activity of aqueous extracts, and the activity of primary antioxidant enzymes had not been changed under the influence of zinc, or changes are within very narrow limits.

The four different types of technological stress produced generalized changes in all productive and safety parameters of Spirulina, Nostoc, and Porphyridium biomass, affecting productivity, biochemical composition, antioxidant activity, and the quantity of MDA.

### 5. OXIDATIVE STRESS IN THE NANOBIOTECHNOLOGY OF CYANOBACTERIA AND MICROALGAE

The research described in this chapter was focused on the biosynthesis of silver and selenium nanoparticles by live cultures of microalgae and cyanobacteria. Various aspects of the

interaction between engineered nanoparticles and microalgae and cyanobacteria were also studied. In terms of toxicity, the interactions between phycological objects and different types of nanoparticles (quantum dots and Au and Ag nanoparticles) were investigated. Biofunctionalization of nanoparticles as a response to stress induced by them represents the third aspect investigated at this stage. Samples were collected at 24-hour intervals in bionanoparticle synthesis and biofunctionalization studies, and at the end of the cycle in toxicity studies. Monitored parameters included: culture productivity, biomass composition (biochemical indicators), antioxidant activity (antiradical tests and oxidative stress markers). Additionally, biosynthesis and biofunctionalization direct indirect identification processes were monitored through the and of synthesized/biofunctionalized nanoparticles (Figure 11).



Fig. 11. The design of oxidative stress effects study in nanophycobiotechnology

### 5.1. Oxidative stress in cyanobacteria and microalgae during bionanosynthesis

Biological synthesis of nanoparticles has several advantages, with the most important being its environmentally friendly nature. Various living organisms, cells, and cellular derivatives are used for biogenic nanoparticle synthesis, including photosynthetic microorganisms. Cyanobacteria and microalgae are interesting objects for nanoparticle biosynthesis research because they serve as a source of metabolites with reducing properties, that can ensure the efficiency of the process. The research presented in this subsection aimed to highlight the possibility of achieving silver and selenium nanoparticle biosynthesis in the living cells of microalgae and cyanobacteria, while preserving the quality and biological value of their biomass. Such an approach is valuable when applying an integrated vision for the use of nanomaterials, especially in the field of theranostics, where the properties of nanoparticles obtained through biosynthesis and the biological matrix, in which they are included, are used for diagnosis and treatment in a single procedure. The conducted research has demonstrated the feasibility of obtaining nanoparticles from different chemical forms of the elements of interest. At the same time, a balance was sought between nanobiosynthesis and biomass quality. As an example, some of the obtained results are presented below. Figure 12 reflects the changes in Porphyridium biomass during the biosynthesis of silver nanoparticles from silver nitrate solution.



Fig. 12. Changes in the content of certain components in *Porphyridium cruentum* biomass during nanobiosynthesis (a-d) and antioxidant activity (e); SEM image of the biomass (f).

The biochemical tests conducted on the main parameters of *Porphyridium cruentum* biomass demonstrate the presence of a toxic effect, which manifests in the first hours of exposure of algal biomass to silver nitrate. The significant decrease in protein, phycobiliproteins, and lipid content indicates the degradation of Porphyridium biomass. The toxic effect is further confirmed by the decrease in the antioxidant capacity of the biomass. At the same time, the microalga *Porphyridium cruentum* can be an important matrix for the biosynthesis of silver nanoparticles, a process, which is based on the activation of protective mechanisms against stress, caused by the presence of silver ions, especially during a 24-hour exposure to silver ions.

The same principle was identified in the biosynthesis of silver nanoparticles by live cultures of *Arthrospira platensis*, *Nostoc linckia*, and *Dunaliella salina*, as well as in the biosynthesis of selenium nanoparticles from cobalt selenite solution by cultures of *Arthrospira platensis* and *Nostoc linckia*. The phenomenon of biosynthesis of nanoparticles by live algal and cyanobacterial

cultures is based on the cells' response to oxidative stress, generated by the presence of synthesis precursors (in this study, silver nitrate and cobalt selenite). In order to protect against the harmful effects of xenobiotics in microalgal and cyanobacterial cells, protective antioxidant mechanisms are activated, reducing oxidative degradation effects, and mechanisms that ensure the biotransformation of harmful elements. As demonstrated in all studied cases, theprocess of nanoparticles biosynthesis involves clear redox processes, that interact with antioxidant protection or are an integral part of it.

# 5.2. Oxidative stress induced by different nanoparticles in microalgae and cyanobacteria

In this section of the study, nanomaterials were treated similarly to other stress factors for algal cultures, monitoring of the productive and safety parameters of the biomass obtained under contact with these specific materials. The conclusion about the stress level was drawn based on a comprehensive analysis of the changes in biomass production, the composition of the obtained biomass, and its antioxidant activity. The study included nanoparticles with widespread practical applications – luminescent quantum dots (CdSe, ZnSe, and ZnS) used in various engineering fields, and noble metal nanoparticles (Au and Ag) used in different industries and medicine.

In the study were used CdSe quantum dots with a size of 3-5 nm, obtained through the high-boiling-point solvent synthesis method, ZnSe particles with a size of 40 nm, and ZnS particles with a size of 30-35 nm obtained through the hydrothermal method by researchers from the Institute of Engineering and Nanotechnology D. Ghiţu. The nanoparticles were added to the culture media of Spirulina and Porphyridium in three series of experiments with different nanoparticle concentrations ranging from 0.01 to 10 mg/l. The objects in this series of experiments were *Arthrospira platensis* CNMN-CB-02 and *Porphyridium cruentum* CNMN-AR-01.

Toxicity of the three types of nanoparticles for Spirulina and Porphyridium cultures decreases in the sequence ZnSe > CdSe > ZnS. The antioxidant activity of water-soluble components in algal biomass varies depending on the type and concentration of particles. The specific method for determining the low-density lipid peroxidation process allows establishing a strong negative correlation (r = -0.61 to -0.90) between the accumulated biomass quantity and MDA in the range of concentrations under which nanoparticles exhibit a toxic effect (Table 1). These strong correlations highlight one of the major effects of nanoparticles, which involves the degradation of biological membranes, changes in its permeability, and disruption of vital processes. Campared to other algal objects, Spirulina culture is more sensitive to the presence of

nanoparticles with potential toxicity in the cultivation medium, and presented results support the idea of using spirulina for xenobiotic toxicity tests.

 Table 1. Significant correlation coefficients between the quantity of Spirulina and

 Porphyridium biomass and the malondialdehyde content in biomass under the action of

 quantum dots

Type of NPs/Concentrations	Arthrospira platensis				
range	CdSe 0,1-4,0 mg/l	CdSe 0,01-0,1 mg/l	ZnSe 0,01-0,1 mg/l		
Correlation coefficients	-0,9054	-0,8211	-0,8980		
between MDA and biomass	(p<0,001)	(p<0,01)	(p<0,001)		
Type of NPs/Concentrations	Porphyridium cruentum				
range	CdSe 1-12 mg/l	ZnS 1-8 mg/l	ZnSe 0,01-0, 06 mg/l		
Correlation coefficients	-0,7837	-0,8563	-0,6087		
between MDA and biomass	(p<0,01)	(p<0,01)	(p<0,05)		

Another objective of the conducted study was to determine the capacity of AgNP and AuNPs nanoparticles, stabilized with organic polymers, to induce a stress state in the cyanobacterium *Arthrospira platensis* and the microalga *Porphyridium cruentum* during cultivation in a closed system. Silver and gold nanoparticles in polyethylene glycol, in the concentration range of  $0.025-0.5 \mu$ M, stimulate the growth of Spirulina biomass by 31.6% and 35.8%, respectively. Both types of nanoparticles did not alter significantly the protein, carbohydrate, and pigment contents. The lipid content increased at nanoparticle concentrations up to 0.1  $\mu$ M, and decreased at a concentration of 0.5  $\mu$ M. Some changes related to the photosynthetic mechanism were observed. The chlorophyll/carotenoid ratio served as an indicator of stress. The values obtained for the chlorophyll/carotenoid ratio in this research are presented in Table 2. For AgNPs at concentrations of 0.025–0.25  $\mu$ M, an increase in this ratio compared to the control was observed, indicating high photosynthetic activity. These data correlate with the increase in biomass. At AgNPs concentration of 0.50  $\mu$ M, a decrease in the ratio compared to the control was observed, which could indicate stress state for the Spirulina culture.

		С	hlorophyll	α/β-caroter	ne ratio			
Type of NPs		Concentration of NPs, µM						
	0 (M)	0.025	0.05	0.10	0.25	0.50		
AgNP	4.57	4.86	5.21	5.07	5.04	4.17		
AuNP	4.57	5.03	5.05	5.13	5.18	5.05		

Table 2. Effec	t of nanoparticles o	n chlorophyll α/β-caro	tene ratio in spirulina
	1	1 2 1	1

Exposure of the Spirulina culture to AgNP and AuNP at a concentration of 0.5  $\mu$ M involves several elements of toxicity in the Spirulina culture. Among these, lipid content decrease, MDA level increase, and  $\alpha$  chlorophyll/ $\beta$ -carotenoid ratio decrease were observed. Maintaining a high level of productivity at this concentration was ensured by an adequate level of antioxidant activity in the biomass. Exposing the *Porphyridium cruentum* culture to the action of AgNP with sizes of 10 and 20 nm within the concentration range of 0.01 to 0.1  $\mu$ M resulted in a decrease in lipid content (by 32-41%) of the biomass (Figure 13).



Fig.13. The effect of different concentrations of silver nanoparticles with sizes of 10 and 20 nm on the lipid content and MDA in the biomass of *Porphyridium cruentum* (\*p < 0.001 for the difference compared to the control)

Higher concentrations significantly increased lipid content, exceeding control by 2.1-3.0 times. Within the applied concentrations of AgNPs, there is a strong dependence between the concentration of AgNP in the microalgal cultivation medium and the lipid content in the biomass. The Pearson correlation coefficient calculated for 10 nm - size AgNPs is r=0.856, and for 20 nm – size AgNPs it is r=0.819. Additionally, the quantity of MDA increases, exceeding normal values by up to 3.4 times. The quantity of malondialdehyde correlates with the applied nanoparticle concentration, with correlation coefficients of r=0.823 for 10 nm - size AgNsP 10 nm) and r=0.899 for 20 nm – size AgNPs. The same response pattern of the Porphyridium culture is observed in the case of citrate-stabilized gold nanoparticles. The coefficients of correlation between the values of malondialdehyde determined in microalgal biomass and the concentrations of AuNPs with size 10 and 20 nm in the culture medium confirmed the existence of a strong positive dependence between these variables (r = 0.744 and r = 0.817).

# 5.3. Oxidative stress during the biofunctionalization of gold and silver nanoparticles by microalgae and cyanobacteria

In this study we used small-sized 5 nm silver and gold nanoparticles, stabilized in polyethylene glycol. Spirulina and Porphyridium were cultivated in the presence of nanoparticles, which were introduced into the culture medium at different stages of the life cycle. It highlighted

the possibilities of control of the natural processes of biofunctionalization of metallic nanoparticles in live cultures, and maintaining the quality of the biomass involved in these processes. The adding of nanoparticles was performed in three experimental series, differing in the age of the culture: 1) lag phase; 2) the beginning of the exponential growth phase, and 3) the end of the exponential growth phase. Biofunctionalization of nanoparticles was demonstrated indirectly (recording shifts in absorption maxima), and directly (visualization of nanoparticles in the algal biomass). In the process of nanoparticle biofunctionalization in algal biomass, the same type of modifications as those described earlier (subsection 5.2) were observed. The idea of the involvement of oxidative stress response mechanisms in the biofunctionalization process is supported by data, related to the reducing capacity of algal biomass. Thus, we demonstrated, that the reducing capacity of the nitric oxide radical, determined for extracts obtained from algal biomass cultivated in the presence of nanoparticles, stabilized in polyethylene glycol and supplemented to the culture medium in the latency phase, was increased significantly. Figure 14 presents the results obtained for extracts from Porphyridium biomass as an example. AuNPs and AgNPs s induced an increase in the reducing capacity of nitric oxide in extracts from Porphyridium cruentum biomass by 36-89%, and the dependence was dose-effect type (r=0.989 for AgNPs and r=0.893 for AuNPs). The age of P. cruentum and A. platensis is an important factor in shaping the culture response to oxidative stress generated by the presence of gold and silver nanoparticles, as well as in modeling the process of nanoparticles biofunctionalization. The exponential growth phase is highlighted as a suitable term for obtaining biofunctionalized nanoparticles but may be associated with accumulation of oxidative lipid degradation products.



Fig. 14. The reducing capacity of the nitric oxide radical by ethanol extracts from P. cruentum biomass increased in the lag phase (\*p<0.01; \*\*p<0.001)

### 6. OXIDATIVE STRESS IN THE PROCESSES OF WATER CONTAMINATED WITH METALS BIOREMEDIATION

The research described in this chapter aims to fill the gap in data regarding the potential application of live cyanobacterial cultures (*Arthrospira platensis* CNMN-CB-02 and *Nostoc* 

*linckia* CNMN-CB-03) as bioremediators for wastewater containing heavy metals, under repeated contact with contaminated effluents. In the studies presented in this chapter, the cyanobacteria *Nostoc linckia* and *Arthrospira platensis* were used to remowe the heavy metals from contaminated effluents. Two types of synthetic effluents were investigated - one with Cu(II) as the dominant element, and the other with Cr(VI). Four bi- and polymetallic systems were modeled, each applied at three different concentrations. The bioremediation process was investigated using cyanobacterial culture in the lag phase and in the exponential growth phase. Bioremediation was carried out over three iterative cycles of cyanobacterial growth (Figure 15).



Fig. 15. Design of the effects of oxidative stress in bioremediation study

# 6.1. The specific response of spirulina culture to oxidative stress during treatment of effluents contaminated with heavy metals and repeated cultivation cycles

*Arthrospira platensis* is a convenient model organism for bioremediation studies, being an extremophile that thrives in challenging conditions such as high alkalinity, elevated temperatures, and high salt concentrations in the culture medium. Moreover, Spirulina can survive not only in environments with high levels of heavy metals, but also accumulates a substantial amount of biomass under these conditions. In this subsection, the results obtained in the study of heavy metal removal from polymetallic systems with copper and chromium content in repeated cultivation cycles using the cyanobacterium *Arthrospira platensis* are summarized.

The first polymetallic system tested was based on copper. In addition to copper, the systems included: I – Fe; II – Fe and Ni; III – Fe and Zn; IV – Fe, Zn and Ni. Polymetallic systems with different metal concentrations (Cu – from 2.5 to 10 mg/l; Fe – 1.25 - 5.0 mg/l; Ni and Zn – 0.5 –

2.0 mg/l) were modeled. In systems containing the maximum number of metals, Spirulina survived only one cultivation cycle, and all productivity and biomass quality parameters were below the acceptable limit. Depending on the system composition, biomass production decreased by 32.8-52.6% compared to the control (Fig.16,b), protein content decreased by up to 69%, and carbohydrate content decreased by up to 29.2% compared to controls. Phycobiliproteins decreased from 14% of biomass in the control to 0.32-0.88% in the experimental variants. The presented results indicate extreme toxicity of polymetallic copper – containing systems with metal concentrations of 10 mg/l for Cu, 5.0 mg/l for Fe, and 2.0 mg/l for Ni and Zn.



Fig.16. The amount of metals accumulated and the biomass production of Spirulina in polymetallic systems I) Cu/Fe, II) Cu/Fe/Zn, III) Cu/Fe/Ni, and IV) Cu/Fe/Zn/Ni (Concentrations, mg/l: Cu – 10.0; Fe – 5.0; Ni – 2.0; Zn – 2.0)

According to the neutron activation analysis (NAA) data (Figure 16a), in the Cu/Fe system, Spirulina biomass accumulated  $6.0\pm0.3$  mg/g of copper, which is 200 times higher than in the control biomass, and  $7.2\pm0.3$  mg/g of iron 1.6 times more compared to the control. With the addition of zinc ions to the system, the accumulation capacity of the biomass for copper and iron remained at the same level, while the amount of accumulated zinc was  $1.60\pm0.05$  mg/g (26 times higher than in the control). Comparing the Cu/Fe/Zn and Cu/Fe/Ni systems, we observed, that Spirulina accumulated approximately 7 times more zinc than nickel, despite being added to the nutrient medium at the same concentration. In the Cu/Fe/Ni system, the accumulated amount of copper increased, while the iron absorption was significantly reduced. In the quaternary system Cu/Fe/Ni/Zn, the highest accumulation was observed for all elements. Iron, copper, and zinc are important elements necessary for living organisms, being involved in many cellular processes. However, when their concentrations exceed the physiological norm, they can become toxic to cells. In the analyzed systems, Spirulina survived only during one cultivation cycle.

In polymetallic systems with a dominant copper content and low concentrations of other metals, it was possible to cultivate Spirulina for three iterative cycles, monitoring the amount of biomass obtained, the level of accumulated metals and the biochemical composition of the biomass in each cycle. The reduction in biomass quantity was 28.1-42.6%, compared to the control, and significant improvements in the quality of the produced biomass were recorded. It was expressed in the reduction of protein and pigment quantities, as well as in the modification of the content of all studied biomass fractions. Metal accumulation varied from cycle to cycle, and a common pattern was observed for all polymetallic systems. The amount of accumulated copper in the biomass was maximum in the first cultivation cycle, after which it gradually decreased in cycles II and III. In contrast, the amount of iron decreased in the first cycle, after which it returned to normal or even increased in the next two cycles. The adaptation of Spirulina culture to effluents with relatively low metal concentrations indicates the potential use of Spirulina biomass as a regenerable accumulator for the treatment of moderately polluted effluents, or for post-treatment of wastewater.

A similar study was conducted using Spirulina for the bioaccumulation of metals from polymetallic systems with chromium content. In addition to monitoring the metal accumulation capacity and biochemical changes in Spirulina biomass, we aimed to evaluate the dependence of the accumulation process on the age of the culture, contacting with effluents containing the metals of interest. Synthetic effluents with different chemical composition and concentrations of metal ions were modeled based on real galvanic effluent data. The accumulation of metals by Spirulina was investigated in synthetic effluents with the following chemical composition: Cr/Fe; Cr/Fe/Ni; Cr/Fe/Ni/Zn, and Cr/Fe/Ni/Zn/Cu during three cultivation cycles. Metals at different concentrations (in mg/l: Cr (VI) -10.0 - 5.0; Fe -5.0 - 1.25; Zn, Cu, and Ni -2.0 - 0.5) were added to the culture medium in the exponential growth and stationary phases, and their accumulation in biomass was monitored using NAA. The effect of metal ions on biomass and key biochemical parameters (proteins, carbohydrates, lipids, phycobilins, and β-carotene) was monitored. When metal ions were added in the stationary growth phase, Spirulina maintained a high metal accumulation capacity over 2-3 cultivation cycles. By adding metals in the exponential growth phase at the following concentrations: 10 mg/L Cr(VI), 5 mg/L Fe, 2 mg/L Zn, Ni and Cu, Spirulina acted as a regenerable sorbent only in the Cr/Fe system. Spirulina maintained its metal accumulation capacity over three cultivation cycles when exposed to lower concentrations of metal ions. The ability of Spirulina to accumulate metal ions over multiple cultivation cycles was ensured by maintaining an optimal level of proteins and lipid content. According to NAA data (Table 3), Spirulina biomass accumulated 7-20 times more chromium (VI) during three cultivation cycles, than its content in the control biomass. In all studied systems, the maximum accumulation of chromium (VI) occurred in the second growth cycle. The highest amount of chromium (VI) was accumulated in the Cr/Fe/Ni/Zn/Cu system. Spirulina was more sensitive to the adding of metals in the exponential growth phase.

		- 0	-			
		Metal content, µg/g				
System	Metal	Control	Cycle I	Cycle II	Cycle III	
Cr/Es	Cr	9±0.3	62±4.5	104±8.3	82±6.5	
Cr/Fe	Fe	4610±270	6190±380	9530±570	11300±680	
	Cr	9±0.3	76±2.3	111±3.3	105±3.1	
Cr/Fe/Ni	Fe	4610±270	6620±260	8900±360	13700±550	
	Ni	4±0.08	53±1.1	66±1.3	115±2.3	
Cr/Fe/Ni/Zn	Cr	9±0.3	96±2.8	182±5.4	129±3.8	
	Fe	4610±270	7620±460	12200±730	15300±920	
	Ni	4±0.08	87±1.7	98±1.9	149±2.9	
	Zn	45±2.2	40.4±2	51±2.5	46±2.3	
	Cr	9±0.3	94±7.5	148±9	139±11	
Cr/Fe/Ni/Zn/Cu	Fe	4610±270	7230±500	10400±630	19300±1350	
	Ni	4±0.08	61±4.3	68±6.8	109±9.8	
	Zn	45±2.2	38±0.3	72±7	97±9	
	Cu	30±2.3	n.d.	n.d.	n.d.	

Table 3. Accumulation of metals by *Arthrospira platensis* biomass in polymetallic systems with chromium (VI) upon the addition of metal ions in the culture medium during the exponential growth phase\*

\*Metals concentrations, mg/l: Cr 2,5, Fe 1,25, Ni 0,5, Zn 0,5 and Cu 0,5

In this case, spirulina survived three cultivation cycles at chromium concentrations of 2.5 and 5.0 mg/l in all studied systems and at a chromium concentration of 10 mg/l in the Cr/Fe system. Chromium and iron were accumulated in larger quantities when metals were added in the exponential growth phase, while nickel, copper, and zinc were accumulated when added in the stationary phase. In all experimental variants, metal accumulation was associated with a significant decrease in the content of phycobiliproteins and  $\beta$ -carotene in biomass, while the increase in carbohydrate content can be considered activation of protective mechanisms against toxic metal ions. The obtained results indicate the prospect of using spirulina as a regenerable accumulator for the treatment of moderately polluted effluents with chromium(VI) or post-treatment of wastewater.

# 6.2. Response characteristics of *Nostoc linckia* cyanobacteria and heavy metal accumulation in multimetallic systems in iterative cycles

Similar research to that described in subsection 6.1 was conducted using cyanobacterium *Nostoc linckia* as the study object. NAA was applied to evaluate the accumulation of Cu, Cr, Fe, Ni, and Zn by Nostoc in polymetallic systems with copper and chromium content. In the case of copper-based systems, the accumulation capacity of Cu ions from multi-element systems by *Nostoc linckia* was high, and increased over two cultivation cycles in Cu-Fe-Ni and Cu-Fe-Zn

systems, and over three cycles in Cu-Fe and Cu-Fe-Ni-Zn systems. The accumulation of Fe, Ni, and Zn also increased from one cycle to another. The metal accumulation process was associated with a significant change in the biochemical composition of the biomass. In the initial exposure to pollutants, high tolerance to heavy metals in the environment was characteristic of the studied cyanobacterial strain. Under these conditions, there was a reduction in protein, lipid, and carbohydrate content, degradation of pigments, and a decrease in the antioxidant capacity of the biomass. Under repeated exposure to pollutants, the culture's tolerance decreased, thus Nostoc should be explored for emergency bioremediation.

In the case of polymetallic systems with Cr(VI), the ability to bioaccumulate Cr(VI) by the cyanobacterium *Nostoc linckia* remained high over three generations, while the absorption of Fe, Ni, Cu, and Zn into biomass increased from one generation to another. Repeated exposure to metals led to a moderate stress condition, expressed by a decrease in biomass quantity and the accumulation of malondialdehyde. At the same time, the quality of Nostoc biomass remained unchanged. Maintaining the biomass quality under stress conditions caused by the presence of metals is ensured by an increase in the content of compounds with antioxidant action in the biomass of Nostoc. Due to its high bioaccumulation capacity and a specific growth pattern with crust formation on the soil surface, the soil-dwelling cyanobacterium Nostoc *linckia* is an important candidate for remediating of the chromium contaminated soil in combination with other metals.

# 6.3. Bioaccumulation capacity of heavy metals and rare earth elements by *Arthrospira platensis*

Using the cyanobacterium *Arthrospira platensis* as the study object, other studies have been conducted for the bioremediation of effluents contaminated with metals and the recovery of technologically valuable elements from the environment. Similar research to those described in subsections 6.1 and 6.2 has been carried out, considering bioremediation in polymetallic systems with a dominant content of zinc and nickel. Additionally, investigations have been conducted for lithium accumulation in Spirulina biomass and the recovery of rare earth elements from diluted solutions. The significant results for the purpose of this chapter are integrated into Table 4.

In the case of mono- and polycomponent systems with a dominant content of nickel, the bioaccumulation performance of metals by *Arthrospira platensis* was monitored for three iterative growth cycles. Alongside the metal bioaccumulation capacity, numerous productive and biochemical parameters of the cyanobacterium were monitored, only two basic parameters of which are presented here – the biomass quantity and the level of malondialdehyde, in terms of

their directional changes. Nickel accumulation in nostoc biomass was directly proportional to its concentration in effluents, reaching maximum absorption (1310 mg/kg biomass) in the Ni/Cr/Fe system. In the same system, biomass accumulated 110 times more chromium and 4.7 times more iron than the control. The highest copper accumulation (2870 mg/kg) was achieved in the Ni/Cu/Zn/Mo system, and highest zinc accumulation (1860 mg/kg) - in the Ni/Cu/Zn/Sr system. The biomass quantity obtained in these metallic systems was at the control level or moderately lower, especially at the end of the first cultivation cycle. Simultaneously, the quantity of malondialdehyde in all experimental variants significantly exceeded the control.

Element /elements	Element concentra- tion, mg/l	The direction of biomass variation	The direction of MDA variation	Bioaccumulation capacity, mg/kg or %	Reference to publication
Zn	2.5-10.0	Without changes	Increases	90 - 16000	
	Zn 2,5-10,0		Increases	Zn 250.0-20000.0	
Zn/Cu/Sr	Cu 1,0-5,0	Decreases		Cu 125.0-500.0	
	Sr 0.5-2,0			Sr 5.0-50.0-	
	Zn 2,5-10,0	Without	Increases	Zn 750.0-20500.0	
Zn/Cu/Ni	Cu 0,5-2,0	significant		Cu 60.0-250.0	[32]
	Ni 0.5-2,0	changes		Ni 40.0-240.0	
	Zn 2,5-10,0		Increases	Zn 120.0-22500.0	
	Cu 0,5-2,0	D		Cu 125.0-500.0	
Zn/Cu/Sr/Ba	Ni 0.5-2,0	Decreases		Sr 5.0-50.0	
	Ba 0,5-1,0			Ba 60.0-25.00	
Ni	2,5-10	Without changes	Increases	50-250	
	NE 2.5.10		Increases	Ni 50.0-1310.0	
	N1 2, 5-10	Desmasses		Cr 1.05-110.0	
N1/Cr/Fe	Cr 1,0-5,0	Decreases		Fe 2500.0-	
	re 1,0-3,0			17000.0	
	Ni 2,5-10		Increases	Ni 60.0-51.00	[7]
NE/Cu/Sa/Za	Cu 0,5-1,0	Decreases in		Cu 70.0-820.0	[/]
INI/Cu/SI/ZII	Sr 1,0-5,0	some variants		Sr105.0-1370.0	
	Zn 0,5-2,0			Zn 20.0-1860.0	
	Ni 2,5-10		Increases	Ni 40.0-480.0	
Ni/Cu/Zn/Mo	Cu 1,0-5,0	Decreases in		Cu 50.0-2870.0	
INI/Cu/ZII/IVIO	Zn 0,5-2,0	some variants		Zn 20.0-1750.0	
	Mo 0,5			Mo 3,5-4,4	
Li	5-500	Without changes	Increases	50.0-2500.0	[8]
La	10-30	Increases	Without changes	88-95%	
Dy	10-30	Increases	Increases	85-90%	
Tb	10-30	Decreases	Decreases	<20%	[33]
Yb	10-30	Decreases	Increases	<20%	
Sm	10-30	Without changes	Decreases	42-97%	]
Nd	10-30	Without changes	Increases	46-86%	

Table 4. The ability of the cyanobacterium Arthrospira platensis to accumulate differentelements from mono- and polycomponent effluents

Thus, productivity parameters and oxidative stress markers, along with the high metal bioaccumulation capacity, allow us to assert that *A.a platensis* can be considered in the bioremediation of wastewater polluted with a complex composition and a dominant content of nickel.

In another series of experiments, the effect of zinc in different combinations with other metals on the biomass accumulation capacity of *Arthrospira platensis*, as well as on the productivity and biochemical composition of the obtained biomass, was investigated. In the biomass of *Arthrospira platensis*, grown in the presence of multimetallic systems containing Zn, the specific oxidative stress indicator MDA was expressed. Even in the case of effluents contaminated with metals, with zinc being dominant, Spirulina can be considered an efficient accumulator and bioremediator.

A separate case presents the research focused on obtaining Spirulina biomass enriched with lithium. In bioaccumulation experiments, lithium was added to the nutrient medium of *Arthrospira platensis* in the concentration range of 5-500 mg/l at inoculation, and on the third day of cultivation (exponential growth phase). The effect of lithium on the biomass quantity and its biochemical composition was studied. The biomass quantity was not influenced by the action of lithium, while the content of malondialdehyde increased. Other biochemical parameters of Spirulina were within normal limits, so we can state, that *Arthrospira platensis* biomass proved to be an excellent matrix for the production of dietary supplements containing lithium.

The growth of *Arthrospira platensis* and physiological changes in biomass under the effects of six rare earth elements were evaluated. According to the analysis of rare earth element quantities in Spirulina biomass, the accumulation capacity of the cyanobacterium for the studied elements decreases in the following order: La > Dy > Nd > Sm > Yb > Tb. The results show that Dy and La ions stimulate biomass growth, while Yb ions inhibit it, and Sm, Tb, and Nd ions do not affect biomass accumulation. Rare earth elements cause an increase in the MDA content in Spirulina biomass. Changes in antioxidant activity reveal moderate stress in Spirulina exposed to contact with rare earth elements, indicating that the cyanobacterium *A. platensis* can be successfully used for the bioremediation of natural waters contaminated with these elements, as well as for their recovery from mildly polluted industrial effluents.

### 7. MANAGEMENT OF OXIDATIVE STRESS IN THE CULTIVATION TECHNOLOGIES OF INDUSTRIALLY RELEVANT PHYCOLOGICAL OBJECTS. GENERALITIES, MECHANISMS, APPLICATIONS

Industrial cultivation of cyanobacteria and microalgae is associated with oxidative stress, which can compromise the quality of biomass. Stress related to the accumulation of free radicals and oxidative degradation products is considered a major challenge, posing economic and health risks. At the same time, moderate stress can provide benefits by increasing biomass production, faster accumulation of polysaccharides, and necessary modifications in pigment content, among other factors. Using moderate oxidative stress as a biotechnological tool, it is crucial to maintain a proper balance between the benefits offered and associated safety risks.

#### 7.1. Common mechanisms of establishment of oxidative stress of different etiology

In the previous chapters, data on the changes in productivity levels, biochemical composition, and antioxidant activity under various types of stress in algal cultures are presented. Under certain conditions of massive accumulation of oxidative degradation products of macromolecules, morphological changes occur at both macro and microstructural levels. The color change of the culture due to modifications in pigment composition, alterations in cell or filament dimensions, and the formation of atypical cellular conglomerates are common phenomena, easily observed and described in sufficient detail. In this study, our focus is on identifying common ultrastructural changes under different type of stress in the culture of *Arthrospira platensis*. Three different states of oxidative stress were analyzed: oxidative stress in nanobiosynthesis, hypothermic oxidative stress, and oxidative stress generated by xenobiotics (metals - Zn, Co, Cu, Mo, Ni, gold and silver nanoparticles, ethanol). Figure 17 illustrates the changes occurring in the ultrastructure of *A. platensis* cells during the process of bionanosynthesis of selenium nanoparticles.

In Figure 17 A, B, and C, the typical ultrastructure of spirulina can be observed. The most significant characteristic structures of the cell cytoplasm are tightly arranged, well-structured thylakoids with easily visible membranes. A large number of carboxysomes are observed polyhedral inclusion bodies containing the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO), responsible for carbon dioxide fixation in spirulina. The cytoplasm is dense and closely adheres to the cytoplasmic membrane. The cell wall is dense and visible, tightly attached to the cytoplasmic membrane. The polysaccharide capsule is compact and thin. During the nanobiosynthesis process of selenium nanoparticles (SeNPs), changes in the structure of spirulina occurred, including a decrease in the compactness of thylakoids, the appearance of translucent spaces between the cytoplasmic membrane and the cell wall, the emergence of large polyphosphate bodies and polyhydroxyalkanoates (which are a form of carbon storage), and de-compaction of the cell wall.



Fig. 17. Ultrastructure of A. platensis cells in the process of nanobiosynthesis. A, B, C - control, D, E, F - cells where nanobiosynthesis of SeNPs occurs. Cs - carboxysomes; T - thylakoids; M - membrane; CW - cell wall; L - lipid inclusions; PHA - polyhydroxyalkanoates; PP - polyphosphate bodies; S - septum; black circles - disorganization of thylakoids, red circles - space between the cell membrane and the cell wall/septum, blue circle - modification of the cell wall/exopolysaccharide density.

The images presented above reflect the typical changes identified during the monitoring of the ultrastructure of *Arthrospira platensis* under oxidative stress conditions. In total, 251 images were analyzed for various oxidative stress conditions, and 60 images were analyzed for the spirulina culture under optimal conditions. The results of deviations from the norm are summarized in Table 5.

From the observations, we can state that only moderate hypothermia does not induce significant ultrastructural changes in spirulina, while stress during nanobiosynthesis, exposure to nanoparticles and heavy metals, and interaction with ethanol, lead to multiple structural deviations from the norm. The most frequent modifications involve disorganization of the photosynthetic membrane system. Considering that practically in all stress variants there is a significant increase in the content of malonic dialdehyde in cells, which is a final product of oxidative degradation of lipids, the visualization of thylakoidal disorganizations confirms, that the primary targets of free radicals formed as a result of stress, are the cell membranes.

			% of modifications					
T C		Нуро-	Xenobiotics			Nano-		
modifications	Control, N=60	thermy, 20°C, N=45	Etha- nol, N=18	NPs, N=39	Havy metals, N=44	syn- thesis N=45	Control	Stress condi- tions
Disorganization	3	2	16	24	38	21	5%	40.2%
of thylakoids							(3/60)	(101/251)
Lack of	4	5	2	12	18	14	6.7%	20.3%
carboxysomes							(4/60)	(51/251)
Damage to cell	2	3	4	6	14	8	3.3%	13.9%
membranes							(2/60)	35/251)
Plasmolysis	0	0	0	4	5	17	0	10.4%
								(26/251)
PHA	8	4	2	28	28	21	13.%	33.1%
							(8/60)	(83/251)
Vacuolization	4	6	4	31	26	19	6.7%	34.3%
							(4/60)	(86/251)

 Table 5. Frequency of Ultrastructural Changes in Arthrospira platensis under

 Oxidative Stress Conditions

In the present study, one of the tasks was to highlight the modification of the expression of genes associated with a generalized response to stress conditions. For this purpose, under stress conditions, the transcriptional abundance of genes for heat shock protein (hsp90), glutamate synthase (also known as glutamine oxoglutarate aminotransferase - GOGAT), iron-superoxide dismutase (*FeSOD*), peroxidase (*POD*), large subunit of Rubisco (*rbcL*), peroxiredoxin (*per*), and iron absorption regulatory protein (*fur*) were measured. The gene expression level of interest was studied under different stress conditions: light stress; moderate hypothermic stress; stress induced by various xenobiotics (rare earth elements and ethanol); and stress during SeNP nanobiosynthesis (Figure 18). In the case of nanosynthesis, according to data referring to the productivity and biochemical composition of biomass, there is no evident state of stress, while a fivefold increase in the expression of *FeSOD* compared to the control can be considered evidence of unfavorable oxidative stress in A. platensis cells synthesizing SeNPs (Figure 18, a). The situation may become more complex over time, as the expression level of peroxidases has remained at the control level, these enzymes being responsible for removing the reaction product (H<sub>2</sub>O<sub>2</sub>) formed as a result of superoxide dismutase activity. Under SeNPs synthesis conditions in A. platensis cells, an increase in the transcriptional abundance of hsp90 (4.4 times compared to the control) was observed, indicating the need to maintain the stability of cellular proteins under stress conditions. At the same time, the transcriptional abundance of the *rbcL* gene, on the contrary, decreased significantly, by 7.7 times compared to the control. Thus, the change in the expression of three out of the five

studied genes associated with stress confirms that the culture of *A. platensis* was exposed to oxidative stress, although apparently, the cyanobacterial culture developed normally.



Fig.18. The difference in relative expression of genes associated with oxidative stress response between control and stress conditions in *Arthrospira platensis*: a - biosynthesis of SeNPs; b – light stress (24 hours of dark); c - ethanol stress (4 ml/l); d - hypothermic stress  $20^{\circ}$ C (\*\* - p < 0.01, \*\*\* - p < 0.001)

Under dark stress conditions lasting 24 hours (Figure18, b), a significant increase in the transcriptional abundance for all five analyzed genes is observed. This condition is associated with the disruption of photosynthesis, the disappearance of carotenoid pigments, and disruption of all assimilation and removal processes of reactive oxygen species. An opposite situation is observed in the case of ethanol-induced stress, added in a quantity of 4 ml/l to medium. In this case, no statistically significant deviations from the normal expression level of the five genes were observed (Figure18, c). In the case of moderate hypothermic stress, the expression level of two of the monitored genes was changed - the transcriptional abundance of the POD gene decreased by almost 3 times, and the expression of the *rbcL* gene increased by 7.5 times (Figure 8, d).

The expression of genes associated with antioxidant protection in spirulina was studied in accumulation of rare earth elements in biomass (table 6).

The stress factor	Relative gene expression difference compared to control							
	FeSOD	POD	GOGAT	rbcL	Hsp90	per	fur	
Gd	0.91	0.61*	0.08***	4.01***	0.27**	0.26**	0.50	
Но	1.25	0.80	0.07***	5.80***	0.38**	0.37**	0.17**	
Nb	1.47***	0.88	0.28***	4.83***	0.84	0.53	0.18***	
Pr	1.52**	0.94	0.21***	4.79***	0.39**	0.47*	0.35*	
Y	1.68***	0.60*	0.10***	7.00***	0.44**	0.36**	0.09***	

 Table 6. The relative expression of genes associated with stress under the conditions of contact of spirulina with rare earth elements

\* p<0,05; \*\* p<0,01; \*\*\*p<0,001

For two of the genes, whose expression was quantified in all five experimental variants, the transcriptional abundance changed according to the same algorithm: *GOGAT* expression decreased by 3-12 times, while rbcL expression increased by 4-7 times. The expression of *POD*, *hsp90*, *per*, and *fur* genes also decreased in all experimental variants, although in some cases, the differences were not statistically significant.

It is evident, that all changes occurring in cells subjected to stress of any origin are based on complicated molecular mechanisms intervening in general metabolic pathways. The purpose of this study was to identify specific elements of these mechanisms with biotechnological value, i.e., elements that can be presumed, identified, and managed within technologies for obtaining valuable and safe phycological biomass for consumption.

In some of the examined cases, the effects of different types of stress are explained by an imbalance in the antioxidant status of spirulina through the overexpression of genes, encoding enzymes in the first line of antioxidant protection, and the underexpression of other genes in the same group. Particularly noteworthy is the increased expression level of *FeSOD*, whose translational product's activity is the formation of hydrogen peroxide, and simultaneously, the decreased expression level of genes, whose products ensure cell detoxification by removing peroxides. Schematically, the appearance of this imbalance and its consequences can be observed in Figure 19.

The superoxide radical is among the first reactive species formed in response to various factors and as a result of normal physiological processes within the cell. The most active centers for the formation of this superoxide radical in microalgae and cyanobacteria are the cytoplasmic

membrane oxidoreductase, apoplastic peroxidase, FeS clusters, and the ferredoxin of photosystems I and II. Some of the superoxide radicals are consumed in signal transduction reactions, while another portion is used as a substrate for superoxide dismutase. As a result of this reaction, hydrogen peroxide is formed, which is neutralized by catalase, as well as by a series of other enzymes (glutathione peroxidase, ascorbate peroxidase, peroxiredoxin), involved in neutralizing various types of peroxides, including hydrogen peroxide.



Fig.19. The appearance and consequences of the imbalance in the activity of primary antioxidant enzymes in *Arthrospira platensis* (explanations are in the text)

In the case of high superoxide dismutase activity without a coordinated increase in the activity of enzymes involved in the degradation of  $H_2O_2$ , the last generates the hydroxyl radical through redox reactions involving metals with variable oxidation states. In the presence of metals with variable valence in the cellular environment, especially Fe and Cu, some of the superoxide radicals are not taken up by superoxide dismutase, but are involved in Fenton and Haber-Weiss reactions, leading to the formation of the hydroxyl radicals. Second-line antioxidants can remove excess superoxide radicals under normal physiological conditions. Under stress conditions, an excess of superoxide radicals attacks all types of macromolecules crucial for the cell, leading to

the formation of deeply oxidized biopolymer products and redox imbalance, ultimately resulting in oxidative stress (Figure 19).

The alteration of the expression of genes, associated with the stress response, amplifies the physiological effects by affecting multiple vital processes in cyanobacterial cells (Figure 20).



### Fig. 20. Changes in the expression of genes associated with stress and their physiological effects in the cyanobacteria *Arthrospira platensis* (explanations are in the text)

In 7 out of the 9 stress situations studied in this work the *hsp90* gene of the heat shock protein was characterized by reduced transcriptional abundance compared to the control. As the translational product of this gene is responsible for the stability of multiple structural and functional proteins, and the correct formation of tertiary and quaternary protein structures, we assume that its underexpression has effects on all other products of genes associated with stress. The reduced expression of *hsp90* can lead to reduced stability and misassembly of higher protein structures, including enzymes of basic metabolic pathways, and could significantly contribute to the manifestation of generalized stress effects in affected cells.

Another gene that showed reduced expression compared to the control was the iron uptake regulatory protein gene - *fur*. The iron uptake regulatory protein is responsible for maintaining metal balance in cells, and can affect the expression of other genes that have a specific position for iron binding in their regulatory site. Among the stress-associated genes we studied, this category includes the *FeSOD* gene, which may be affected by metal imbalance as a result of *fur* gene underexpression. Under stress conditions, the expression level of *GOGAT* decreased in most of the studied cases, with an increase observed only in the case of light stress. The significant

decrease in the transcriptional abundance of GOGAT is associated with a decrease in nitrogen assimilation efficiency and, consequently, a reduction in primary production of photosynthetic microorganisms. In conditions of increased GOGAT expression, efficient nitrogen assimilation occurs, leading to an increase in protein content in biomass, which is crucial for spirulina, known as an ideal protein superproducer. The increase in the transcriptional abundance of rbcL, whose product is responsible for the assimilation of inorganic carbon, can be evidence of the redirection of cyanobacterial metabolism towards carbohydrate synthesis, both as an energy reserve and a protective structure. However, the transcriptional product of rbcL is also responsible for photorespiration. As the gene's transcription level can both increase and decrease, the generated physiological effects can be very different. Regardless of the direction of change in the transcriptional abundance of rbcL, when the intensity of the carbon fixation process dominates over the intensity of the photorespiration process, carbohydrate reserves accumulate in the cells.

# 7.2. Identification and characterization of stress based on the intensity of oxidative processes

The classification of stress states is an extremely challenging task, addressed by researchers from various fields - from molecular biology to personalized medicine, on which there is still no consensus. According to the intensity level of stress, three types of stress are outlined: high-intensity stress, intermediate-intensity stress, and low-intensity stress. The stress intensity level is determined by the signaling pathways involved in triggering stress, the molecular product, and the physiological product, generated under stress conditions. Signaling pathways are considered to exist in low and intermediate-intensity stress. Numerous studies have identified signaling pathways under various oxidative stress conditions for cyanobacteria and microalgae. Currently, signaling factors such as Sigma factors, two-component systems, transcriptional regulators, and regulatory RNAs, acting either separately, or in combination, are considered stress signals.

Understanding stress signaling pathways and mechanisms is important for providing a solid foundation for biotechnological research, but they are still far from completion. Therefore, in practical terms, stress intensity classification is based on the other two criteria. According to these criteria, *low-intensity oxidative stress* is characterized at the molecular product level by changes in the quantity and activity of antioxidant enzymes and, at the physiological product level, by the manifestation of an adaptive response.

*Intermediate-intensity oxidative stress* is characterized at the molecular product level, in addition to changes in antioxidant enzyme activity, by the alterations in the expression of heat

shock proteins, and other prooxidant factors. At the physiological product level, a combined response is observed, involving both adaptation and damage.

*High-intensity oxidative stress* results in cell death as the physiological product, and is not of interest for biotechnological applications. Therefore, low and intermediate-intensity stress situations were considered when developing biotechnological procedures. Figure 21 presents patterns of oxidative stress progression over time (a-c), or stress factor intensity (d-f), based on the results presented in this study.

Figures 21a-c reflect situations, in which a specific stress factor is applied to the phycological culture to obtain a final product (biomass or biomass components) in a larger quantity, representing the essence of the stimulation procedures currently applied in phycobiotechnology. During the cultivation cycle of microalgae and cyanobacteria, depending on the nature of the applied factor, three different situations are observed: an increase in the productive parameter of interest up to a certain level, maintained until the end of the cultivation cycle (Figure 21a); an increase in the factor value to a certain level, followed by a decrease to the initial or near-initial level (Figure 21b); and an increase in the productive factor value up to a certain level, followed by a drop below the acceptable limit (Figure 21c). All described situations imply the possibility of implementation in phycobiotechnology, providing a time interval, during which the predicted quantity and desired quality of the product can be collected. The optimal intensity of the applied factor in these situations can be identified through experiments with variable values.

Figures 21d-f reflect three other response patterns of phycological cultures to the action of stress factors with variable intensity. Thus, it is possible that the tested factor may not have a stimulating potential, and then the situation described in Figure 21,d unfolds. Increasing the intensity of the factor within certain limits does not produce a change in the parameter level, after which the generated effect becomes negative, and the value of the parameter of interest decreases.

The situations described in Figure 21,e-f highlight a zone of the intensity of the factor where beneficial reactions occur, leading to an increase in the value of the parameter of interest. This zone can be identified under the application of a genuine stimulator (Fig. 21,e). After this zone, an increase in the intensity of the factor leads to a decrease in the values of the parameter of interest until the initial value or a value close to it. In this situation, it is possible to delineate low-intensity stress from intermediate-intensity stress by identifying the point of return to the initial value of the parameter. Additionally, a zone of preferential concentrations is defined when the hormetic effect of the tested substance occurs. After the stimulation zone, there is a decline in the parameter values below their initial value (Fig. 21,f). The situations described in Figures 21,e, and 21,f are interesting when values of the stress factor intensity can be identified to ensure a maximum quantity of the product.



Figure 21. Patterns of oxidative stress progression in cyanobacteria and microalgae: a, b, c
– over time, following the action of a known intensity stress factor; d, e, f – at the end of the life cycle depending on the intensity of the stress factor.

In Figure 21, the patterns most favorable for application in phycobiotechnology for obtaining a qualitative and safe product are outlined in the green frame. The horizontal green line indicates the parameter level before the application of the stressor/stimulus factor. The red dashed line represents the time limit or intensity of the stress factor, at which the monitored parameter returns to the pre-stress value. Blue arrows indicate points of return to the initial values of the parameter, serving as delineation points between low-intensity stress (LIS) and medium-intensity stress (MIS). The characteristic zone of low-intensity stress is the domain where biotechnologies for obtaining native phycological products, such as quality-predefined biomass, can be applied. The characteristic zone of intermediate-intensity stress also has its applications in biotechnology, where processes such as biofunctionalization, bionanosynthesis, and bioremediation occur.

### 7.3. Applications of oxidative stress in phycobiotechnology

The principles of installation and progression of oxidative stress, identified and described earlier, have been applied in the development of several biotechnological processes:

- Process for directing the content of bioactive compounds (phycobiliproteins and polysaccharides) during the industrial cultivation of spirulina;
- Biosynthesis of silver (AgNPs) and selenium (SeNPs) nanoparticles using microalgae and cyanobacteria as objects of interest;

• Biofunctionalization process of silver nanoparticles (AgNPs) with the cyanobacterium *Arthrospira platensis*;

• Processes for obtaining phycological biomass with high lipid content (Patents 4542MD, 4543 MD, 4714 MD, 4796 MD);

- Bioremediation of moderately polluted effluents with metals, using the cyanobacteria *Arthrospira platensis* and *Nostoc linckia*;
- Process for determining the toxicity of nanoparticles using the red microalga *Porphyridium cruentum* (Patent 4200 MD).

All these procedures are based on the results presented in chapters 4-7, and have formed the basis of invention patents, obtained and implemented in the practice of phycological production at the biotechnological and Ficotehfarm pharmaceutical enterprise. The novel procedures for obtaining valuable biotechnological products (biomass with high phycobiliprotein, carbohydrate, and lipid content; biosynthetic nanoparticles, biofunctionalized nanoparticles), dwhich were eveloped based on the application of phycological cultures' response to oxidative stress, are efficient and easily achievable under industrial production conditions.

### CONCLUSIONS AND RECOMMENDATIONS

The doctoral thesis **"Oxidative stress in phycobiotechnology – mechanisms and regulation procedures"** is dedicated to studying phenomena associated with oxidative stress in microalgae and cyanobacteria of biotechnological interest, and the possibility of applying them as a useful tool in the development of procedures for the cultivation of phycological cultures to obtain biomass and other valuable products, including nanoparticles.

Oxidative stress is inevitable when referring to the industrial cultivation of microalgae and cyanobacteria. It is triggered by the components of the nutrient medium, high cell density in growth reactors, and biotechnological procedures, such as agitation, changes in light intensity, temperature, humidity, and the application of stimulants, among others. As a result, microalgae

and cyanobacteria cells produce more reactive oxygen species, than would be typical under natural conditions for these organisms.

Within certain limits, low and medium-intensity stress can have beneficial effects on phycological cultures, associated with the intensification of specific biosynthetic processes, leading to higher productivity and increased content of valuable biomass compounds. Antioxidant protection systems manage to eliminate toxic by-products, ensuring superior quality of both biomass and its derived products. In this context, oxidative stress can be considered as a convenient biotechnological tool, enabling the production of phycological biomass with a programmed content of bioactive compounds.

Even under optimal conditions, microalgae and cyanobacteria exhibit different levels of resistance to oxidative stress, depending on the life cycle phase of the culture. This variability can be successfully applied in biotechnology, allowing attenuation or amplification of effects induced by stimulants, inhibitors, or physical factors. Understanding of the cellular response peculiarities at different stages of the culture's live cycle is essential for the development of effective and safe biotechnological procedures for obtaining high-quality phycological biomass. However, the application of oxidative stress as a biotechnological tool must be approached with a great care, as the balance between benefits and harms is delicate and easily distorted.

Oxidative stress is an important tool in bionanotechnology. The production of reactive oxygen species upon contact of microalgae and cyanobacteria with metal ions activates mechanisms, involved in their reduction, leading to the formation and sequestration of nanoparticles in various cellular compartments. The ability of microalgae and cyanobacteria cultures to mitigate the effects of oxidative stress, caused by various pollutants, including heavy metals, forms the basis for the sequestration of pollutants inside the cells. Thus, the studied phycological objects can be considered natural accumulators and serve the purpose of polluted aquatic environments bioremediation.

The same detoxification mechanisms, in the case of cell contact with industrially produced nanoparticles, ensure their biofunctionalization into phycological biomass, a process that modifies the properties, biodisponibility, and biocompatibility of nanoparticles with animal cells and tissues. Biofunctionalized nanoparticles hold great potential for diagnosis and treatment of severe diseases, such as cancer. Phycological biomass containing biofunctionalized nanoparticles offers multiple benefits, harnessing the healing properties of biomass, and the unique properties of nanomaterials.

The completion of this work has resulted in the identification of common elements of specific responses of the studied microalgae and cyanobacteria to different intensities of stress.

These new insights revealed stress application points to achieve predicted results and the development of procedures and technologies. That enable the production of phycological biomass, characterized not only by valuable biochemical composition, but also by an adequate level of safety for human and animal use.

The elucidated aspects throughout the completion of the doctoral thesis can be summarized in the following conclusions:

- 1. Monitoring of the oscillations in antioxidant activity and biochemical parameters of biomass throughout the life cycle of microalgae (*Haematococcus pluvialis, Dunaliella salina, Porphyridium cruentum*) and cyanobacteria (*Arthrospira platensis, Nostoc linckia*) has allowed to identify period of vulnerability in cultures, largely associated with changes in the functional state of the culture, especially corresponding to transitions between different phases of the life cycle. This knowledge forms the basis for shaping and implementing various biotechnological procedures to enhance the cultivation of phycological cultures for the production of valuable biomass, safe for human consumption.
- 2. Analysis of the results obtained during the study of the influence of various types of stress (thermal, illumination, osmotic, and chemical) on multiple phycological objects (*Arthrospira platensis, Nostoc linckia, Haematococcus pluvialis, Dunaliella salina, Porphyridium crientum*) has revealed common elements in cellular response, manifested by changes in quantifiable parameters. All studied stress types are associated with an increase in the amount of MDA (damage-associated molecular patterns) in biomass, altering antioxidant activity, and affecting the enzymes in the first line of defense against oxidative stress. Depending on the strain, stress type, and its intensity, the values of parameters reflecting the antioxidant status of biomass may increase or decrease.
- 3. Correlation between beneficial effects of induced stress (e.g., light stress) expressed through technological advantages, such as increased biomass production and high quantities of phycobiliproteins and carbohydrates, and stress marker values, indicates the need for rigorous control of phycological biomass safety. When the goal is not the use of the entire biomass, but the extraction of specific bioactive components (e.g., phycobiliproteins, polysaccharides), moderate stress can be successfully applied as a simple, cost-effective, and efficient technological solution.
- 4. The study of the influence of nanoparticles of different nature (quantum dots CdSe, CdS, ZnS; gold and silver nanoparticles stabilized in organic polymers) on microalgae and cyanobacteria cultures has highlighted the same common features in the stress response, expressed by changes in the parameters of the antioxidant status of biomass, indicating the

role of these structures as stress factors. Inducing a state of stress as a result of the contact between nanoparticles and microalgae and cyanobacteria cells, triggers cellular protection mechanisms, forming the basis for the biofunctionalization process of industrially synthesized nanoparticles.

- 5. The obtained results have demonstrated the ability of live cultures of microalgae (*Haematococcus pluvialis, Dunaliella salina, Porphyridium crientum*) and cyanobacteria (*Arthrospira platensis, Nostoc linckia*) to biosynthesize nanoparticles from potentially toxic ions (e.g., Ag<sup>+</sup>, SeO<sub>3</sub><sup>-2</sup>). Biochemical changes and antioxidant activity alterations in phycological biomass during the process eloquently demonstrate, that nanoparticle biosynthesis is based on the reducing power of the matrix used for synthesis.
- 6. The high accumulation of heavy metals from wastewater with low pollutant content, based on stress counteraction mechanisms of *Arthrospira platensis* and *Nostoc linckia*, highlighted in research using polymetallic systems containing Cu, Cr, Ni, Zn, Fe in iterative cycles allows naming these cyanobacteria as natural bioaccumulators, capable of concentrating heavy metals in cells, with a concentration factor of x1000. Thus, modeling of bioremediation systems by changing process parameters (chemical composition of effluents, culture age, number of iterative cycles) ensures the success of post-treatment technologies for effluents contaminated with heavy metals.
- 7. The results obtained during the monitoring of the transcriptional abundance of genes associated with cellular stress response in *Arthrospira platensis* culture have highlighted a clear imbalance in the activity of the first-line antioxidant protection system. With the increased transcriptional abundance of the FeSOD gene, the relative expression of peroxidase (*POD*) and peroxiredoxin (*per*) genes decreases. Additionally, there is a reduction in the relative expression of the iron absorption regulatory protein gene (*fur*), heat shock protein (*hsp90*), glutamate synthase gene (*GOGAT*), and the modification of the relative expression level of the large RUBISCO subunit gene (*rbcL*), resulting in changes in carbon and nitrogen metabolism, disorganization of higher protein structures, alteration of metal intake, and, ultimately, a decrease in the adaptability of spirulina, confirmed by significant ultrastructural changes.
- 8. The correlational analysis based on normalized data from multiple stress induction experiments in microalgae cultures (*Haematococcus pluvialis, Dunaliella salina, Porphyridium crientum*) and cyanobacteria (*Arthrospira platensis, Nostoc linckia*) highlighted, that various types of relationships between the level of the oxidative stress marker malondialdehyde (MDA) and the biochemical parameters of biomass (proteins,

lipids, carbohydrates), including antioxidant activity, can be considered as indicators of oxidative stress in phycological cultures. The inverse correlation between MDA levels and the antioxidant activity of hydro-ethanolic extracts from biomass is useful in identifying optimal concentrations of stimulants, used in biotechnological processes, involving microalgae and cyanobacteria.

- 9. Generalizing of the results obtained in this study allowed identification of parameters that underlie the classification of stress types, based on their intensity. For the field of phycobiotechnology, low-intensity oxidative stress, characterized by changes in the expression of first-line antioxidant enzyme genes and physiological adaptive reactions, is relevant. Additionally, moderate-intensity oxidative stress is characterized by alterations in the expression of multiple stress-associated genes and moderate destructive manifestations at the physiological level.
- 10. Based on the analysis of the obtained results, as a demarcation line between low-intensity oxidative stress and intermediate-intensity oxidative stress (which poses a danger to phycological biomass production technologies), it is useful to apply the moment in time, or the intensity of the factor, at which the positive effect on the monitored parameter decreases to a level equal or close to that, which was characteristic for culture before the stress factor application.
- 11. The conducted study confirms the hypothesis, that formed the basis of this work: the response to induced oxidative stress is a useful tool for obtaining valuable phycological biomass with controlled composition, for the biosynthesis and biofunctionalization of nanoparticles, and for the bioremediation of polluted environments. The application of this tool is opportune, while maintaining a balance between the obtained beneficial effects and the accumulation of reactive species.

### **Practical Recommendations:**

 Correlation of the procedures for stimulating productivity and the accumulation of biologically active substances by microalgae and cyanobacteria with physiological characteristics, depending on the cultural cycle phase, in which the intervention occurs is recommended. The mandatory introduction of safety control in the phycological biomass production biotechnological flow, based on oxidative stress markers, in tandem with closely identified biochemical indicators is recommended.

- 2. We recommend to implement four procedures of silver nanoparticle biosynthesis using live cultures of *Arthrospira platensis, Nostoc linckia, Dunaliella salina*, and *Porphyridium cruentum*, according to the developed schemes.
- 3. We recommend to implement two procedures of selenium nanoparticle biosynthesis, using live cultures of *Arthrospira platensis* and *Nostoc linckia*, according to the developed schemes.
- 4. We recommend to implement four procedures of phycological biomass production with high-lipid content, using nanoparticles as stimulants, according to patents MD 4542, 4543, 4714, 4796.
- 5. We recommend implementation of the nanoparticle toxicity testing procedure for aquatic organisms according to invention patent MD 4200.
- 6. We recommend implementation of the model for effluents, polluted with moderate amounts of heavy metals decontamination using cultures of cyanobacteria *Arthrospira platensis* and *Nostoc linckia*.

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### LIST OF OWN PUBLICATIONS ON THE THESIS TOPIC

### 1. Monographs

**1.1.single author monographs** 

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- 13. RUDI, L., **CEPOI, L.**, CHIRIAC, T., ş.a. Unele aspecte ale aplicării nanoparticulelor de aur în biotehnologia microalgei *Porphyridium cruentum*. În: *Buletinul Academiei de Ştiinţe a Moldovei. Ştiinţele vieţii.* 2021, nr. 2 (344), p. 126-132. ISSN 1857-064X.
- 14. **CEPOI, L.**; RUDI, L.; CHIRIAC, T., ş.a. Dialdehida malonică un potențial marcher al toxicității nanoparticulelor în mediul acvatic. In: *One Health and Risk Management*. 2020, nr. 1, pp. 64-71. ISSN 2587-3458

### 3. Articles in scientific collections

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1. **CEPOI, L.,** RUDI, L., CHIRIAC, T; et al. Silver nanoparticles as stimulators in biotehnology of *Porphyridium cruentum*. In: *International Conference on Nanotechnologies* 

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- 4. Theses in scientific collections

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1. RUDI, L., CHIRIAC, T., **CEPOI, L.,** ş.a. Metode de analiză în ficobiotehnologie. Ghid metodic. 2020. Chişinău: Arpoligraf. 101p. ISBN 978-9975-3462-9-0.

### ADNOTARE

### Cepoi Liliana, "Stresul oxidativ în ficobiotehnologie – mecanisme și procedee de reglare", teză de doctor habilitat în științe biologice, specialitatea 167.01 – Biotehnologie, bionanotehnologie, Chișinău, 2023

**Structura tezei**: Teza conține introducere, 7 capitole, concluzii și recomandări, bibliografie cu 502 titluri, 8 anexe, 268 pagini text de bază, 98 figuri, 16 tabele. Rezultatele sunt reflectate în 75 publicații științifice.

**Cuvintele cheie:** stres oxidativ, microalge, cianobacterii, biomasă, markeri ai stresului, componența biochimică, ultrastructură, expresia genelor asociate stresului, intensitatea stresului.

**Scopul lucrării:** Fundamentarea aplicării stresului oxidativ în calitate de instrument în ficobiotehnologie prin elucidarea elementelor comune ale răspunsului microalgelor și cianobacteriilor la diferite tipuri de stres indus.

**Obiectivele lucrării:** Evidențierea particularităților de manifestare a stresului oxidativ indus de factorii fizici și chimici la cianobacterii și microalge de interes biotehnologic; Elucidarea posibilității de aplicare a răspunsului la stresul oxidativ indus în scopul obținerii biomasei ficologice cu componență prognozată; Estimarea implicării stresului oxidativ în procesele de bioremediere de către microalge și cianobacterii a apelor contaminate cu metale, în sisteme iterative; Conturarea principiilor de realizare a nanobiosintezei și biofuncționalizării nanoparticulelor cu ajutorul microalgelor și cianobacteriilor, în baza mecanismelor de protecție contra stresului oxidativ; Fundamentarea posibilității și a limitelor de aplicare a răspunsului la stresul oxidativ în calitate de instrument în ficobiotehnologie; Elaborarea procedeelor ficologice, bazate pe aplicarea răspunsului la stresul oxidativ indus.

Noutatea și originalitatea științifică: Originalitatea lucrării constă în abordarea răspunsului microalgelor și cianobacteriilor de interes biotehnologic la stresul oxidativ ca instrument eficient pentru dirijarea proceselor în ficobiotehnologie. Utilizând răspunsul culturilor de microalge și cianobacterii la stres, au fost elaborate tehnologii de obținere a biomasei ficologice cu conținut valoros dirijat, procedee de biosinteză și biofuncționalizare a nanoparticulelor și de bioremediere a mediului ambiant. Au fost identificați indicatori noi ai stresului oxidativ de intensitate joasă și formulate principii de apreciere a intensității stresului.

**Rezultatele obținute, care contribuie la soluționarea problemei științifice importante** constau în fundamentarea prin dovezi a posibilității aplicării răspunsului microalgelor și cianobacteriilor la stresul oxidativ indus în calitate de instrument biotehnologic, ceea ce a condus la elaborarea procedeelor originale de biosinteză a nanoparticulelor, inclusiv a celor biofuncționalizate; obținere a biomasei ficologice calitative și sigure, cu un conținut dirijat de compuși bioactivi; bioremediere a efluenților contaminați cu metale grele, ceea ce a conturat o direcție nouă de cercetare: *stresul ca instrument în ficobiotehnologie*.

**Semnificația teoretică:** Au fost formulate reperele conceptuale pentru utilizarea stresului oxidativ de diferită intensitate în calitate de mecanism de dirijare a proceselor ficobiotehnologice. Au fost argumentate principiile de aplicare a unor indicatori noi ai stresului și regulilede apreciere a intensității stresului oxidativ. Au fost identificate elementele comune ale reacțiilor de răspuns a microalgelor și cianobacteriilor la starea de stres. Au fost stabiliți indicatori noi pentru controlul de siguranță în condițiile aplicării stresului oxidativ de intensitate joasă în ficobiotehnologie.

Valoarea aplicativă a lucrării: În baza cunoștințelor noi acumulate au fost elaborate tehnologii bazate pe răspunsul microalgelor și cianobacteriilor la stres oxidativ indus, orientate spre obținerea de biomasă ficologică prețioasă, spre îndepărtarea/acumularea metalelor grele din mediul contaminat, spre biosinteza și biofuncționalizarea nanomaterialelor.

**Implementarea rezultatelor științifice:** Tehnologiile elaborate au fost implementate la întreprinderea de producere cu profil biotehnologic și farmaceutic FICOTEHFARM SRL(3 acte de implementare).

### ANNOTATION

Cepoi Liliana, "**Oxidative stress in phycobiotechnology – mechanisms and methods of its regulation**", dissertation for the degree of Doctor Habilitatus of Biological Sciences, specialty 167.01 – Biotechnology, bionanotechnology, Chisinau, 2023

Dissertation structure: The dissertation consists of an introduction, 7 chapters, conclusions and recommendations, a bibliographic list of 502 titles, 8 appendices, 268 pages of the main text, 98 figures, 16 tables. The results are presented in 75 published scientific papers.

**Keywords:** oxidative stress, microalgae, cyanobacteria, biomass, oxidative stress markers, biochemical composition, ultrastructure, stress-associated gene expression.

**Purpose of the work:** Identification of the common responses of microalgae and cyanobacteria to various types of induced oxidative stress and grounding the possibility of using the oxidative stress as a phycobiotechnology tool.

**Objectives of the work:** To identify the peculiarities of the oxidative stress induced by physical and chemical factors in cyanobacteria and microalgae; To estimate possibility of using the response to the induced oxidative stress for obtaining phycological biomass with predicted composition; To estimate the possibility of using microalgae, cyanobacteria and oxidative stress in remediation of waters contaminated by heavy metals in the iterative systems; To ground the principles of nanobiosynthesis and biofunctionalization of nanoparticles based on the protective mechanisms against of oxidative stress in microalgae and cyanobacteria; To assess the possibilities and limitations of applying the response to the induced oxidative stress; To elaborate phycological procedures based on the application of the induced oxidative stress.

**Scientific novelty and originality**: The originality of the work lies in utilizing the response of microalgae and cyanobacteria to oxidative stress as an efficient tool for directing processes in phycobiotechnology. By leveraging the response of microalgae and cyanobacteria cultures to stress, technologies have been developed for obtaining phycological biomass with directed valuable content, as well as methods for biosynthesis and biofunctionalization of nanoparticles and environmental bioremediation. New indicators of low-intensity oxidative stress have been identified, and principles for assessing stress intensity have been formulated.

The obtained results, which contribute to addressing the important scientific problem, consist in providing evidence for the possibility of applying induced oxidative stress as a biotechnological tool, leading to the development of original procedures for the biosynthesis of nanoparticles, including biofunctionalized ones; production of high-quality and safe phycological biomass with a controlled content of bioactive compounds; bioremediation of effluents contaminated with heavy metals, which outlined a new research direction: *oxidative stress as a tool in phycobiotechnology*.

**Theoretical significance**: Conceptual landmarks have been formulated for utilizing oxidative stress of varying intensity as a mechanism to guide phycobiotechnological processes. Principles for applying new stress indicators and rules for assessing the intensity of oxidative stress have been established. Common elements of the response reactions of microalgae and cyanobacteria to stress have been identified. Furthermore, new safety control indicators have been implemented for the application of low-intensity oxidative stress in phycobiotechnology.

**Applied significance**: on the basis of the new accumulated knowledge we developed technologies based on induced oxidative stress, focused on obtaining valuable biomass, removal/accumulation of heavy metals from the contaminated environment; and biosynthesis and biofunctionalization of nanomaterials.

**Implementation of scientific results**: The developed technologies were implemented by the FICOTEHFARM production company, specialized in biotechnological and pharmaceutical products (3 acts of implementation).

### LIST OF ABBREVIATIONS

А	Antioxidant
NAA	Neutron activation analysis
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
AgNPs	Silver nanoparticles
APX	Ascorbate peroxidase
AuNP	Gosd nanoparticles
CAT	Catalase
MDA	Malondialdehyde
DPPH	2,2-diphenyl-1-picrylhydrazyl
FeSOD	Ferric superoxide dismutase gene
FeSOD	Ferric superoxide dismutase
FUR	Ferric Uptake Regulator proteine
fur	Ferric Uptake Regulator proteine gene
GOGAT	Glutamate synthase gene
GOGAT	Glutamate synthase (also known as Glutamine oxoglutarate
	aminotransferase)
GPX	Glutathione peroxidase
GSH	Reduced glutathione
hsp90	Heat shock protein 90 gene
HSPs	Heat shock proteins
SEM	Scaning electron microscopy
PEG	Polyethylene glycol
per	Peroxiredoxin gene
PER	Peroxiredoxin
PHA	Polyhydroxyalkanoates
PAONA	Products of advanced oxidation of nucleic acids
PAPO	Products of advanced protein oxidation
pod	Peroxidase gene
POX	Peroxidase
PP	Polyphosphate bodies
RCPMR	The reducing capacity of the phosphomolybdenum reagent
PSI	Photosystem I
PSII	Photosystem II
rbcL	Large subunit of ribulose bisphosphate carboxylase gene
RuBisCo	Ribulose-1,5-bisphosphate carboxylase/oxygenase
SeNP	Selenium nanoparticles
SOD	Superoxid dismutase
ROS	Reactive Oxygen Species
TBARS	Thiobarbituric acid reactive substance

### **CEPOI LILIANA**

### OXIDATIVE STRESS IN PHYCOBIOTECHNOLOGY -MECHANISMS AND REGULATION PROCESSES

### 167.01 – BIOTECHNOLOGY, BIONANOTECHNOLOGY

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